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**Research Article**

# Detection of Potentially Novel Dengue Lineages in the EMRO Region Through Integrated Phylogenomic and Protein Analysis

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**Abstract**

Despite decades of confirmed dengue circulation in the Eastern Mediterranean region (EMRO), genomic data remain limited and underrepresented. This research aimed to examine the DENV genome, focusing on nucleotide and E protein levels, using various computer simulation methods, so as to analyze DENV transmission dynamics closely. Using multiple computational approaches and custom Python pipelines, we examined nucleotide and envelope (E) protein sequences to assess evolutionary relationships, divergence, and substitution patterns. Our results show that recent strains collected between 2023 and 2025 from Pakistan, the UAE, and Djibouti are genetically diverse and closely aligned with global variants from Cuba, India, and China. Nucleotide and amino acid phylogenies supported by PCA and time-resolved tree models revealed tight clustering among the collected isolates. They suggested a potential regional convergence with newly reported lineages from Asia and the Americas. In particular, a new lineage of DENV-3 (AII) of Cuban origin was found to be related to Saudi and Israeli strains, raising concern about silent spread in other regions. Moreover, amino acid analyses of the envelope protein (E) identified common substitution sites within 2025 samples, with some mutations potentially associated with viral fitness and antigenic variation, although their functional effects require experimental validation. Our data suggest a pattern of changing transmission routes and emerging silent lineages, but a crucial limitation is the lack of realtime surveillance and the limited number of contributed sequences from important countries in the EMRO region. Finally, we emphasize the pressing need for genomic surveillance, detailed analyses of functional mutations, and data sharing on DENV spread to monitor and control potential outbreaks before they escalate into large-scale outbreaks.

**Keywords:** Dengue virus, EMRO region, Vector-borne diseases, Genomic surveillance

## *1. Background*

Dengue virus (DENV) is a mosquito-borne pathogen of global importance that has expanded its geographic range and clinical impact over the past decades, it became another major public health issue in subtropical and tropical regions (1, 2). DENV is a member of the Flaviviridae family that belongs to the Orthoflavivirus denguei species; it's an acute infection transmitted primarily by *Aedes aegypti* and *Aedes albopictus* mosquitoes and has four main serotypes (DENV-1, DENV-2, DENV-3, DENV-4); with each type providing lifelong immunity to itself but not to other serotypes (3). Another type (DENV-5) was discovered in Malaysia in October 2013, yet The International Committee on Taxonomy of Viruses (ICTV) has not officially recognized it as a distinct serotype; fortunately, unlike the other types, DENV-5 has not been associated with widespread human outbreaks as it follows a different transmission cycle that primarily involves non-human hosts and forest-dwelling mosquitoes (1, 4). Dengue occurs with major outbreaks typically occurring every 3–4 years;

however, the COVID-19 pandemic disrupted this cycle, leading to lower transmission in some areas and a buildup of people lacking immunity to specific variants (1). Dengue disease is often asymptomatic or causes mild symptoms (e.g., fever, headache, rash) but if a second infection occurs, it can lead to severe or potentially life-threatening illness with signs such as acute abdominal pain, persistent vomiting, bleeding, rapid breathing and extreme fatigue, where infection with a different subtype can cause a wide range of effects thereby predisposing individuals to sequential infections that can lead to a serious disease manifestations, such as dengue hemorrhagic fever (DHF) and dengue shock syndrome (DSS) (5, 6). This immunological phenomenon is called Antibody-dependent enhancement (ADE); in dengue, after the first infection, the body creates potent antibodies that protect against the same serotype, but they are short-lived. Once it fades, non-neutralizing antibodies may help the virus infect cells more easily (extrinsic ADE) primarily through Fc gamma receptors that are found on immune cells. This process suppresses normal immune responses, allowing the virus to multiply and possibly cause critical illness during reinfection (6). A spike in 2023 occurred where the number of virus cases increased globally across (80+) countries and five WHO regions including Eastern Mediterranean, with about 80% of reports in America (1). DENV possesses a single positive-sense RNA genome of approximately (10–11) kb, which encodes a single polyprotein that is cleaved into three structural proteins (capsid, pre-membrane/membrane, and envelope) and seven nonstructural proteins (NS1, NS2A, NS2B, NS3, NS4A, NS4B, and NS5) (5). However, there's limited structural data regarding the molecular aspects of the non-structural proteins NS1, NS2A, NS4A, and NS4B. It's known that NS1 supports viral RNA replication and inhibits complement activation, aiding immune evasion, while NS2A and NS4B contribute to the formation of the replication complex. Although these proteins are very important for the viral lifecycle, they are not as prominently involved in the host-pathogen interaction process as the envelope (E) protein (7, 8). The envelope protein has been characterized by its role in receptor binding, membrane fusion, and antigenicity (5). This protein has been found to hold an integral part within the viral infectivity process since it is the main glycoprotein responsible for viral attachment and the primary target for neutralization. This property of the protein renders it the main antigen for the immune response within natural infections, vaccine generation, and mutation analysis (9, 10).

In addition, urbanization, climate change, and the rise in international travel are all contributing to the transmission of dengue fever. Global warming, in particular, increases the efficiency of the mosquito vector in transmitting the disease, thereby prolonging the dengue season in endemic and non-endemic areas, such as the Middle East (3). Dengue is a vector-borne disease that is spread by blood-feeding arthropods such as mosquitoes, ticks, and fleas (6). Transmission occurs when a mosquito bites an infected person and later transmits the virus to another individual during a subsequent blood meal (11). The Aedes aegypti mosquito is adapted to urban environments, breeds in standing water, and typically bites during daylight hours (6, 11). Given the increasing global concern over dengue virus emergence and its accelerated spread in warmer climates, multiple recent studies have documented significant outbreaks and the introduction of novel viral variants. For instance, a 2025 study conducted in Makkah, Saudi Arabia, examined the prevalence of dengue virus serotypes among patients with dengue fever; they found positive DENV samples but with no specific classification, raising the suspicion of novel variants introduced from other regions (1). Another one is from Cub, which describes a new lineage of DENV-3 that is likely to have originated from the Indian subcontinent and is now being distributed across the Americas, possibly due to rising international travel and the vulnerability of surveillance systems (11). Despite the several countries in the EMRO that have reported instances of dengue infections, only very few have made available genomic data or carried out thorough viral characterization. Based on the ongoing observations, the purpose of this study is to examine the transmission

patterns and genetic properties of the dengue virus to better comprehend its distribution and possible evolution. Moreover, this is the first genomic and phylogenetic study that targets the subtypes of the dengue virus in the EMRO region, specifically in the Middle Eastern countries, where the data sources have been obtained from public databases available online. We used our custom Python workflow to investigate the virus's diversity and evolution at the nucleotide and amino acid levels. Our approach consisted of phylogenetic reconstruction, analysis of amino acid changes, heatmap analysis of mutation severity, PCA, and identification of the most affected countries, understanding the extent to which dengue resurgence threatens public health, and providing foundational data to strengthen future dengue surveillance and response strategies across the region.

## *2. Results and Data Analysis*

### 2.1 Phylogenetic Tree Analysis

**DENV Nucleotide Sequences.** We analyzed complete and partial viral sequences using the Neighbor-Joining (NJ) method to better understand the evolutionary relationships and regional clustering patterns of circulating dengue virus strains in the EMRO. First, we selected 26 available EMRO sequences from the total 47 collected strains to compare them with 4 NCBI RefSeq reference sequences (RefSeq) for each dengue type to establish baseline divergence and clustering behavior. As seen in **Figure 1**, the resulting tree has 30 sequences in total, separating all strains into four major serotypes (DENV-1, DENV-2, DENV-3, and DENV-4), each type clustered with its corresponding RefSeq, confirming accurate variant classification and validating the dataset's integrity. Within the DENV-1 group, Pakistani isolates (2022–2024), Djibouti (2001), Saudi Arabia (2014), and Israel (2023) all cluster closely together with high bootstrap support (96%>), revealing a conserved lineage that has circulated across the Middle East and neighboring regions over two decades. DENV-2 formed the most diverse and densely populated clade, with isolates from Pakistan (2013–2025), UAE (2024), Djibouti (2021–2023) and Somalia (2021, 2023) grouped firmly together with high bootstrap support; interestingly, one Pakistani sample collected in 2025 appeared closely related to other samples from the same country collected in 2022 and 2024, as well as to a UAE isolate from 2024. Dengue type 3 also showed a strong geographic linkage where the Somali strain (OK605766.1 from 2022) is close to those from Saudi Arabia (2014) and Israel (2024), suggesting the long-term persistence of this lineage across the region. By contrast, DENV-4 shows the least variability, with only two sequences grouped into a highly conserved clade. Pakistan dominates the phylogenetic tree, contributing to the highest sequences across all four serotypes. In the second part, we analyzed the identical sequences alongside recently reported (2024/2025) global isolates to explore broader phylogenetic relationships. **Figure 2** shows a tree of 47 sequences, with each dengue subtype clustered together with no outgroups seen. Within the DENV-1 clade, recent Pakistani isolates (2023–2024) are tightly placed with sequences from Israel (2023), Cuba (2023), and India (2025); within DENV-2, sequences from Pakistan (2025), India (2025), UAE (2024), Somalia (2023), and China (2025) forming multiple well-supported lineages. DENV-3 cluster included recent isolates from Israel (2024), Saudi Arabia (2021), and Cuba (2022), where the small group of DENV-4 all paired together, pointing to Pakistan (2013), which was linked to India (2025). All recent sequences (2024–2025) did not form entirely new or divergent clades but were integrated within existing lineages.

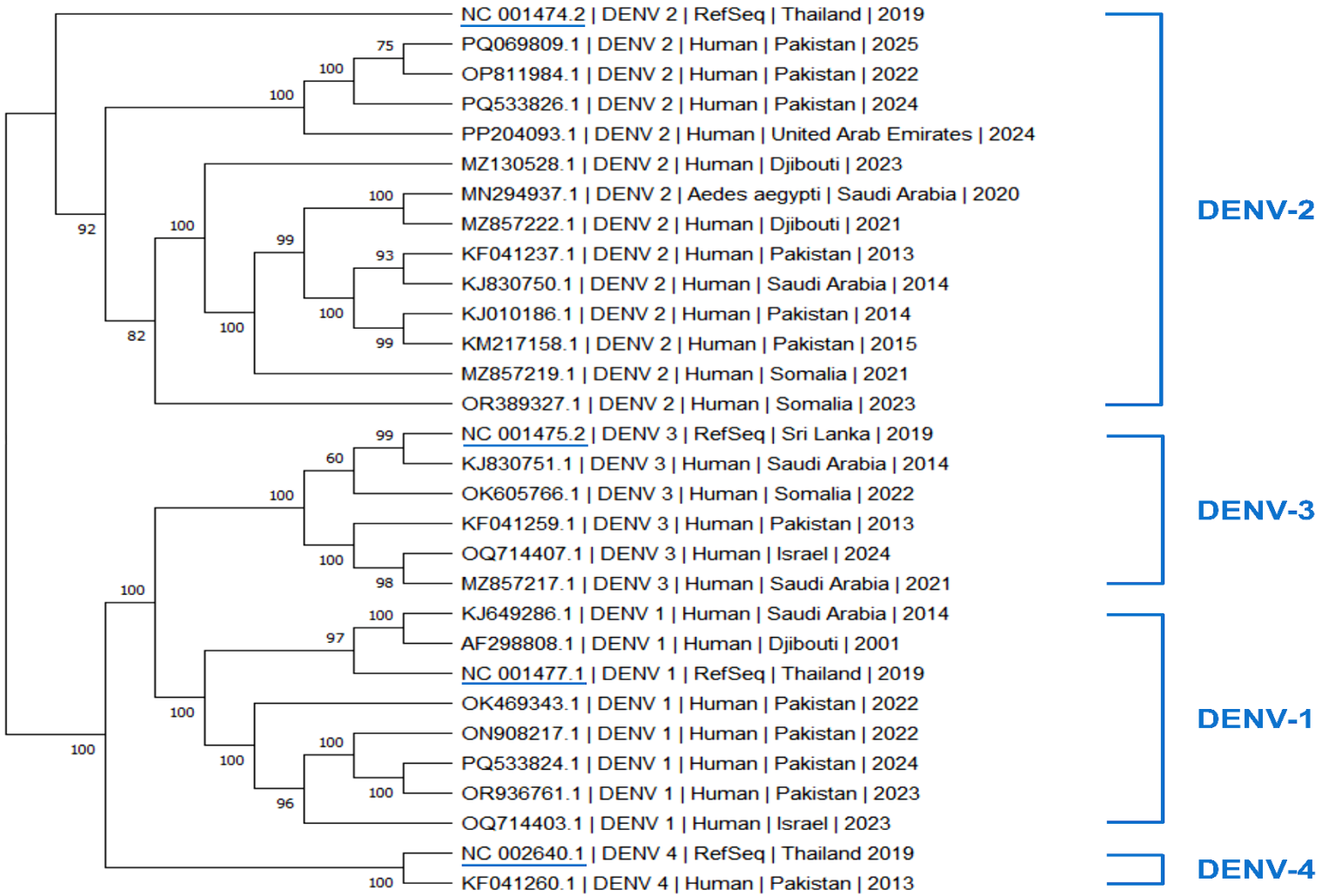


**Figure 1.** Shows the evolutionary relationships of a total of 30 different dengue virus subtypes. The strains were collected from the EMRO region with 4 reference sequences from NCBI in a Neighbor-Joining phylogenetic tree. The Sequences are grouped by serotype (DENV-1 to DENV-4) as indicated by bracketed labels. High bootstrap values support the branches at most nodes, and the reference sequences are underlined.

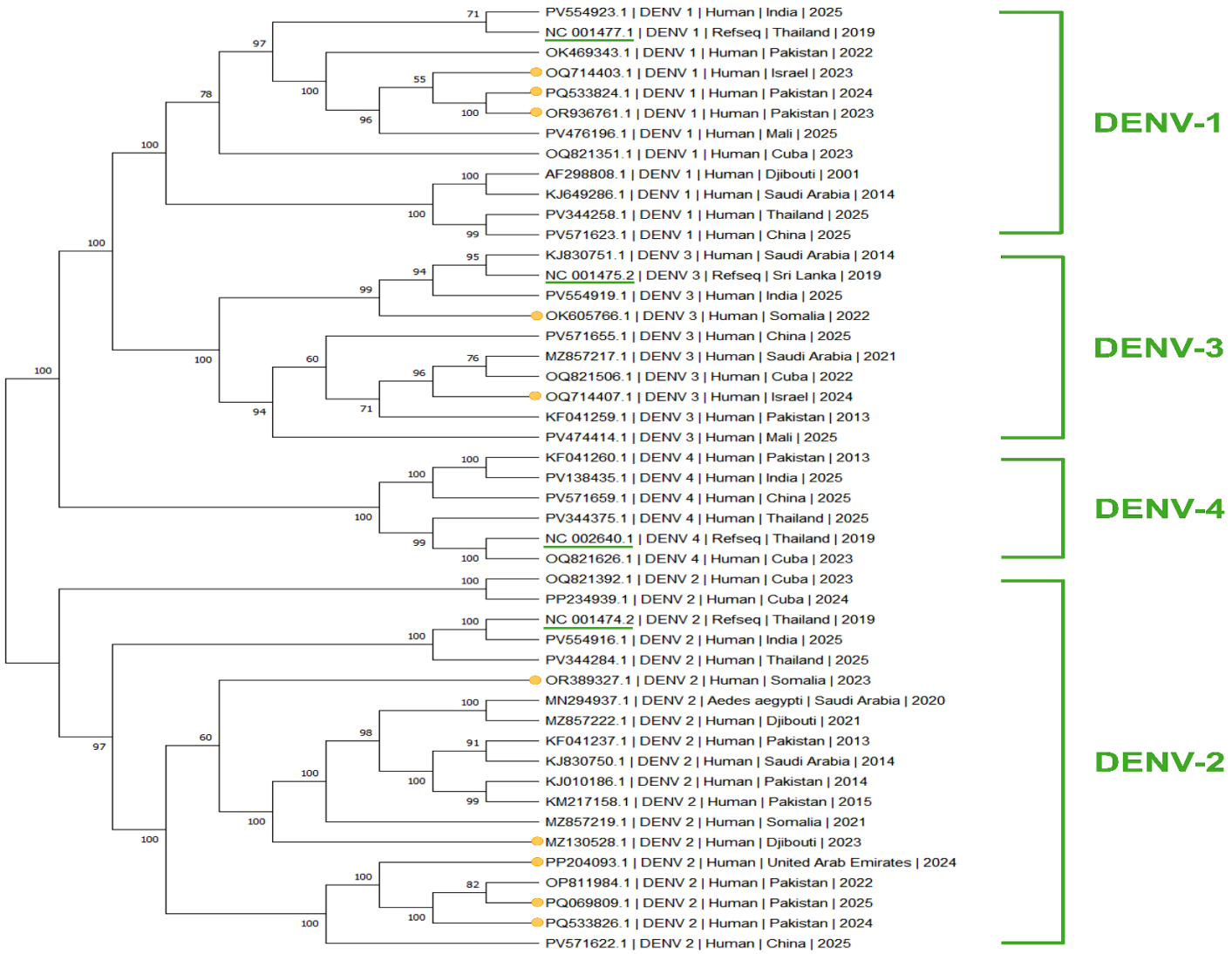


**Figure 2**. Shows the evolutionary relationships between 43 DENV strains and 4 reference sequences from NCBI in a Neighbor-Joining phylogenetic tree. All 47 sequences were grouped by serotype (DENV-1 to DENV-4) as indicated by bracketed labels, and the branches are well-supported at most nodes. The reference sequences are underlined, while the recent EMRO samples collected in 2023 or later are marked with yellow circles.

**DENV Amino Acid Sequences.** Subsequently, we analyzed the envelope (E) protein's translated amino acid sequences, further assessing the evolutionary relationships among DENV strains beyond nucleotide-level resolution. In **Figure 3**, we represent a circular inverted-phylogenetic tree of the translated DENV envelope protein sequences, where each type is grouped. A sequence was added (PQ508793.1) GIIIAII Cuban and previously proposed as a new DENV-3 lineage in the Millan et. al. study (11). This sequence was linked to other DENV-3 isolates from Israel, Saudi Arabia, and Mali between 2021 and 2025. Notably, the China 2025 DENV-3 strain formed an outgroup within its serotype, which was not manually selected, underscoring its huge sequence divergence. The tree shows strong conservation within each group, where recent sequences (2023–2025) remain interlinked mainly with global strains, though subtle divergence is observed across time and geography. To complement the amino acid-based circular phylogeny, a follow-up, time-resolved phylogenetic tree was constructed to examine the divergence and molecular clock dynamics of the DENV E protein, as seen in **Figure 4**. This tree supports the serotype-level structure observed in the circular tree, with DENV-1 to DENV-4 forming distinct and well-supported clades. Again, the new Cuban strain PQ508793.1 (DENV-3, GIIIAII, 2024) stands out, creating a close relationship with the Saudi Arabia strain (2021), Israel (2024), and Mali (2024); while all of them are close to the chosen root (China 2025), as the most recent and divergent sequence within all serotypes. Sequences that branch closer to the root are inferred to have diverged earlier; several key nodes across the tree exhibit high posterior support (100%), especially within recent clades such as the DENV-1 and DENV-2 Pakistani and regional EMRO strains, resulting in strong confidence in the inferred relationships and timing of divergence. Some older sequences, particularly those from 2013 to 2015 (e.g., KF041237.1, KM271158.1, KT239363.1, and KJ649286.1), exhibit long branch lengths and are positioned at greater genetic distances from the rest of their respective clades; these are associated with higher node heights such as 5.40, 6.67, or 9.52. In contrast, the 2023–2025 sequences cluster tightly within each serotype; for example, the DENV-2 strains from Pakistan (2022–2025), Somalia (2023), and the UAE (2024) form a close compact. In conclusion, the tree illustrates the temporal depth and contemporary connectivity of DENV evolution.

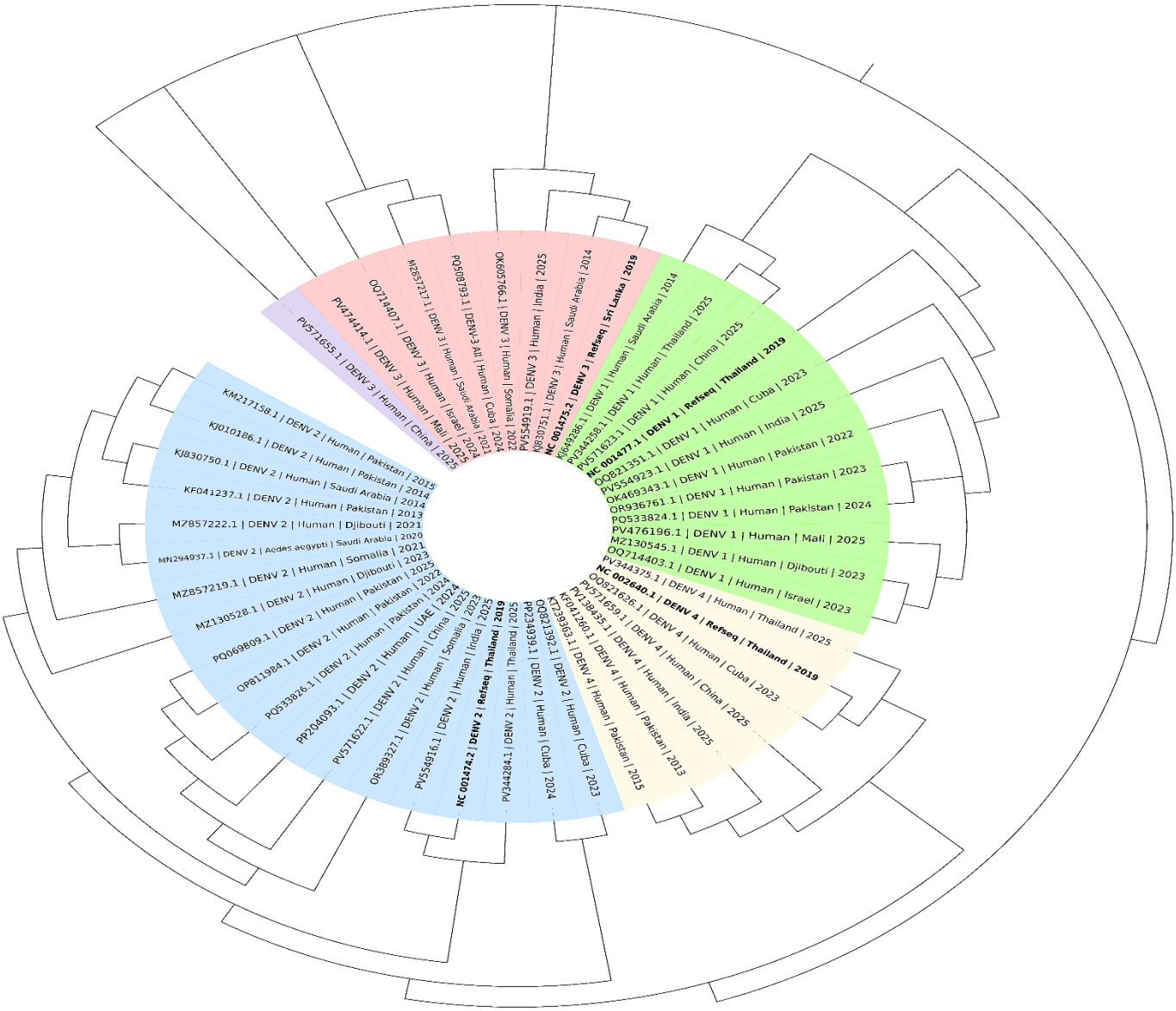


**Figure 3.** Inverted circular Neighbor-Joining phylogenetic tree based on full-length DENV envelope (E) protein amino acid sequences. Sequences are color-coded by serotype: DENV-1 (green), DENV-2 (blue), DENV-3 (red), DENV-4 (yellow), and purple (the outgroup), while reference sequences (RefSeq) are shown in bold. The tree reveals strong intra-serotype clustering, with recent strains from 2023–2025 forming tight, well-supported groups within each serotype.

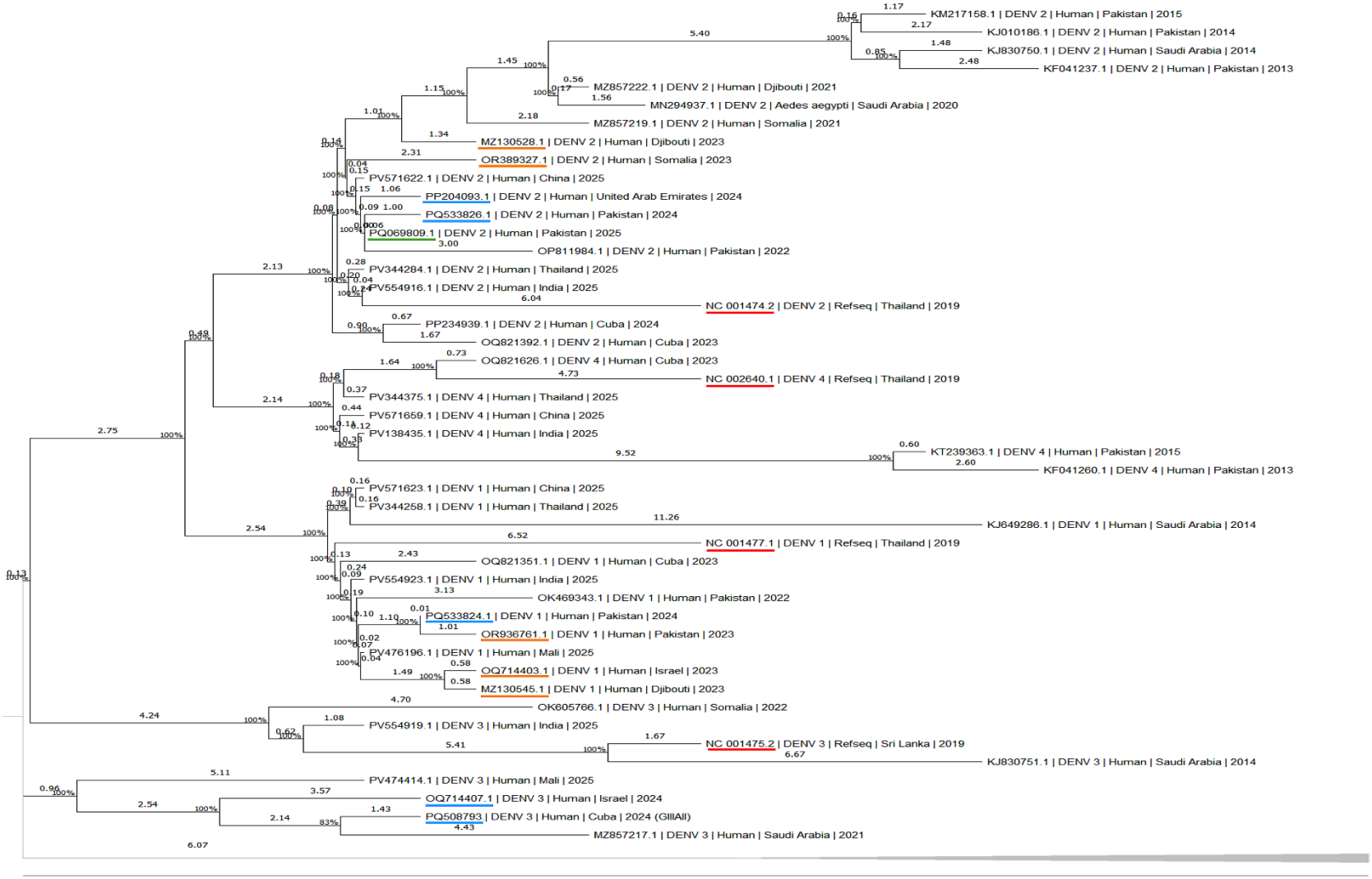


**Figure 4.** The time-calibrated phylogenetic tree of DENV envelope (E) protein sequences was inferred using the RelTime method in MEGA11. The tree is based on full-length amino acid sequences with 100% coverage, branch lengths representing relative divergence times, and node labels indicating substitution rate estimates. Reference sequences are underlined in red, while other underlined sequences are color-coded by year of collection from the EMRO region: orange for 2023, blue for 2024, and green for 2025.

### 2.2 Amino Acid Substitution Patterns in the Envelope Protein

**Heatmap of Substitution Severity.** The mutational profile for the DENV envelope protein was assessed by creating a comparison heat map for amino acid substitutions among various strains isolated in different geographic locations at various times, as indicated in Figure 5. This was undertaken using information in **Supplementary Table 1**, which required a manual comparison of aligned full-length E protein sequences, with each sequence examined amino acid by amino acid for substitutions relative to a corresponding reference strain, check Table 1 for the sequence data summary. The substitution severity was classified based on the type of alteration: if the change was to the same polarity or charge, it was classified as low. They were considered minor if the charge remained unchanged but significantly differed in chain size. If the change was in polarity, it was classified as either moderate or minimal, depending on the extent of the side chain change. Alterations with a massive difference in charge and polarity were labeled as high substitutes and could greatly influence the three-dimensional protein structure due to their potential effect on the interactions as well as the stability of the residue (12-14). The heatmap outcomes were normalized to facilitate color-scaled representation (0: none to 1: severe substitutions), allowing visualization of relative mutation severity across strains; this normalization approach was used for comparative visualization purposes and was based on physicochemical differences between amino acids. The most mutated were the latest strains of DENV from the EMRO countries of Pakistan, UAE, Djibouti, and Cuba (2023-2025), showing the most similar substitutions to those of the 2025 strains of India and China. These strains share unique changes or shared substitutions not observed in earlier isolations, as the reference and older isolates show broadly conserved amino acid profiles. For example, the DENV-2 of Pakistan 2025 and India 2025 had severe substitutions of R→T at amino acidic positions (120) and (149), while for China 2025 DENV-3, the E→A substitution was detected at amino acidic (406) also present in DENV of the EMRO countries of Saudi Arabia in 2021 and Israel in 2024; position 120 lies close to the fusion loop region of Domain II of the envelope protein, which is essential for membrane insertion and viral entry, whereas residues in the C-terminal stem region, including those near position 406, contribute to membrane fusion and virion assembly (15, 16). Nevertheless, the added Cuban GIIIAII strain (2024) also shared moderate-to-severe substitutions with EMRO and Asian isolates (A→T at 221 and E→A at 406).

**Table 1:** Summary of Dengue Virus Envelope Protein Sequences and Key Amino Acid Substitutions

| *Virus Type* | Accession Number | Country | Year | Severe Substitution | Moderate Substitution |
|---|---|---|---|---|---|
| *Dengue I* | NC001477.1 (Ref) | Thailand | 2019 | *Baseline* | *Baseline* |
| | KJ649286.1 | Saudi Arabia | 2014 | K203E | I293T |
| | MZ130545.1 | Djibouti | 2023 | D73N<br>K203E | T161I<br>I293T |
| | OQ714403.1 | Israel | 2023 | D73N<br>K203E | T161I<br>I293T |
| | PQ533824.1 | Pakistan | 2024 | D73N<br>K203E | T161I<br>I293T |
| | PV554923.1 | India | 2025 | K203E | T161I<br>I293T |
| | PV571623.1 | China | 2025 | K203E | I293T |
| | OQ821351.1 | Cuba | 2023 | D73N | T161I |

| | | | | K203E | I293T |
|---|---|---|---|---|---|
| Dengue II | NC001474.2 (Ref) | Thailand | 2019 | *Baseline* | *Baseline* |
| | MN294937.1 | Saudi Arabia | 2020 | E71A<br>R120T<br>H149N | |
| | MZ130528.1 | Djibouti | 2023 | E71A<br>R120T<br>H149N | |
| | PP204093.1 | UAE | 2024 | E71A<br>R120T<br>H149N | |
| | PQ069809.1 | Pakistan | 2025 | E71A<br>R120T<br>H149N | |
| | OR389327.1 | Somalia | 2023 | E71A<br>R120T<br>H149N | |
| | PV554916.1 | India | 2025 | E71A | |
| | PV571622.1 | China | 2025 | E71A<br>R120T<br>H149N | |
| | PP234939.1 | Cuba | 2024 | E71A<br>R120T | |
| Dengue III | NC001475.2 (Ref) | Sri Lanka | 2019 | *Baseline* | *Baseline* |
| | MZ857217.1 | Saudi Arabia | 2021 | E406A | A221T |
| | OQ714407.1 | Israel | 2024 | E406A | A221T |
| | KF041259.1 | Pakistan | 2013 | E406A | A221T |
| | OK605766.1 | Somalia | 2022 | E406A | A221T |
| | PV554919.1 | India | 2025 | E406A | - |
| | PV571655.1 | China | 2025 | E406A | A221T |
| | OQ821506.1 | Cuba | 2022 | E406A | A221T |

***Table 1:*** *A summary of the dengue virus envelope (E) protein sequences analyzed in this study, including GenBank accession numbers, country of origin, and year of isolation. The reference sequence (Ref) represents the baseline for comparison of amino acid substitutions. Severe (Sev) and moderate (Mod) effects indicate substitutions classified based on physicochemical differences in charge, polarity, and side-chain size; the complete mutation dataset is provided in Supplementary Table 1*

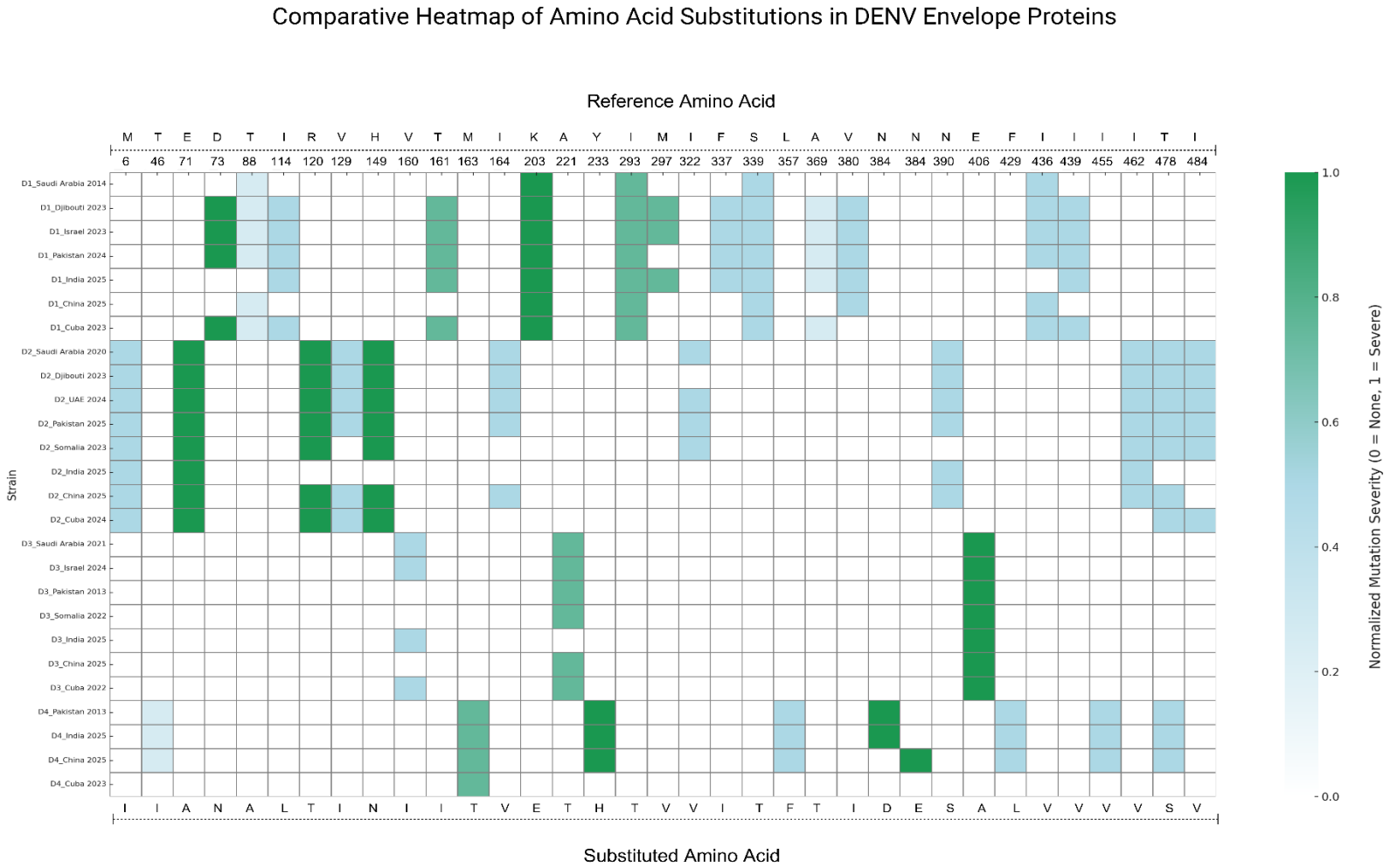


**Figure 5.** Comparative heatmap showing amino acid substitutions across DENV envelope (E) protein sequences from various global and EMRO strains. Reference amino acids are listed across the top X-axis, corresponding to positions in the E protein. In contrast, substituted amino acids are shown along the X-axis, with the chosen sequences on the Y-axis. Each cell indicates the presence and severity of substitution, with color intensity representing normalized mutation severity (0 = no change, 1 = severe). Severity classification was based on physicochemical differences between amino acids, including changes in polarity, charge, and side-chain size, where substitutions preserving polarity/charge were considered low severity and substitutions involving major polarity or charge changes were considered high severity, see **Supplementary Table 1** for detailed substitutions.

**Principal Component Analysis.** PCA was the final analysis to visualize DENV envelope protein variation patterns. The biplot in **Figure 6** describes the variability in PC1 and PC2. The PCA obtained four distinct and well-separated groups, and each group represented one of the serotypes of the virus (DENV-Type I to IV). The variants explained by PC1 was 36.08%, while PC2 was 27.19%, and the total captured by the first two components, PC1 + PC2 = 63.27%, explains the variation in envelope protein sequences, which is why the plot clearly separates the clusters. The isolates of DENV Type 1 (green circles in the upper left corner) have the highest coordinates in PC2, and the EMRO strains from 2023 to 2025 cluster together with the strains from India and China. Though the DENV-2 isolates (right cluster, brown triangle) were clumped together with less PC1 variation, hence low variability within DENV-2 isolates despite being geographically diverse, among them, more recent isolates such as UAE 2024, Pakistan 2025, and Somalia 2023, overlapped with other recent isolates, including India 2025 and Cuba 2024, which supported the heatmap findings. For the DENV-3 isolates (bottom-left quadrant, blue squares), they clumped together, with this cluster encompassing both old and new isolates. Interestingly, the Cuban GIIIAII strain (PQ508793.1, 2024) and some recent strains isolated in Israel and Mali were located towards the outskirts of the group. Likewise, the DENV-4 strains (pink cross-markings) were clustered together, relatively closely and compactly, yet separate from other types. Overall, the results of the PCA are consistent with previous findings from the heat map and phylogenetic analysis: recent strains, particularly those from EMRO countries, are geographically and temporally clumped.

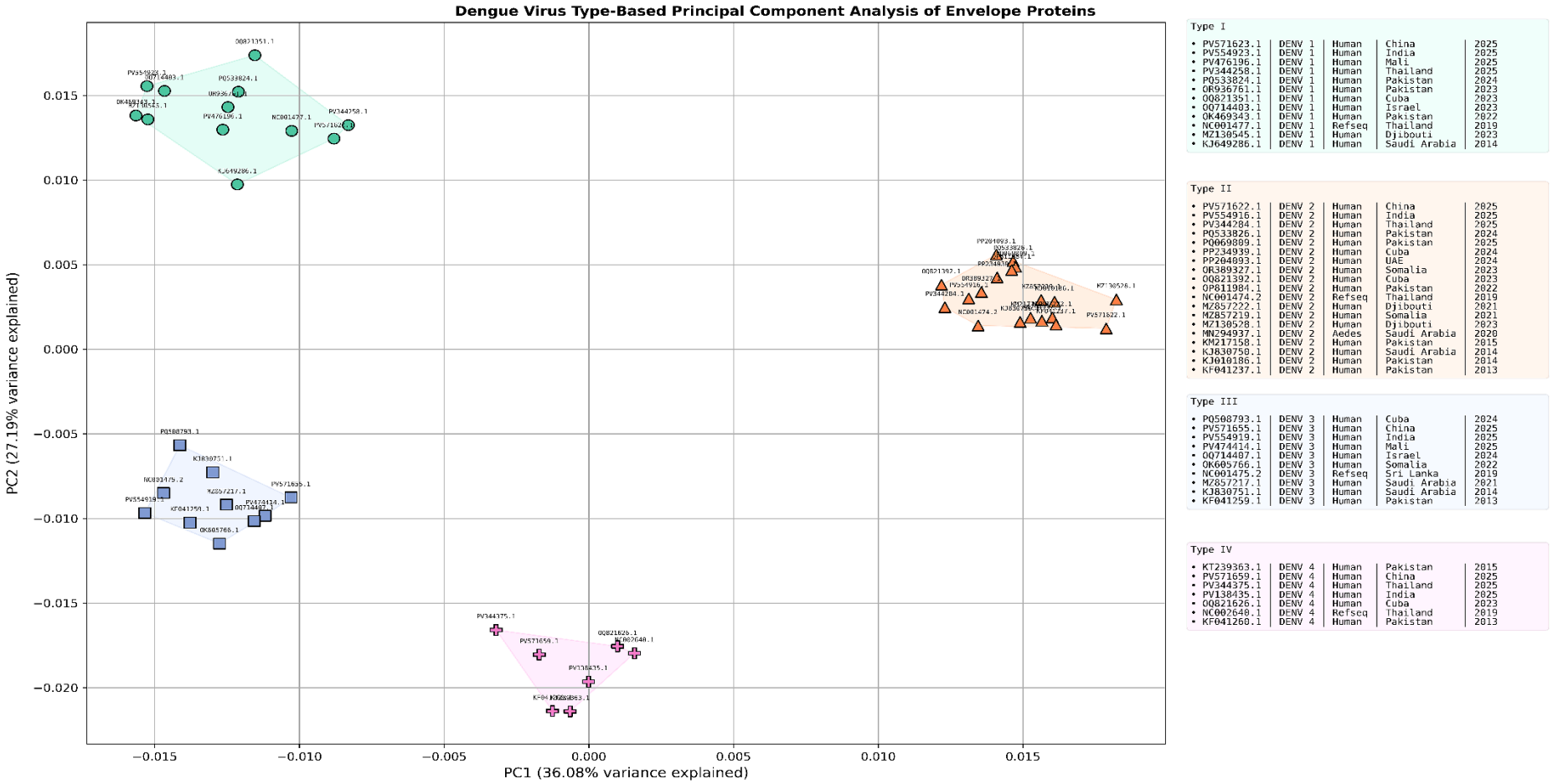


**Figure 6**. Shows a Principal Component Analysis (PCA) of full-length DENV envelope (E) protein amino acid sequences performed using a custom Python pipeline, with each point representing a unique viral strain, plotted based on its variation across two principal components, PC1 (explains 36.08% of the variants) and PC2 (explains 27.19% of the variants). The plot reveals four well-defined clusters corresponding to the four DENV serotypes: Type I (green circles), Type II (brown triangles), Type III (blue squares), and Type IV (pink crosses). The color codes are found in the legend boxes at the right list, which have strain metadata by serotype, including country and year of isolation.

## 3. Discussion

Comprehensive genomic data from the region remains limited and uneven, although the dengue virus has circulated in parts of the Middle East for over two decades. Studies like Hill et al. (2025) and Al-Eitan (2024) highlighted the challenges of monitoring mosquito-borne diseases (MBDs) in the region. Regional research, according to Hill et al., tends to be data-based upon sporadic outbreaks and a phylogenetic structure, thereby being unable to trace a distinct pattern for Arthropod-borne viruses (17, 18). In this analysis, it was found that Pakistan has the most up-to-date DENV sequence in the EMRO area, whereas the remaining countries have underrepresented sequences in public databases despite having confirmed cases, including Iran, Egypt, and Jordan—the issue poses a challenge for monitoring mutations within the virus and emerging strains in the region. First, we found that the DENV-2 sub-clade represents the greatest diversity in this area with newly found strains from Pakistan in 2023-2025 grouping together with strains from Somalia, UAE, and Djibouti aligning with existing data in the field, for example, genetic structure and distribution in Kenya of dengue virus-2, proving the exchange of genes between it and other countries through gene flow in East Africa (19). Research on *Aedes aegypti* mosquitoes in the Arabian Peninsula has shown that populations in Saudi Arabia originated from colonization by both the ancestral African form (*A. aegypti formosus*) and the global domestic form (*A. aegypti aegypti*), suggesting a viable transportation route for the dengue vector from Africa to the Arabian Peninsula (20). A 2024 study by Mashlawi et al. supported this by providing further genetic evidence of this dual origin, underscoring significant admixture between the two forms across different Saudi regions (21). Finally, a previous systematic review analysis from 2016 by Humphrey et al. discussed numerous reports that came from the Red Sea subregion and Pakistan, scoring the highest seroprevalence estimates for dengue, which indicated the significant virus circulation and potential for cross-border transmission in these areas (22). This research, together with our analysis results, supports the concept that East Africa and the Arabian Peninsula are intercontinental hubs for dengue movement. On a broader scale, integration of recent global sequences revealed no entirely new clades but rather a subtle shift in regional connectivity; note in the figure how the EMRO strains increasingly overlap with newer sequences from India, Cuba, and China 2024-2025, particularly in DENV-2 and DENV-3-meaning towards a possible recent transmission patterns and ongoing regional spread. Some India and China articles in 2025 support our observation of increased regional connectivity in dengue virus transmission, particularly involving DENV-2 and DENV-3 serotypes; for instance, India accounts for a substantial portion of the global dengue burden as the virus load is escalating due to the climate change; while China reported that dengue cases were found to be expanding and spreading across new area with imported cases primarily originating from Southeast Asian countries (23, 24). The inclusion of the Cuban DENV-3 sequences was classified under the emerging GIIIAII lineage in the Guzman et al. (2025) study, this new lineage, which was first detected in Cuba amid a spike in cases and supported by national molecular surveillance data, was shown to cluster closely with sequences from the USA, Puerto Rico, and notably, India (11). More importantly, the Cuban 2022 sequences exhibited close phylogenetic proximity to EMRO strains when placed in our broader phylogenetic framework, particularly the 2024 Israeli and 2021 Saudi Arabian isolates, indicating a possible shared transmission network or regional dissemination pattern not previously documented. In Saudi Arabia, a cross-sectional study by Melebari et. al. conducted between April 2023 and May 2024 in Makkah hospitals confirmed 238 dengue cases and found 11 unclassified samples that didn't fit into any dengue category, which highly supports the possibility of having novel or divergent strains (1). As of June 2025, there have been no reports of new dengue outbreaks or surveillance findings in Israel, aside from their previously reported imported cases, although some sequences have still been submitted. In addition, their most recent information from Israel came in October 2023,

when their Ministry of Health reported 30 dengue cases, all of which had traveled to other countries (25). The danger of possible local transmission in such occurrences is underscored, especially knowing that the presence of competent vectors in the region increases the danger of regional transmission to other neighboring nations.

In terms of protein analysis, the amino acid tree and analysis identified sequences with pivotal substitutions not present in their previous sequences (Figures 4, 5, and 6). As we mentioned previously, E protein sequence variations can affect the virus's fitness, tropism, and immune evasion (26-28). However, the present study did not experimentally validate the functional effects of the identified amino acid substitutions, and the findings should be interpreted as hypothesis-generating observations that require confirmation through functional and structural studies. From our analysis, we find significant patterns of amino acid substitution across the whole E protein sequences of recent DENV isolates, pointing to a recent rapid phase of molecular evolution that may have been driven by immune selection pressures or vector/host factors. Notably, these patterns are similar to or closely resemble those within the 2025 India/China E protein sequences, which may suggest convergent evolutionary processes or may reflect transmission association. On a more universal scale, it is clear through these findings, as well as those preceding and recent, that the role of antigenic drift, particularly for the E protein, persists as a non-neutralizing process, as shown both in vitro and vaccine models independently (29-32). Given this, our results highlight the need for continued surveillance of E protein variants and functional studies to assess the phenotypic consequences of these substitutions. Such evidence is provided by PCA, in which serotyping is more significant, whereas after 2023, these data show an association between the E protein in the Asia Pacific and those in EMRO, particularly for DENV-2 and DENV-3. Genetic drift is the second major cause of these mutations, and it determines the evolution of DENV; this selection pressure could continuously flip the shape of DENV sequences, leading to sudden outbreaks or the silent transmission of the disease (33). The emergent pattern analyzed may raise concern if a new form is silently spreading across regions due to travel, changes in vector populations driven by climate change, or reduced genomic surveillance. However, given the current data limitations, especially the lack of sequences from critical EMRO countries post-2022, we cannot yet confirm whether this represents a new regional lineage or coincidental parallel evolution; therefore, continued genomic surveillance, supported by broader sequence sharing and regional coordination, will be essential to clarify these trends and detect true emergent lineages before they result in major outbreaks.

## 4. Biosafety and Biorisk Management Recommendations

The lack of antivirals and effective medications against the dengue virus makes it a considerable biological threat; as it spreads worldwide, well-thought-out strategies must be implemented to lessen the danger (34, 35). Vector control remains a viable process to decrease the spread of the dengue virus, done by the use of insecticides such as larvicide and adulticide, insect repellent, and/or chemical agents, like picaridin and N, N-Diethyl-meta-toluamide, to keep waters sterile (34). Only Denvaxia has been granted licensing as a vaccine for dengue, yet it is still not recommended for use on patients with no prior dengue virus exposure; several initiatives are also being undertaken to develop an effective vaccine against dengue using techniques such as live-attenuated chimeric recombinant virus, live-attenuated virus, inactivated virus, recombinant protein, and mRNA vaccines. The National Institute of Allergy and Infectious Diseases' TV003/TV005 vaccine is currently going through the clinical trials phase (34, 36). The World Health Organization (WHO) created a multidisciplinary plan consisting of 5 core components divided into 11 pillars dubbed the 5Cs, focusing on emergency coordination to prevent future spreads and develop a robust way to mitigate risk; it also aims to create a collaboration in surveillance to control its spread. Further ensures that the laboratories have access to rapid and reliable tests for suspected dengue virus patients; it also includes community protection and access to safe and scalable care

and countermeasures (37). WHO also reported that the virus spread to Africa by travelers from Southeast Asia and Latin America; this led to the spread of the disease in highly dense urban areas which points out the need for more efficient travel surveillance and vector control (e.g., screening or educating travelers) (38).

## 5. Study Limitations

While our paper showed how the dengue virus's circulate in the EMRO region, there seems to be a notable lack of complete/partial genome sequences in many of its countries including Afghanistan, Bahrain, Egypt, Iran (Islamic Republic of Iran), Iraq, Israel, Jordan, Kuwait, Lebanon, Libya, Morocco, Oman, Qatar, Somalia, Sudan, Syrian Arab Republic, Tunisia, Yemen, and the Occupied Palestinian Territory; even though the WHO reported cases of dengue virus related infections in these countries. Despite this challenge, we tried to include a diverse list of countries from the EMRO region with several countries from other areas and continents for better resemblance, still a majority of our sequences came from Pakistan which could in turn, create a geographic bias that may result in the overrepresentation of the clusters found in Pakistan at the expense of other countries found in the region. Furthermore, a few recent sequences came from the Eastern Mediterranean, as only one sequence was found from 2025. In the protein-level study, only a handful of data points were represented in the viral envelope protein. Therefore, we focused on the envelope (E) protein because of its well-known roles in viral entry, immune system recognition of the virus, and its relevance to vaccines. Although the non-structural proteins NS1, NS2A, and NS4B are recognized for their importance in viral replication and immune modulation, they were excluded from the study to maintain the manuscript's focus; including them would have substantially extended the scope and complicated the analysis, potentially diluting the primary objective of tracking transmission dynamics through envelope protein variation. Although this study presents new information on the variation of dengue virus types in their sequences, further study using more complete genomic information from across the EMRO region could provide insights into dengue virus circulation within and beyond the region, which could aid in the prevention of outbreaks in the future.

## 6. Future Direction

The next steps should focus on expanding the dengue virus genomic database, especially in EMRO and surrounding regions that were underrepresented in the past. This would highlight the spread of the dengue virus globally and on its evolution over time, and its possible by developing a "centralized digital platform" like VectorSurv system-like centralized digital platform (37), where all EMRO countries can upload their sequence data, and this platform will automatically monitor mutation trends and generate dynamic risk assessments for vector-borne disease outbreaks. Functional analyses would also be required to evaluate the potential biological consequences of the most significant amino acid changes, particularly those occurring at conserved or immunologically important positions. Such research might include structural modeling studies, epitope analyses, and neutralization tests. Finally, a coordinated surveillance effort with standardized sequencing techniques would allow for real-time surveillance for new variants, which could help prevent dangerous outbreaks in the future.

## 7. Conclusion

This work offers a detailed and informative overview of the dengue virus (DENV) evolution in the Eastern Mediterranean Region (EMRO) and worldwide. Through the combination of genomics and protein-based analysis, we show that the latest DENV strains (2023-2025) in regions like Pakistan, the UAE, Djibouti, and Cuba display a considerable degree of genetic diversity while being closely related to the latest global strains. Phylogenetic trees based on the analysis of either the genetic and/or protein sequences clearly display the close grouping of the EMRO strains, in particular for DENV-2 and DENV-3; these patterns suggest regional and cross-continental transmission dynamics that are not always visible through traditional surveillance. Other methods, such as PCA clustering and time-dated Phylogenetic trees,

further supported that the newer strains represented the cutting edge of recent evolution in the case of the recent evolution of the virus. The entry of the Cuban GIIIAII strain and the identification of other unclassified serotypes in other countries clearly point to the incompleteness of the classification system and the need to have real-time genomic data, especially when the possibility of its undetected evolution increases due to high temperatures and vector populations. All together, these findings emphasize that without robust genomic data and coordinated surveillance across borders, the region may fail to detect or contain emerging variants early enough to intervene; however, given the current data limitations, especially the lack of sequences from critical EMRO countries, we cannot yet confirm whether this represents a new regional lineage or coincidental parallel evolution. Therefore, future work should prioritize standardized sequencing, regional data platforms and functional studies of key mutations to assess their impact on antigenicity and outbreak potential, allowing real-time tracking of emerging variants, and help mitigate the risk of dangerous future outbreaks.

## 8. Methods

### 8.1 Inclusion and Exclusion Criteria

To investigate dengue virus diversity and evolutionary trends in the Eastern Mediterranean Region, we accessed sequences from the NCBI Virus database, which is known for its high-quality and trustworthy genomic data (14). The following keywords were used to specify the dengue virus and its types: "Dengue virus", "Orthoflavivirus denguei", "Dengue virus type 1", "Dengue virus type 2", "Dengue virus type 3", "Dengue virus type 4", "Dengue virus 4", and "Dengue virus group", we obtained a total of 52,675 sequences; therefore, a set of filtration methods were followed to refine the dataset. First, we identified the EMRO region according to the World Health Organization (WHO) classification (17), and found 958 dengue sequences from Djibouti, Israel, Pakistan, Saudi Arabia, Somalia, and the United Arab Emirates. The sequence length parameter was set to 9,000 base pairs (bp) to take only complete and near-complete genomes between 10,000 and 11,000 (bp) to ensure analytical consistency and resolution across comparative analyses. Then, we selected sequences from 2013 onward, reflecting a critical period in dengue virus evolution following the emergence of the DENV-5 lineage first reported in 2013 (4). Sequences containing ambiguous nucleotides or large unresolved gaps were corrected or removed entirely. In rare cases where a key sequence could not be fully resolved but was critical for regional representation or lineage continuity, we substituted closely matched, high-quality sequences. For example, the Indian isolate (PV476196.1) was substituted with (OR259174.1), a cleaner representative from the same outbreak and genomic cluster. We also included the main 4 types of reference sequences (RefSeq) in the NCBI virus database, leading to 30 sequences to use. In addition, sequences from Cuba were included due to their role in the emergence of new dengue lineages, along with strains from India, China, Thailand, and Mali based on their geographic proximity and relevance to regional transmission dynamics; these additions help fill gaps caused by limited sequence availability across parts of the EMRO. Finally, a total of 47 sequences were used for further analysis. All sequences analyzed in this study were retrieved from publicly available records in the NCBI Virus database available at the time of data collection. The years reported throughout the manuscript (including 2025) refer to the collection year or database annotation associated with each sequence record. The final dataset used in this study is a retrospective comparative genomic study of the publicly available EMRO and non-EMRO strains used to illustrate the regional cluster findings.

### 8.2 Software and programs

Our results were produced using a range of software tools. MAFFT v7 was first used for sequence alignment to perform a general clean-up and organize the sequences before phylogenetic analysis, followed by re-alignment in MEGA11 using the MUSCLE algorithm with default parameters. We applied a gap open penalty of −400.00 and a gap extension of 0.00, with UPGMA used for clustering in both iterations; this approach ensured speed and accuracy for multiple sequence alignment across viral datasets (25, 39). All phylogenetic analyses

were performed in MEGA11, where the nucleotide tree was constructed using the Tamura-Nei model under the Neighbor-Joining method with 1000 bootstrap replicates; the model incorporated a gamma distribution (G = 5.0) to account for evolutionary rate variation among sites with pairwise deletion used to handle gaps (40, 41). Codon positions 1st, 2nd, 3rd, and noncoding regions were all included in the tree-building process. The dataset was trimmed for amino acid-based analyses to include only the envelope (E) protein, removing all non-relevant regions. The remaining sequences were codon-aligned using MUSCLE in MEGA11 under default protein alignment settings (gap open = −2.90; hydrophobicity multiplier = 1.20). The resulting amino acid phylogenetic tree was built using the Neighbor-Joining method with the p-distance model to ensure direct and straightforward evolutionary distance measurements that calculate the proportion of nucleotide differences between sequences (21), also using gamma distribution (G = 5.0) with gaps treated by pairwise deletion, and bootstrap analysis repeated 1000 times; trees were rooted using the outgroup method to clarify directional divergence across strains (42). Lastly, we generated a molecular clock tree using the RelTime method to evaluate divergence timing and possible temporal clustering. We chose the China 2025 sequence as the outgroup based on its clear phylogenetic distance from all other strains. Sampling years were extracted from sequence names and assigned as tip dates, allowing MEGA to infer divergence times concerning known collection points and enabling a temporal view of the virus evolution in the dataset (43, 44).

### 8.3 Protein Level Analysis

The same dataset used for the nucleotide phylogenetic tree was translated into amino acid sequences using MEGA11; however, we added a newly reported DENV-3 Cuba strain, suspected to represent a distinct lineage, to evaluate whether it showed any relation to sequences in our dataset. After translation, amino acid substitutions were manually extracted from the alignments and documented. Check Table 1 for a summary of the sequences used and Supplementary Table 1 for each substitution point that was labeled with its position and categorized by severity. The finalized FASTA file of the translated protein sequences was then used to generate a Principal Component Analysis (PCA) plot and a mutation severity heatmap, both created using Python pipelines. The custom Python workflow was used only for downstream visualization and comparative feature extraction after sequence preprocessing and alignment. Specifically, translated E protein FASTA sequences were converted into numerical feature vectors using a k-mer frequency–based encoding approach, which represents each sequence as a fixed-length numerical vector based on amino acid subsequence frequencies (45). Subsequently for the heatmap, the classification was based on physicochemical differences between amino acids, including polarity, charge, and side-chain properties, which are widely used indicators of the potential structural and functional impact of protein substitutions in computational protein analysis.

### *Abbreviations*

| | |
|---|---|
| **EMRO** | Eastern Mediterranean Region Office |
| **DENV** | Dengue Virus |
| **GIIIAII** | A novel genotype classification for DEI |
| **MBDs** | Mosquito-Borne Diseases |
| **BLAST** | Basic Local Alignment Search Tool |
| **PCA** | Principal Component Analysis |
| **ADE** | Antibody-Dependent Enhancement |
| **TV003/TV005** | Tetravalent Dengue Vaccine Candidate |
| **ICTV** | International Committee on Taxonomy |

**CRediT authorship contribution statement:**
**Laith N. AL-Eitan:** Writing – review & editing, Writing – original draft, Visualization, Validation, Supervision, Project administration, Methodology, Investigation, Funding acquisition, Conceptualization.

**Diana L. Almahdawi:** Writing – review & editing, Writing – original draft, Software, Resources, Methodology, Investigation, Formal analysis, Data curation.

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