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ORIGINAL ARTICLE

A Study of Genetic Diversity of Dengue Virus Circulating in Saudi Arabia

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ABSTRACT
Dengue fever causes significant morbidity and mortality globally including in Saudi Arabia. The genetic diversity of the dengue virus in the country has not been well characterized, which hinders the development of effective prevention and control strategies. A retrospective study of dengue virus isolates collected and sequenced in Saudi Arabia between 2000 and 2019. The whole genome sequence of DENV-1, 2, and 3 isolates was used to perform phylogenetic and conserved domain analyses to infer the evolutionary relationships and fitness among the viral strains. The DENV isolates could be divided into four distinct genotypes, with genotype IV being the most prevalent. Within each genotype, multiple sub-lineages diverged over time. The genetic diversity of DENV strains increased, with the emergence of a specific conserved domain in the DENV-1 isolate. Our study provides new insights into the genetic diversity of the dengue virus in Saudi Arabia. The findings suggest that ongoing surveillance and monitoring of dengue virus diversity and evolution in the region are necessary for effective management and containment of dengue fever. Additionally, the genetic characterization of dengue virus strains can lead to valuable understanding and knowledge of effective vaccines and antiviral therapies.
Keywords: *Dengue; Virus; Retrospective study; Genetic diversity; Saudi Arabia*

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INTRODUCTION

Dengue fever, a viral infection, is spread to humans by vector Aedes species mosquitoes. It is prevalent in tropical and subtropical areas [1]. Dengue fever typically begins with a sudden onset of high-grade fever, often reaching 104 °F (40 °C), usually accompanied by severe headache and body aches. A characteristic rash may appear on the skin during the early phase of the illness. It is usually a maculopapular rash, which means it consists of flat, red spots and raised bumps. Dengue fever often leads to fatigue and weakness, which can persist even after the acute phase of the illness has resolved. In some cases, dengue fever can cause bleeding manifestations, such as nosebleeds, bleeding gums, or easy bruising. These symptoms are more commonly observed in severe dengue cases [2]. Additionally, patients with dengue who recover without experiencing any serious consequences are still considered to have the disease, according to the updated WHO categorization system [3]. Dengue fever can vary in severity, from mild, resembling a flu-like illness to a severe life-threatening form known as dengue hemorrhagic fever (DHF). Severe dengue is characterized by plasma leakage, bleeding, and organ impairment [4]. DENV, a member of the Flavivirus family, is divided into four serotypes (DENV-1 to DENV-4) [5]. The most diverse of these serotypes is DENV-1, which is further categorised into five different genotypes: genotype I, genotype II, genotype III, genotype IV, and genotype V [6]. These genotypes are further broken down into four lineages (lineages I through lineages IV), with genotype I being one of them [7]. The single-stranded, +-sense RNA virus DENV has around 10,000–11,000 nucleotide bases long genome, which also includes 5′ and 3′ untranslated regions (UTRs) and a single open reading frame that codes for a polyprotein.

The polyprotein is made up of seven nonstructural proteins (NS1, NS2A, NS2B, NS3, NS4A, NS4B, and NS5) and three structural proteins (capsid [C], membrane [PrM/M], and envelope [E] [8, 9].
DF is an epidemic illness that tends to spread in tropical and subtropical regions including **S**outh America, southern Europe, Southeast Asia, the western Pacific, north Africa, Australia, the eastern Mediterranean, and the islands in the Indian Ocean [10]. The morbidity of DF has rapidly increased in recent years as a result of the spread of the DENV [11]. More than 100 nations have recorded DF instances, and each year, 390 million individuals worldwide contract the disease, including 96 million cases of severe dengue [12, 13]. Three different serotypes of DENV have been identified in Saudi Arabia; however, a robust phylogenetic analysis to compare the genetic diversity and viral fitness of DENV-1, 2**,** and 3 strains that have been prevalent in Saudi Arabia.

MATERIAL AND METHODS

Multiple Sequence alignment and phylogenetic analysis
The complete viral genome sequences of DENV-1 (GenBank accession number KJ649286.1), DENV-2 (GenBank accession number KJ830750.1; MN294937.1) and DENV-3 (GenBank accession number KJ830751.1) along with reference sequences of DENV-1 (GenBank accession number NC_001477.1), DENV-2 (GenBank accession number NC_001474.2), DENV-3 (GenBank accession number NC_001475.2) and DENV-4 (GenBank accession number NC_002640.1) were obtained from NCBI (Table 1 & 2).

Table 1: Dengue virus types included in the present study

Name	Accession	Base pairs	percent GC
Dengue virus type I	KJ649286_1	10622	46.63905
Dengue virus type 2	KJ830750_1	10718	46.03471
Dengue virus type 3	KJ830751_1	10635	46.56323
Dengue virus type 2	MN294937_1	10723	45.86403
Dengue virus type 2	NC_001474_1	10723	45.8174
Dengue virus type 3	NC_001475_1	10707	46.70776
Dengue virus type I	NC_001477_1	10735	46.66977
Dengue virus type 4	NC_002640_1	10649	47.1218

Table 2: GenBank accession of nucleotide and protein sequences of dengue virus types

GenBank Accession. Nucleotide	GenBank Accession. Protein
KJ649286.1	AIG59667.1[1..3392]
KJ830751.1	AIH13925.1[1..3390]
KJ830750.1	AIH13924.1[1..3391]
MN294937.1	QEV86381.1[1..3391]
NC_001477.1	NP_059433.1[1..3392]
NC_001474.2	NP_056776.2[1..3391]
NC_001475.2	YP_001621843.1[1..3390]
NC_002640.1	NP_073286.1[1..3387]

BLAST software (http://www.ncbi.nlm.nih.gov/BLAST/) was used to compare the whole genome of the DENV-1-3 circulating in Saudi Arabia. ClustalW was used to multiplex align the sequences, and the identity matrix of the nucleotide sequences as well as amino acid substitutions were examined. The MEGA 7.0 program was utilized for phylogenetic analysis and distance calculations employing the Neighbor-Joining method of the Maximum Composite Likelihood model, with gamma-distributed rates among sites. The analysis was performed with 1,000 bootstrap replicates to assess the robustness of the inferred phylogenetic relationships. Pairwise comparisons of nucleotide sequences were performed using the Genome-BLAST Distance Phylogeny (GBDP) method, as described by Meier-Kolthoff et al. (2013). The GBDP method, specifically designed for prokaryotic viruses, was employed with the settings recommended by Meier-Kolthoff and Göker (2017) [14, 15]. The GBDP analysis yielded intergenomic distances, which were then utilized to construct balanced minimum evolution trees. The trees were constructed separately for three different formulas: D0, D4, and D6. The branch support for each tree was inferred using FASTME with SPR postprocessing for each of the formulas D0, D4, and D6, respectively [16]. To assess the reliability of the inferred branches, 100 pseudo-bootstrap replicates were generated for each formula. To establish a root for the trees, the midpoint method proposed by Farris (1972) was

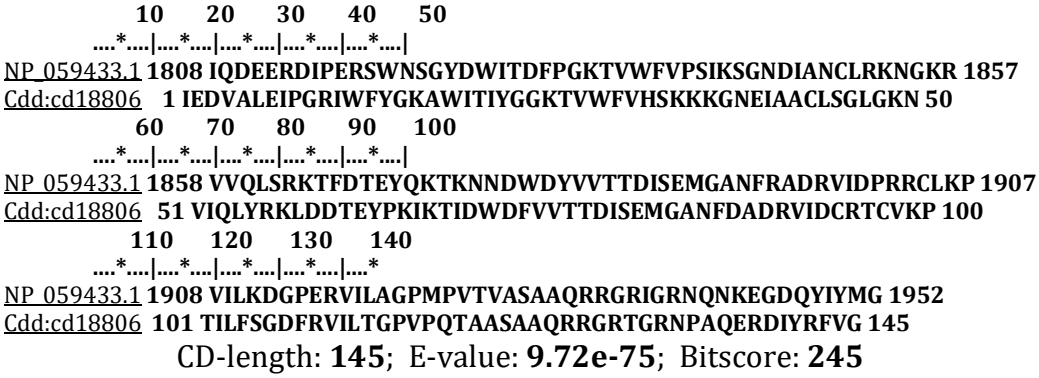
applied. The resulting trees were visualized using the tree software developed by Yu (2020) [17, 18]. To estimate taxon boundaries at various levels (species, genus, and family), the OPTSIL program developed by Göker et al. (2009) was employed. The recommended clustering thresholds provided by Meier-Kolthoff and Göker (2017) were utilized, along with an F value of 0.5 (fraction of links required for cluster fusion), as described by Meier-Kolthoff et al. (2014). This allowed for the delineation of taxonomic boundaries within the analyzed dataset [15, 19, 20].

Conserved Domain Analysis

To identify conserved domains in a protein, the analysis involved multiple sequence alignments. These alignments were performed using either a protein sequence in FASTA format or the GI or Accession of a protein sequence from the Entrez Protein database, serving as the query sequence. The CD-Search tool was utilized to conduct the domain analysis. The results obtained from CD-Search included concise and full displays, which provided valuable information about the identified domains. In both displays, colored bars were used to depict the domain footprints. Notably, these colored bars were interactive hotlinks that enabled users to access the corresponding CD summary pages. The CD summary pages contained detailed information about the identified domains. Moreover, they included the query sequence embedded within the multiple sequence alignment of proteins used to create the domain model. This allowed for a comprehensive understanding of the conserved domains and their distribution among related proteins. The Conserved Domain Database (CDD) served as the source for the domain information. CDD incorporates domains from various databases, including NCBI Curated Domains, NCBIfams, Pfam, SMART, COG, PRK, and TIGRFAMs. By leveraging these diverse domain repositories, the analysis aimed to capture a wide range of conserved domains in the protein of interest.

RESULTS

Phylogenomic GBDP (Genome BLAST Distance Phylogeny) trees inferred using the formulas D0 (**Figure 1**), D4 (**Figure 2**), and D6 (**Figure 3**) yielded average support of 51%, 100%, and 78%, respectively. The numbers above branches are GBDP pseudo-bootstrap support values from 100 replications. The branch lengths of the resulting VICTOR (VIral ClassificaTiOn Resource) trees are scaled in terms of the respective distance formula used (**Figure 4**). The OPTSIL clustering yielded three (D0), eight (D4), and four (D6) species clusters, respectively. At the genus level, one (D0), four (D4) and one (D6) clusters resulted, respectively. The number of clusters determined at the family level was one (D0), four (D4), and one (D6), respectively.



A specific conserved domain pfam00948, short name Flavi_NS1 accession 279316 was found in Flavivirus non-structural Protein NS1 from 777 to 1130 with an E value 0 and bitscore 728.761. The NS1 protein, found in flaviviruses, demonstrates a high degree of conservation across different members of the virus family. It possesses a characteristic feature of 12 cysteines within its structure. Similar to other NS proteins, NS1 also undergoes glycosylation, a process where sugar molecules are added to the protein.

Extensive mutational analyses have provided strong evidence suggesting the involvement of NS1 in the initial stages of RNA replication. These studies have highlighted the functional significance of NS1 in facilitating and regulating the replication process of viral RNA. By analyzing the effects of specific mutations within the NS1 protein, researchers have gained valuable insights into the functional domains and residues critical for its role in early RNA replication events.

However, this CD was only found in Dengue virus 1 isolate DENV-1-Jeddah, ACCESSION KJ649286, VERSION KJ649286.1


```
                10      20      30      40      50
          ....*....|....*....|....*....|....*....|....*....|
NP_059433.1   777 SGCVINWKGRELKCGSGIFVTNEVHTWTEQYKFQADSPKRLSAAIGKAWE 826
Cdd:pfam00948   1 QGCAINFGGRELKCGDGIFIFNDSDDWLEKYKFQADDPKKLAAAIGAAFE 50
                60      70      80      90      100
          ....*....|....*....|....*....|....*....|....*....|
NP_059433.1   827 EGVCGIRSATRLENIMWKQISNELNHILLENDMKFTVVVGDVSGILAQGK 876
Cdd:pfam00948  51 EGKCGINSADRLEHEMWKQIADEINAIFEENDMDFSVVVGDPKGILAQGK 100
                110     120     130     140     150
          ....*....|....*....|....*....|....*....|....*....|
NP_059433.1   877 KMIRPQPMEH-----KYSWKSWGKAKIIGADVQNTTFIIDGPNTPECPDN 921
Cdd:pfam00948 101 KMIRPHPFEHirdglKYGWKSWGKAKIFGADRKNGSFIIDGKNRKECPDN 150
                160     170     180     190     200
          ....*....|....*....|....*....|....*....|....*....|
NP_059433.1   922 QRAWNIWEVEDYGFGIFTTNIWLKLRDSYTQVCDHRLMSAAIKDSKAVHA 971
Cdd:pfam00948 151 NRAWNIFEIEDFGFGIFTTNIWLDARDEYTIDCDGRILGAAIKDKKAAHA 200
                210     220     230     240     250
          ....*....|....*....|....*....|....*....|....*....|
NP_059433.1   972 DMGYWIES-EKNETWKLARASFIEVKTCIWPKSHTLWSNGVLESEMIIPK 1020
Cdd:pfam00948 201 DMGFWIEShEKNETWKIARAEAIDVKECEWPKSHTIWGNGVEESEMFIPK 250
                260     270     280     290     300
          ....*....|....*....|....*....|....*....|....*....|
NP_059433.1  1021 IYGGPISQHNYRPGYFTQTAGPWHLGKLELDFDLCEGTTVVVDEHCGNRG 1070
Cdd:pfam00948 251 IIGGPISQHNHIPGYFTQTAGPWHLGKLELDFDACEGTSVIIDEHCDGRG 300
                310     320     330     340     350
          ....*....|....*....|....*....|....*....|....*....|
NP_059433.1  1071 PSLRTTTVTGKTIHEWCCRSCTLPPLRFKGEDGCWYGMEIRPVKEKEENL 1120
Cdd:pfam00948 301 KSLRSTTDSGKTIHEWCCRSCTLPPLRFHGEDGCWYGMEIRPRKEHEEHL 350
                360
          ....*....|
NP_059433.1  1121 VKSMVSAGSG 1130
Cdd:pfam00948 351 VKSMVSAGEG 360
```

Further phylogenetic analysis revealed that the DENV isolates from Saudi Arabia could be divided into three distinct genotypes (I-III). Within each genotype, multiple sub-lineages may diverge over time, indicating ongoing viral evolution. The evolutionary dynamics of DENV-1 in Saudi Arabia are characterized by the emergence of new sub-lineages and genetic mutations that are associated with changes in viral fitness and disease severity. Further, the study identified a novel Dengue virus 1 isolate DENV-1-Jeddah, ACCESSION KJ649286, VERSION KJ649286.1 harboring conserved domain pfam00948, Superfamily: cl03032 which was not found in any other DENV-2 or DENV-3 isolate.

DISCUSSION

Southeast Asia, the western Pacific, and America are the most afflicted regions globally, with dengue incidence rates increasing in recent decades [21]. Multiple sequence alignment (MSA) is a fundamental technique used in phylogenetic analysis to compare and align multiple sequences of DNA, RNA, or protein sequences. It is a crucial step in inferring the evolutionary relationships and constructing phylogenetic trees. The goal of MSA is to identify homologous positions among the sequences, which are believed to have descended from a common ancestor. By aligning these homologous positions, researchers can identify similarities and differences between sequences, which provide insights into their evolutionary relationships.

The ClustalW algorithm method and iterative method PSI-BLAST were used to perform MSA in the current study. These algorithms used different strategies to optimize the alignment based on maximizing sequence similarity or minimizing the number of gaps. DENV 1, 2, and 3 whole genome sequences from Saudi Arabia were used to generate MSA using maximum likelihood or Bayesian inference. These methods used the aligned sequences to estimate the evolutionary distances leading to the construction of a phylogenetic tree that represents the evolutionary relationships among the sequences. The present results provide a foundation for inferring evolutionary relationships and studying sequence conservation, functional motifs, and evolutionary constraints across related sequences.

The present study conducted Conserved Domain Analysis (CDA) utilizing multiple sequence alignments (MSA) of protein sequences derived from four whole DENV genomes from Saudi Arabia and Four

reference genomes of DENV 1 to 4 to identify and analyze conserved domains, a region within a protein sequence that exhibits a high degree of sequence similarity across different organisms or protein families [22]. These domains often correspond to functional units or structural motifs that are important for the protein's activity. A specific conserved domain pfam00948, short name Flavi_NS1 accession 279316 was found in Dengue virus 1 isolate DENV-1-Jeddah, ACCESSION KJ649286, VERSION KJ649286.1. The NS1 protein plays a crucial role in the replication and pathogenesis of flaviviruses. It is involved in various functions, including immune evasion, viral replication, and modulation of host cell responses. Due to its essential functions, the NS1 protein is under selective pressure to maintain its structural and functional integrity across different flaviviruses. The conservation of the NS1 protein implies that its amino acid sequence remains relatively unchanged or shows minimal variations among different flavivirus strains. This conservation is attributed to the importance of the NS1 protein in the virus life cycle and its interactions with the host immune system. The preservation of the NS1 protein sequence allows it to perform its functions efficiently across different flaviviruses. The conservation of the NS1 protein among flaviviruses also has implications for diagnostics, vaccine development, and therapeutic interventions. The presence of conserved regions within the NS1 protein can be exploited for the design of broad-spectrum diagnostic tests capable of detecting multiple flaviviruses. Furthermore, conserved epitopes or regions in the NS1 protein can be targeted for the development of vaccines or antiviral drugs that confer protection against multiple flavivirus infections [23].

Conserved Domain Analysis (CDA) is essential for understanding the molecular mechanisms of viral replication, pathogenesis, and host-virus interactions. More importantly, CDA can be used to probe evolutionary Relationships. Viruses evolve rapidly, and their genetic diversity can be vast. By comparing the conserved domains across different viral species or strains, CDA allows us to infer evolutionary relationships, in understanding viral evolution, transmission patterns, and the emergence of new viral strains.

An equally important application of CDA is the vaccine and Antiviral Target Discovery by identifying conserved regions crucial for viral replication or virulence. Presently found specific conserved domain pfam00948 (accession 279316) in nonstructural protein 1 (NS1) plays a crucial part in viral replication, and controls the host immunological response. By targeting such conserved domains, interventions that have broad-spectrum efficacy against multiple viral strains or species can be developed. CDA can also be utilized for the design of diagnostic assays to detect and identify viral infections. By targeting conserved regions, sensitive and specific tests that can detect a wide range of viral strains, even those with high genetic variability can be developed.

Fig. 1: VICTOR nt tree with suggested taxa (trimming, D0)

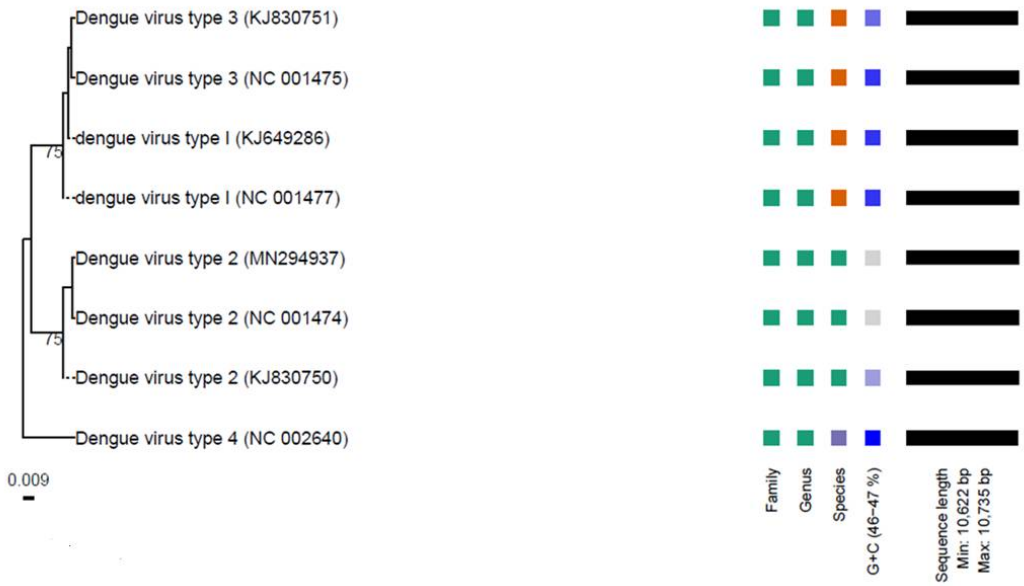


Fig. 2: VICTOR nt tree with suggested taxa (trimming, D4)

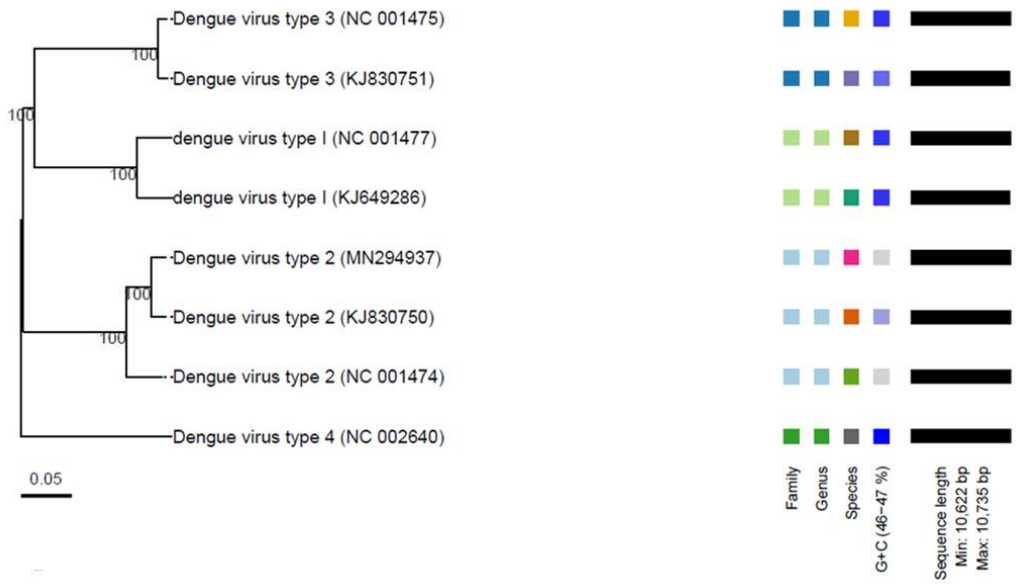


Fig. 3: VICTOR nt tree with suggested taxa (trimming, D6)

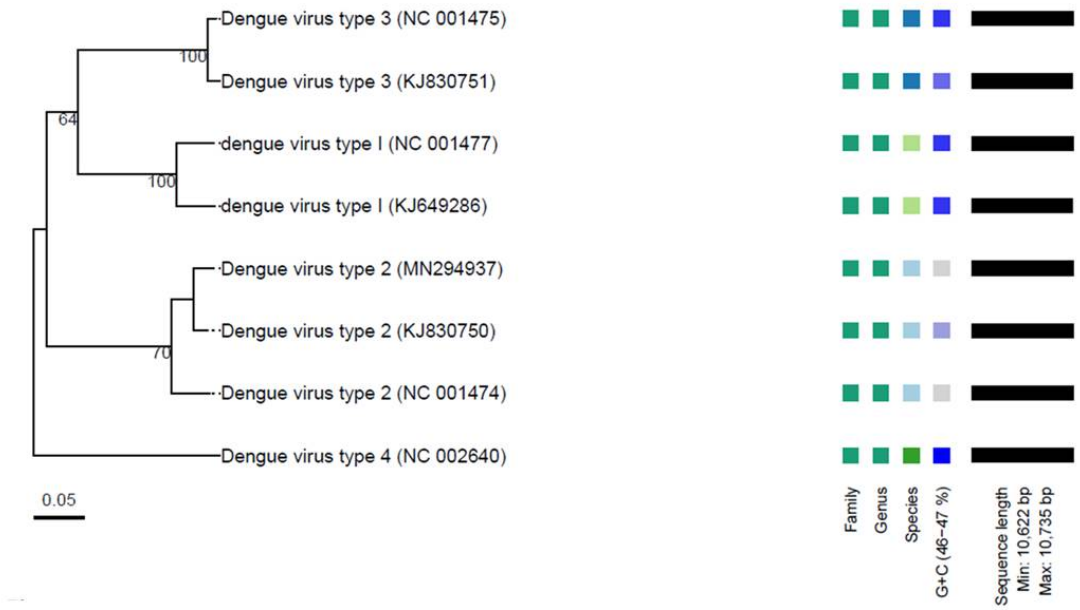
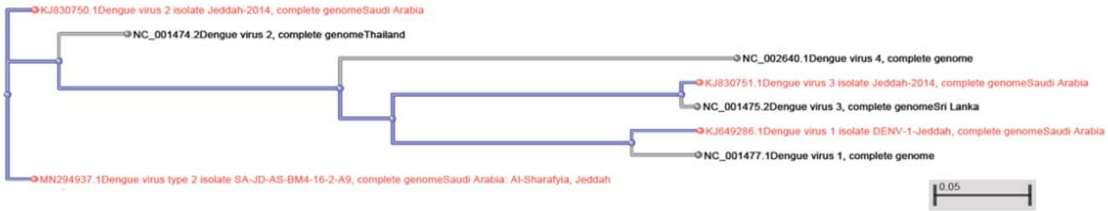


Fig. 4: The branch lengths of the resulting VICTOR trees in terms of the respective distance formula



CONCLUSION

The current study provides new insights into the genetic diversity and evolutionary dynamics of the dengue virus in Saudi Arabia. The findings suggest that ongoing surveillance and monitoring of dengue virus diversity and evolution in the region are necessary for effective prevention and control of dengue fever. Additionally, Conserved Domain Analysis using MSA plays a crucial role in virology by uncovering

important functional and structural aspects of viral proteins. It may lead to an improved understanding of viral evolution, identifying targets for intervention, developing diagnostics, and facilitating comparative genomics studies. Overall, the genetic characterization of dengue virus strains can provide valuable information for the development of effective vaccines and antiviral therapies.

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Conflict of interests: No conflict of interest related to this work

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