EVOLUTION AND VACCINE EFFICACY ANALYSIS OF HAEMAGGLUTININ 1 (HA1) DOMAIN OF INFLUENZA A VIRUSES THAT CIRCULATED IN KENYA BETWEEN THE 2007 AND 2013 SEASONS

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Evolution and Vaccine Efficacy Analysis of Haemagglutinin 1 (HA1) Domain of Influenza A Viruses that Circulated in Kenya Between the 2007 and 2013 Seasons

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DECLARATION

This thesis is my original work and has not been presented for an award a degree in any other University

Signature…….Date……

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DEDICATION

I dedicate this to My late father Thomas Nyang'au and my late grandfather David Onsarigo who always believed in me and thought me the values of education and hard work. May they continue resting in peace

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ABBREVIATIONS AND ACRONYMS

BEAST	Bayesian Evolutionary Analysis Sampling Trees
CDC	Centre for disease control
cRNA	complementary Ribonucleic acid
dN	Non-synonymous substitution
dS	Synonymous substitution
FEL	fixed effects likelihood
HA1	Haemagglutinin 1
HAI	Hemagglutinin Inhibition Assays
HRSV	Human respiratory syncytial virus
KEMRI	Kenya Medical Research Institute
MCMC	Markov Chain Monte Carlo method
mRNA	Messenger Ribonucleic acid
NA	Neuraminidase
NCIRS	National centre for immunization research and surveillance
NIC	National Influenza Centre
NS1	Non-structural protein 1
NS2	Non-structural protein 2

PA	Polymerase acidic protein
PB1	Polymerase basic protein 1
RNA	Ribonucleic acid
RNP	Ribonucleoproteins
SLAC	Single likelihood Ancestor counting
US	United States
VE	Vaccine efficacy
vRNA	viral ribonucleic acid
WHO	World health organization

ABSTRACT

The influenza virus is one of the most infectious and contagious pathogens in the world. The pathogens evolve rapidly causing regular seasonal epidemics in humans and other mammalian species and also posing a challenge to effective vaccination. The study sought to evaluate the evolutionary dynamics of the human influenza A viruses that circulated in Kenya just before, during, and after the 2009 pandemic. A/H3N2, A/sH1N1, and A/H1N1pdm09 HA1 nucleotide sequences, previously deposited in GenBank and GISAID genetic databases were retrieved including those of corresponding WHO influenza virus vaccine strain compositions. A total of A/H3N2 (N=122); A/sH1N1 (N=126) and A/H1N1pdm09 (N=176) Kenyan and global reference sequences virus isolates the three datasets were analysed using bioinformatics approaches. The Kenyan A/H3N2 viruses revealed mutations across the antigenic sites (A-E) whereas the A/sH1N1displayed mutations at the antigenic site Cb and Ca_2 and Sb. However, the mutations revealed by A/H1N1pdm09 did not occur at any designated antigenic site. Phylogenetic analysis showed that the A/H3N2- viruses clustered with A/Brisbane 10/2007 like, with a drift to A/Perth/16/2009 (H3N2)-like viruses in 2013. The majority of A/H1N1pdm09 (2009-2011) belonged to clade 7 while a minority of clade 5 and 6A and the Kenyan A/sH1N1 belonged to clade B. Vis-à-vis N-linked glycosylation all the isolates in the three categories retained all the potential N-linked glycosylation sites. The Kenyan A/H3N2 evolved mainly through purifying selection while the A/H1N1pdm09, and A/sH1N1 evolved under negative selection. The Kenyan A/H3N2, A/H1N1pdm09 & A/sH1N1 isolates shared tMRCA at 7.5 (95% HPD = 6.4-8.9), 5.9 (95% HPD = 4.7-7.1) and 2.7 (95% HPD = 2.3-3.2) years ago, respectively. The A/H3N2 revealed modest vaccine efficaciousness during 2008, and 2010 influenza seasons, whilst sub-optimal effectiveness was registered in 2007, 2009, 2012, and 2013. The A/H1N1pdm09 estimates indicate 80% effectiveness for the years 2009-2011 and the worst case for the subsequent year (below 40%,). Likewise, A/sH1N1 efficaciousness was modest in the 2008 season, declining in the following season (below45%). The mean evolutionary rate of the Kenyan A/H3N2, A/H1N1pdm09 and A/sH1N1 isolates was $4.6x10^{-3}$ (95% HPD = $3.0x10^{-3}$ to $6.3x10^{-3}$), $5.4x10^{-3}$ (95% HPD = $4.0x10^{-3}$ to $7.2x10^{-3}$) and $5.1x10^{-3}$ (95% HPD = $3.3x10^{-3}$ to $7.0x10^{-3}$) nucleotide substitutions per site per year, respectively.In conclusion, constant evolution and viral amino acid changes at the HA1 domain play a vital role in vaccine efficaciousness shaping the evolutionary dynamics of local influenza A viruses. Our findings underscore the importance and need for consistent surveillance and molecular characterization of influenza viruses, to inform decision-making and enhance early detection of strains with epidemic/pandemic potential as well as benefit in guiding decisions regarding the appropriate annual influenza vaccine formulation

CHAPTER ONE

INTRODUCTION

1.1 Background Information

Influenza, commonly known as "the flu" is an infectious disease caused by the influenza virus. This disease causes three to five million(3,000,000-5,000,000) morbidities and a quarter to half a million (250,000 to 500,000) mortalities annually (Cauchemez et al., 2012). The Influenza viruses are single-stranded, negative-sense RNA viruses belonging to the family *Orthomyxoviridae*. The family has four genera: Influenzavirus A, Influenzavirus B, Influenzavirus C, and Thogoto virus. Only the first three genera cause illness in humans while the last genera cause infections in cattle (Kochs *et al*., 2000). Among the genera causing infections in humans, Influenzavirus A exhibits the greatest genetic diversity and can infect a great number of animals causing the most severe form of the disease. This influenza A viruses have been characterized by some of the great pandemics in humans, birds, and other mammalian species (Blackburne, Hay, & Goldstein, 2008; Nelson & Holmes, 2007; Popova *et al*., 2012)

The segmented nature of the human influenza A virus genome and error-prone vRNA trigger two or more subtypes to infect the same host cell resulting in the "mixing" of disparate gene segments to constitute mosaic virus variants that have different constellations of the gene segments, a process called genetic re-assortment resulting to pandemics (Kilbourne, 2006). The first human influenza pandemic appeared in the 16th century and since then, 31 other pandemics have been reported globally. Over the last century, three influenzas A pandemics have occurred. These include the 1918 Spanish flu, the 1957 Asian flu, and the 1968 Hong Kong flu. In this century, the first influenza pandemic occurred in march 2009 (Dawood FS *et al*., 2009).

1.2 Human influenza pandemics

In the spring of 1918 Spanish flu pandemic appeared and caused an unusually severe and deadly influenza disease. The pandemic killed at least 50 million people surpassing by far those who died as a result of World War I, making it the worst pandemic in history(Taubenberger, K, Kash, & C, 2010). In recent years, the genetic material of the 1918 Spanish flu virus has been isolated from archived lung tissues fixed in formalin from influenza victims. This genetic material confirms the influenza A /H1N1 subtype was the cause of the deadly 1918 pandemic. Besides studies indicate that the A /H1N1was entirely avian-like that adapted to humans (Xiao *et al*., 2013).

Influenza A/H2N2 subtype was the cause of the pandemic of 1957 Asian flu. It was introduced by three antigenic shift events that involved the haemagglutinin gene (HA), RNA polymerase PB1 gene, and neuraminidase gene (NA) (Viboud et al., 2016). The pandemic was milder than the 1918 Spanish flu and it caused approximately 1-2 million deaths globally. Amongst these deaths, the majority were children and the elderly *(Viboud et al., 2016*).

The Hong Kong flu pandemic was caused by gene re-assortment. The reassortments lead to novel viruses with hitherto un-encountered antigenic epitopes due to "antigenic shift" In this process, two different influenza virus subtypes combined their surface antigens forming a new subtype of influenza virus with novel antigenicity (Blackburne *et al*., 2008; Daum *et al*., 2005; Kilbourne, 2006; Nelson & Holmes, 2007; Viboud *et al*., 2016). This pandemic was characterized by antigenic shifts in the HA and PB1 genes of A/H2N2 (Furuse et al., 2010). During this pandemic, it was observed that individuals above 65 years old did not severely suffer from the disease, due to pre-existing antibodies from the previous pandemic of 1957 (Furuse *et al*., 2010; J. K. Taubenberger & Morens, 2006; Viboud *et al*., 2016)

In April 2009 a new influenza A virus was reported by CDC. The virus contains gene segments from both North America and Eurasian swine lineages (Simonsen *et al*., 2013).

The 2009 H1N1 pandemic strain resulted from antigenic shifts from the seasonal human H1N1 subtype to the pandemic influenza A H1N1 2009 virus. This was the first influenza pandemic in the millennium, affecting more than 214 countries(Dawood FS *et al*., 2009; Hung, Yuen, Cheng, To, & Herman Tse, 2012). The centre for disease control (CDC) estimated that 43 million people had been infected with the pandemic H1N1 in the period between April 2009 and April 2010 with an estimate of 18300 H1N1-related deaths (Dawood FS *et al*., 2009; Furuse *et al*., 2010)

The adaptation behaviour of the viruses has been of great interest and genetic sequence data of the viruses have been used to study the virus evolution mechanisms. (Furuse *et al*., 2010). The influenza season in Kenya overlaps with the winter period in the Southern hemisphere and as a consequence, the Kenyan government through its Ministry of Health recommends the use of the Southern hemisphere WHO influenza vaccine formulation for her citizens (Bulimo, Mukunzi, *et al*., 2012; Majanja *et al*., 2013). However, the efficacy of the annually recommended WHO vaccines has been seen to be in a decline due to high mutation interplays of the influenza virus resulting in changes in the antigenic character of the major surface glycoprotein, the HA (Shao, Li, Goraya, Wang, & Chen, 2017).

1.3 Evolutionary forces of influenza A viruses

Influenza A virus evolutionary studies have been attributed to the interplay between natural selection, phylogeny, and epidemiology (Boni, 2008; Shao *et al*., 2017). In antigenic drift, the changes in the HA1 antigens of the virus enable the virus to evade host immunity and infect more hosts. In the antigenic shift, the HA1 antigens combine through re-assortment to form a novel HA that is new to the host immunity and co-infects the host cells. This type of evolution is the sole cause of human influenza pandemics (Boni, 2008)

In Kenya, studies have shown that circulating influenza viruses continue to evolve posing challenges to health by engendering pandemic potential. Previous studies have

concluded that the influenza A virus evolves by re-assortment leading to emergent and re-emergent variants that continue to pose a serious health threat to the country(Bulimo, Achilla, *et al*., 2012). This study will bridge this gap by analysing the evolution of human influenza A s/H1N1, A/H3N2, and A/H1N1/ pdm09 viruses that circulated in Kenya between 2007 and 2013

1.4 Problem Statement

Influenza disease is predominantly an infection of the human upper respiratory tract caused by a range of influenza viruses. Each year millions of people worldwide suffer from this highly contagious disease. Studies have indicated that 28,000-111,500 children below five years die from influenza-related illness globally, 99% of the deaths occur in Africa(Emukule, Paget, Velden, & Mott, 2015). This high mortality and morbidities have been a burden to Africa and developing countries. Influenza outbreaks are usually sudden and then spread through the population sometimes creating an epidemic (Shao *et al*., 2017). In Kenya, a tropical country, influenza is present throughout the year but with exacerbations at some times of the year. Influenza incidence tends to be highest in Kenya during the wet months: March-May, October-November, and the cold month of July(Majanja *et al*., 2013). Whereas viruses are genetically stable, influenza viruses evolve very rapidly and mutations in the HA1 gene through antigenic drift can lead to vaccine mismatches necessitating changes in the formulation of vaccines (Tewawong *et al*., 2015). The HA1 segment is the fastest evolving gene segment yet it plays a crucial part in the infection of the host cells given its role in viral attachment and evasion from host immunity rendering the vaccines less effective in protecting the population. The ever-present danger of evolution of these viruses (mostly antigenic drifts and shifts), necessitates routine monitoring and accurate understanding of these viruses to address the mortality, morbidity, and economic losses. In Kenya Complete understanding of the evolutionary dynamics of influenza viruses, HA1 has not been keenly analysed despite great advancements in influenza surveillance. It is, therefore, necessary to study the trends of the evolution of these viruses to understand them for preparedness regarding epidemic and pandemic threats posed by these viruses.

1.5 Justification

Whereas influenza surveillance has been ongoing in Kenya for more than ten years now, a comprehensive evolutionary analysis of influenza viruses identified has not been undertaken. Yet, such analyses are essential to understanding molecular events that may favour the pandemic potential of Kenyan influenza viruses, vaccine formulation appropriateness for the country, as well as the uniqueness of the influenza virus in the country. This information may be crucial to informing the provision of quality public health across the country of Kenya. The findings of the study will be supportive of healthcare stakeholders in understanding and devising appropriate public health policies regarding response to influenza in Kenya. Likewise, the study will support decisions involving the distribution and allocation of the limited resources for influenza programs in the country. Finally, the information forms a critical pillar in pandemic preparedness and the provision of health interventions such as vaccination to mitigate the effects of influenza disease. It is on this basis that this study seeks to understand the evolutionary changes of the viruses' HA1 gene in this crucial period in history (2007-2013) when three strains of influenza A viruses co-circulated in humans in Kenya. Furthermore, the study contributes to capacity building in the field of Molecular Virology and Bioinformatics in Kenya.

1.6 Research questions

i. What are the phylogenetic links of Kenyan influenza A viruses in successive seasons from the same geographical locations?
ii. Are there any discernible selection pressure indications amongst the Kenyan influenza A viruses? If so, what nature of selection pressure is observed over time?
iii. What is the nature of evolution amongst influenza A viruses circulating in Kenya?

iv. Were the WHO inactivated trivalent vaccines recommended for Kenya and the southern region effective for strains that circulated in Kenya just before, during, and post 2009 influenza pandemic?

1.7 Objectives

1.7.1 Main objective

To analyze the evolution and vaccine efficacy of human influenza A viruses that circulated in Kenya between 2007 and 2013 focusing on the HA1 gene.

1.7.2 Specific objectives.

i. To infer phylogenetic links of the Kenyan influenza A virus isolates against world reference strains.
ii. To determine the molecular evolutionary forces (natural selection) of human influenza A Kenyan virus.
iii. To evaluate the efficaciousness of the WHO-recommended vaccine for the Kenyan strains
iv. To estimate the evolutionary rates (tMRCA) of the Kenyan Human influenza A viruses

CHAPTER TWO

LITERATURE REVIEW

2.1 Genome organization of influenza viruses

The influenza virus's genome is approximately 13 kilobases (kb). The segmented genome consists of 8 separate RNA gene segments covered by the nucleo-capsid protein. The segmented genome encodes up to 14 different viral proteins. Segments 1, 4, 5, and 6 each encode a single protein: PB2, HA, NP and NA respectively (Dawson, Lazniewski, & Plewczynski, 2018), whereas segments 2, 3, 7, and 8 have the additional protein-coding capacity. Segments 2 and 3 are responsible for protein polymerase PB1 and PA respectively. Segments 2 and 3 also produce PB1-F2, PB1-N40, and PA-X proteins from a single mRNA species by leaky ribosomal scanning and translation termination- re-initiation (in the case of segment 2) and +1 ribosomal frameshifting (for segment 3). The proteins, PB1, PB2, and PA, are the largest and form a trimeric polymerase complex responsible for viral replication and transcription. The HA protein functions as a receptor that binds to the sialic acid of the host cell and induces the penetration of the virus particle by membrane fusion. NA cleaves sialic acid from the HA molecule and enables penetration of the virus through the mucin layer of the respiratory epithelium. In segments 7 and 8, protein-coding capacity is expanded by differential mRNA splicing (Shi, Lei, Zhu, Sievers, & Higgins, 2010; Wise *et al*., 2012). For segment 8, a single spliced species produces NS2/NEP, while NS1 is produced from the unspliced transcript. NS1 is responsible for exporting a poly-A containing mRNA molecules from the nucleus whereas NS2 mediates the nuclear export of virus RNAs by acting as an adaptor between viral ribonucleoprotein complexes and the nuclear export machinery of the cell, In addition it is associated with the transport of newly synthesized RNPs from the nucleus to the cytoplasm (O'Neill, Talon, & Palese, 1998). The Segment 7 mRNA splicing is highly complex, it contains three spliced transcripts denoted as mRNAs 2–4 and an additional unspliced mRNA1. The Unspliced mRNA1 gives rise to M1 protein while the spliced mRNA2 encodes the M2 ion channel protein. The spliced

mRNAs use a common 39-splice acceptor (SA) site, but a different 59-splice donor (SD) site. (Wise *et al*., 2011; Wise *et al*., 2012) A single mutation in the mRNA2 SD site leads to a mRNA4 which encodes M42 that functionally complements M2. The mRNA3 is produced from the most 59-proximal SD and negatively regulates segment 7 protein expression during early infection(Wise *et al*., 2012).

The surface glycoproteins hemagglutinin (HA) and neuraminidase (NA) are the most functionally important and the most widely researched proteins (Shi *et al*., 2010). Similarly, they form the basis of subtype classification on the antigenic properties. Different HA/NA combinations allow viral subtype combinations (Freidl *et al*., 2015; Latorre-Margalef *et al*., 2014). Currently, there are 18HA and 11NA gene variants of the influenza A virus whose combinations can lead to numerous subtypes (Freidl *et al*., 2015). The most common subtypes that infect humans include H1N1, H2N2, H3N2, H5N1, H7N2, H7N3, H7N7, H9N2, and H10N7. H1N1 and H3N2 have been the main circulating influenza A virus subtypes infecting humans (Iqbal, Yaqub, Reddy, & McCauley, 2009; Jagger *et al*., 2012; Wise *et al*., 2012), However, a new strain of influenza A /H1N1 viruses (the 2009 H1N1 pandemic) (A/H1N1 pdm09) emerged in the human population and caused a global outbreak in the year 2009 (Dawood FS *et al*., 2009). Since then, there's been a continuous circulation of the A /H1N1 pdm09 influenza strains displacing s/H1N1 influenza in many parts of the world (Majanja *et al*., 2013).

The HA and NA on the surface glycoproteins of influenza viruses represent the main immunogenic properties of the viruses. The former is the major target of neutralizing antibodies that has two sub-units HA1 and HA2 (Fan *et al*., 2015). The most evolving domain is the HA1 of the molecule. The HA1 subunit contains all the antigenic sites for the H1 and H3 subtypes (Fan *et al*., 2015). The H1 antigenic sites are denoted as Sa, Sb, and Ca whereas H3 antigenic sites are denoted as A, B, C, D, and E. The antigenic sites of the HA molecule of the influenza B virus occur within loops 120,150,160 and 190 helix (Sun *et al*., 2019). Mutations occurring at these antigenic positions in the HA1 globular head allow the virus to evade the host immune response (Popova *et al*., 2012).

2.2 Replication cycle of influenza A viruses

2.2.1Attachment, endocytosis, and uncoating of influenza A virus.

The surface glycoprotein HAO is essential for virus infectivity. The glycoprotein binds the sialic receptors on the host cell surface leading to the cleavage of HA to HA1 and HA2 peptides. The cleaved HA1 habours the receptor binding sites while the HA2 harbours the fusion peptide(Dou, Revol, Östbye, Wang, & Daniels, 2018). The haemagglutinin(HA) of influenza viruses has different specificity for sialic acid receptors. Human influenza recognizes the α-(2, 6 linked receptors and in avian species preferably bind to the-(2, 3) linked receptor molecule. In pigs, both types of receptors are extant (Greenwood, 2003) and for this cause, they are considered "mixing vessels" for avian and human influenza viruses (Dou et al., 2018; Ma, Kahn, Richt, & research, 2009). After attachment of the viral HA to the specific receptors, the viruses enter the host cells by receptor-mediated endocytosis. The virus HA contains a fusion peptide which at a low pH, undergoes a conformational change to expose the peptide. The M2 ion channel then opens up and causes an influx of H+ ions. The acidic environment triggers a conformational change of the HA in which the HA2 fusion peptide is exposed causing endocytosis between viral and endosomal membranes resulting in to release of vRNP into the cytoplasm of host cells. The acidic pH dissociates the M1 protein is also from the vRNP(Dou *et al*., 2018)

2.2.2 Nuclear import of viral ribonucleoproteins in host cells

The replication and transcription of viral ribonucleoproteins take place in the nucleus. The vRNP transfer to the nucleus following the fusion event is vastly dependent on the host cell machinery and transport pathways(Dou *et al*., 2018; Eisfeld, Neumann, & Kawaoka, 2015). The newly released cytoplasmic vRNPs use the importin-α–importin-β nuclear import pathway to gain entry to the host cell nucleoplasm. To engage this pathway, the vRNPs use the surface exposed nuclear localization sequences from the numerous NP molecules to recruit the adapter protein importin-α(Wang, Palese, &

O'Neill, 1997; Wu, Weaver, & Panté, 2007). Upon binding to the vRNP, importin-α is recognized by the importin-β transport receptor, which directs the vRNP to the nuclear pore complex, where it is transported into the nucleoplasm(Dou *et al*., 2018)

2.2.3 Viral RNA replication and transcription

In the nucleus, the heterotrimeric viral RNA-dependent RNA polymerase carries out the transcription and replication of the vRNAs (York & Fodor, 2013). The genome of the influenza A virus is organized in a negative sense manner, thus the viral RNAs have to be converted into positive sense before they can be transcribed into viral mRNAs. The complementary RNA(cRNAs)are produced by an unprimed process that relies on the correct complementation of free GTP and ATP (Dou *et al*., 2018; Eisfeld *et al*., 2015). The mechanism of viral RNA transcription occurs uniquely. First, the 5' cap from cellular mRNAs is cleaved by a viral endonuclease and used as a primer for transcription by the viral transcriptase. This is followed by transcribing six of eight RNA segments into mRNAs in a monocistronic manner followed by a translation into HA, NA, NP, PB1, PB2, and PA. Contrarily two RNA segments are each transcribed to two mRNAs by splicing. For the M and NS genes, mRNAs are translated in dissimilar reading frames, generating M1 and M2 proteins and NS1 and NS2 proteins, respectively (Webster *et al*., 1992). (Dou *et al*., 2018)

2.2.4 Viral ribonucleoprotein export from the nucleus to cytoplasm in host cells

The synthesized viral mRNAs are then exported to the cytoplasm of the infected cells for them to be translated into viral proteins. Subsequently, the viral mRNAs are incorporated with the polymerase complexes to form vRNPs, viral mRNAs are exported to the cytoplasm with the help of the nuclear export machinery found on vRNPs (Dou *et al*., 2018; York & Fodor, 2013). The nuclear export of vRNPs is mediated through the cellular Crm1/Exportin pathway and two viral proteins, M1 and NS2 (Watanabe *et al*., 2010). The M1 protein facilitates the nuclear export by forming a complex with vRNP in the nucleus of host cells. The NS2 protein, also known as nuclear export protein (NEP)

for the presence of a nuclear export signal (NES) in the protein, associates with the M1-vRNP complex and the cellular Crm1 to mediate the export of vRNP to the cytoplasm (Eisfeld *et al*., 2015)

2.2.5 Virus assembly and budding

The vRNPs and viral proteins synthesized will be localized around the basal membrane, and the viral progeny particles are ready to be packaged and bud off from the host cell. Largely, a viral core of vRNPs becomes trapped in a layer of M1 proteins and buds outward through the cell membrane (Eisfeld *et al*., 2015), enclosing itself within a bubble of the membrane as its envelope, complete with the viral surface glycoproteins(Eisfeld et al., 2015) Interactions between M1 and the cytoplasmic domains of HA, NA or M2 have been projected as signals for budding and NA activity of progeny virions releasing them from the host cell. When the budding process is complete, HA still binds to the sialic acid on the cell surface until virus particles are released by the sialidase activity of NA protein which is anchored to the viral envelope by a transmembrane domain. NA cleaves the terminal sialic acid residues from cell-surface glycoproteins and gangliosides to release progeny virus from the host cell. NA could also remove sialic acid residues from the virus envelope itself, preventing viral particles from self-aggregation, to enhance the viral infectivity (Dou *et al*., 2018)

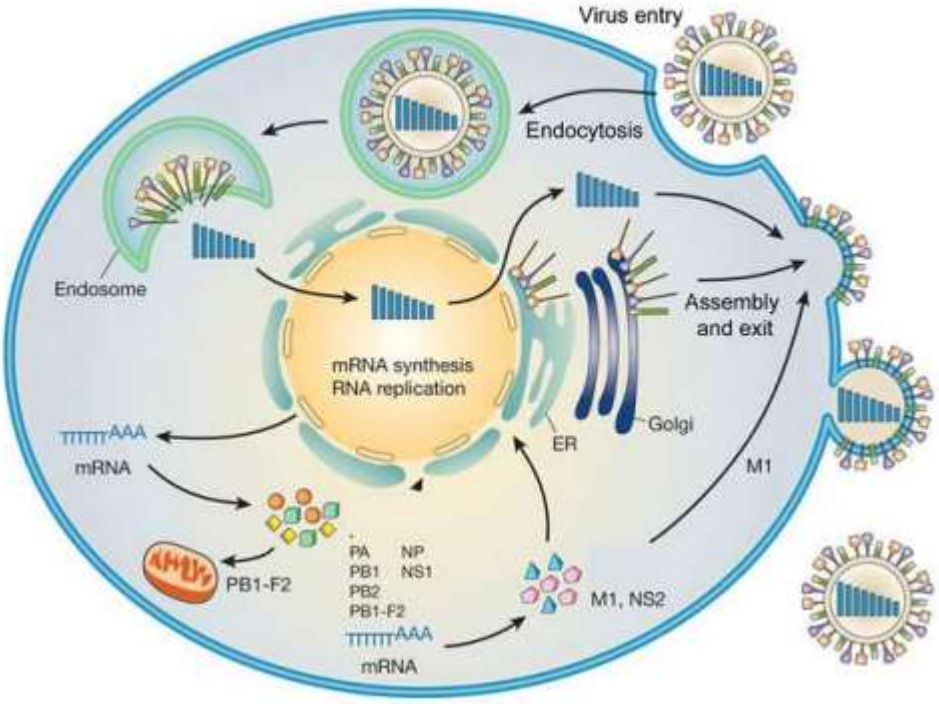

Figure 2.1: An illustration of influenza A virus replication (Neumann et al., 2009)

2.2.6 Transmission and pathogenesis

Transmission of influenza in humans involves both respiratory infections by aerosols and droplets together with some contact transmission from contaminated surfaces. Additionally, several incidents of transmission of influenza viruses from pigs to humans have been described in the past years. After influenza viruses infect pigs, horses, humans, or birds, the method of spread of influenza within the population is mainly inhalation of respiratory droplets containing the virus. Less often, the virus is spread by person-to-person contact or contact with contaminated items.

The pathogenicity of influenza viruses is complicated and may involve viral, host, and environmental factors. Inhaled virus particles are deposited on the mucous membranes lining the respiratory tract or directly to the alveoli. The major site of infection is the

ciliated columnar epithelial cell. The first alteration is the disappearance of the elongated form of these cells which becomes round and swollen. At this stage, the nucleus becomes shrunk. A vacuole of the cytoplasm is formed and the nucleus starts to break. The release of the virus from the cell allows it to spread via the mucus blanket to other areas of the respiratory tract. The cell damage causes an acute inflammatory response with edema and the attraction of phagocytic cells. The earliest response is the synthesis and release of interferons from the infected cells.

2.3 Influenza vaccine; success and continuing challenges

Influenza vaccines have been available and used for over 80 years. These vaccines have contributed greatly to reducing influenza morbidity and mortality. However, there are shortcomings in their effectiveness and efficacy. The efficaciousness of the vaccines has been affected due to the continuous antigenic evolution of seasonal influenza viruses. The principal use of embryonated chicken eggs for vaccine production has restrained the effectiveness of the vaccines(Becker, Elbahesh, Reperant, Rimmelzwaan, & Osterhaus, 2021).

It is estimated that 2%–10% of vaccinated healthy humans do not produce adequate levels of antibodies following vaccination. This is due to their genetic characteristics for instance human leukocyte antigen [HLA] type or single-nucleotide polymorphisms. Vaccines efficacies can also be influenced negatively by lifestyle (eg, stress, nutritional deficiency, obesity, and previous contact with closely related viruses (Osterholm, Kelley, Sommer, & Belongia, 2012). Changes in the immune system triggered by comorbidities like diabetes, immunosuppression, or medication can further weaken the immune response to vaccines.

With these limitations, alternative approaches for vaccine design and production are increasingly pursued with already licensed quadrivalent seasonal influenza vaccines produced in cell cultures. Next-generation influenza vaccines aim at inducing broader and longer-lasting immune responses to overcome seasonal influenza virus antigenic

drift and to timely address the emergence of a new pandemic influenza virus (Becker *et al*., 2021; Osterholm *et al*., 2012). The use of Adjuvants has been recorded to improve the vaccine response by enhancing and modulating the immune response. Adjuvants act through different mechanisms; they can create an antigen depot, activate the innate immune response, induce inflammasomes and cytokines, recruit immune cells, improve antigen uptake, enhance immune cell maturation, or change the activation profile of adaptive immune cells (Elbahesh, Gerlach, Saletti, & Rimmelzwaan, 2019).

Kinases are other modifiers that have been investigated for intervention strategies against influenza viruses. Host kinases regulate influenza virus entry and replication and form integral components of various antiviral and inflammatory pathways (Becker *et al*., 2021; Meineke, Rimmelzwaan, & Elbahesh, 2019).

2.4 Phylogenetic Links between Viruses in Successive Seasons

The evolution of influenza viruses has become a big block in predicting the current and future epidemics and pandemics. Phylogenetic trees have been used to establish the molecular diversity characteristics of circulating strains of influenza viruses and how the genetic diversity of an epidemic relates to the observed epidemiology each season (Nelson *et al*., 2014). Antigenic drift is the mutation process that is responsible for the epidemics whereby the virus undergoes point mutations so that it can evade the immune response of the host. The strains of H1N1 and H3N2 influenza virus that have been in circulation in the past few years have been mutating and they are responsible for the seasonal flu epidemics. In contrast, pandemics of these viruses occur as a result of antigenic shift that results from gene segment re-assortment within the virus to the form to which the immune system of the host has not had exposure previously.

Influenza viruses undergo continuous evolution to form new strains that may not be recognized by the immune system of the host. The application of phylogenetics has been used to illustrate that related species are somehow ecologically similar. This has been applied to influenza viruses from a distinct geographical region (Satheeshkumar, 2011).

This is to say that viruses from a particular region may exhibit some phylogenetic similarities under positive selection pressure. The phylogenetic analysis compares the relationship that exists between strains from different seasons. Adaptive evolution has become a topic of interest and has elicited awareness in Darwin's work. Ecologists are increasingly agreeing with Darwin that closely related species tend to be ecologically similar. Sequencing of the HA1 gene can be performed to analyse how strains from one season are related to strains from the previous season thus knowing the link between the seasons' strains. This analysis can be done with data that is stored in GenBank http://www.ncbi.nlm.nih.gov/genomes/FLU/aboutdatabase.html).

The sequencing of the HA gene from circulating strains has been used to show that the changes that take place at the distinct antigenic sites result in the emergence of new variant strains. These variants can cause severe outbreaks, especially in places where there is reduced efficacy of the influenza vaccine(Michael W Deem, Pan, & Selection, 2009; Naumova, Lofgren, Fefferman, Naumov, & Gorski, 2007). The evolutionary rates of the virus also happen at some consistent intervals in some particular regions. For example, in temperate regions, the evolutionary rate of the virus is found to take place at consistent intervals of 6 months. This is because the epidemics occur at an interval of 6 months. The case is however different for tropical regions which have a more variable epidemic periodicity (Satheeshkumar, 2011). Influenza virus activities are found to peak at similar times in regions at similar latitudes at particular seasons. This shows some relatedness in viral behaviour. It has been established that as viruses evolve, they also spread across geographical locations. In temperate regions, influenza epidemics usually occur in winter, while in the tropical regions, influenza epidemics are witnessed all year round (Rambaut *et al*., 2008)This has been used to imply that the environment plays a role in the evolution of viruses. There is a possibility that the environment influences the adaptive evolution of influenza viruses (Satheeshkumar, 2011).

2.4 Selection Signatures acting on codons/amino acid residues

Detecting the selection type that may have acted on genes/polypeptides is an essential step in the evolutionary analysis. Selection working on protein-encoding genes can be investigated in terms of the nonsynonymous and synonymous substitution rates occurring at the codon level (Duvvuri *et al.*, 2009). The substitutions that induce changes in the code residues are termed Nonsynonymous substitutions, whereas synonymous substitutions do not induce any residue modifications. Selection pressures shape the evolution of protein-encoding genes through purifying or conservative, neutral, and positive or diversifying selections (Kryazhimskiy & Plotkin, 2008) Determining the selection pressures that shape genetic variation is an integral part of many studies involving molecular evolution. When the estimates of nonsynonymous (dN) are significantly different from synonymous (dS), they provide convincing evidence for non-neutral evolution. Thereby estimating the rates of dN and dS substitution is attractive as it does not make any assumptions regarding the demographic history of the population (Kosakovsky Pond, Frost, & evolution, 2005)

The nonsynonymous (dN) to synonymous substitution (dS) rate ratio ω (=dN/dS) is used to differentiate the three selection types. In Purifying selection the change of an amino acid residue at a given position is prevented in multiple alignments, to favour an excess of synonymous versus nonsynonymous substitutions. As a result, ω will have values <1; the lower the value, the higher the purifying selection acting on the residue. In regards to Neutral selection synonymous versus nonsynonymous substitutions gives a value ω that is approximately close to 1, this selection type is mainly driven by mutation and genetic drifts. Positive selection is an excess of nonsynonymous versus synonymous substitutions with a change of residue at a given position. This implies that ω will be >1; the higher the values, the stronger the positive selection acting on the proteins.

During the antigenic drift process, the influenza virus undergoes positive selection to select advantageous amino acids thus avoiding the host immune response. This positive selection process is vital and influences the host in building up immunity. The host

system builds specific antibodies to the surface of HA1 of the virus with the strongest antibody build-up occurring at the first infection with the first variant. During the replication of the HA gene, the RNA polymerase makes errors resulting in the mutation of the antigens of HA (Bulimo, Achilla, et al., 2012; Ebranati et al., 2015; Kryazhimskiy & Plotkin, 2008).

It is expected that dN and dS should occur at the same rate when there are no restrictions on HA proteins such that at the codon positions where dN/dS (ω) =1 indicates that there is a neutral selection. At positions where amino acid alterations lead to lower fitness and dN accumulates at a slower rate, the ratio will be less than one ($\omega < 1$) indicating negative selection. In such cases, the purifying selection can be so strong that there is no room for amino acid changes and the ratio stand at zero ($\omega = 0$). In positive selection, the rate of dN is higher than dS making the ratio greater than one ($\omega > 1$). Therefore, comparisons of fixation of the types of substitutions are important in understanding the mechanisms of gene evolutions (Guéguen & Duret, 2017; Kryazhimskiy & Plotkin, 2008)

2.5 Seasonality of Influenza Viral Infections

Influenza viral infections are seasonal in temperate regions while they occur all year round in the tropical regions. In temperate regions, influenza epidemics usually happen in winter, from November to March in the northern hemisphere, and also between May and September in the southern hemisphere (Nelson & Holmes, 2007). For some time, influenza viruses have been considered to be "cold weather" pathogens. However, the tropics are considered to be permanent mixing pools of viruses from around the world; southeast Asia is known as the epicenter for the emergence of 18 pandemic viruses (Nelson, Simonsen, Viboud, Miller, & Holmes, 2007).

The reasons for the winter seasonality of the influenza virus in the temperate zones are yet to be identified. However, some theories have been proposed to explain this phenomenon. The first hypothesis is that the observed seasons of virus activities are due

to decreased host immunity rather than an outbreak of infection sweeping through the population (Satheeshkumar, 2011). Changes in biological factors contribute to decreased immunity as well as behavioral changes like crowding and the tendency to stay indoors. Tropical regions, on the other hand, are considered ideal source populations for selection pressures due to extended periods of viral transmissions in this population (Rambaut *et al*., 2008)

2.5 Vaccine efficacy Estimation

Influenza remains a serious respiratory disease despite the availability of antivirals and inactivated quadrivalent vaccines. The influenza Vaccines have been recommended as an effective approach to reducing morbidities and mortalities, however, these vaccines need to be updated annually due to antigenic drift (Skowronski *et al*., 2014). The selection of these vaccines depends on surveillance information, hemagglutinin inhibition assays (HAI), and HA and NA gene sequence phylogeny (Michael W Deem *et al*., 2009; Guéguen & Duret, 2017; Staneková & Varečková, 2010). Presently, the WHO-recommended human influenza vaccines contain HA components of A(H1N1)pdm09, A/H3N2, and one lineage of type B (trivalent vaccine) or both lineages of type B viruses (quadrivalent vaccine) that match a great extent antigenic properties of circulating influenza virus strains (Tewawong *et al*., 2015). However, high mutation rates associated with these viruses necessitate not only annual vaccine re-formulation but also limit influenza virus vaccine efficaciousness to about 50% to 60% (Tewawong *et al*., 2015). Influenza A/H3N2 virus HA1 domain contains five major antigenic sites (epitopes) designated A, B, C, D, and E against which host neutralizing antibodies are directed (Michael W Deem *et al*., 2009)

There are several methods for estimating the antigenic distance between influenza vaccine strains and the circulating viruses. The P epitope is one of the methods used to carry out these estimates. The model predicts the differences in the second antigen, in the epitope region after exposure to an original antigen by probability(Michael W Deem *et al*., 2009; Munoz & Deem, 2005). The epitope regions are significantly involved in

immune recognition, thus the percentage of the epitope that changes characterize the antigenic drifts. The severity of seasonal flu is correlated with $p_{\text{-epitope}}$ rather than the total number of amino acids in a protein ($p_{\text{-sequences)}}$ and is both logical and consistent with the observed data to characterize antigenic drift by the number of mutations within the epitope regions (Munoz & Deem, 2005). The $P_{\text{-epitope}}$ model quantifies the number of amino acid changes in dominant epitopes of the vaccine strain and the circulating virus strain, then estimates the antigenic distance (Tewawong *et al*., 2015).

The calculated antigenic distance linearly correlates well with the vaccine efficacy (Michael W. Deem & Pan, 2009; Gupta, Earl, & Deem, 2006). This model is simple and amenable thus it can be used to interrogate the vaccine efficacies of human influenza AWHO-recommended vaccines for the southern and northern hemispheres in a given influenza season.

CHAPTER THREE

MATERIALS AND METHODS

3.1 Study site and Design

This study was carried out at the National Influenza (NIC) laboratories located within Kenya Medical Research Institute (KEMRI). The study is based on human influenza A virus sequence data previously obtained at the NIC laboratories within KEMRI and deposited in genetic databases; the GeneBank and GSAID. It is a retrospective study involving evolutionary analyses of influenza A virus isolates obtained in Kenya between 2007 and 2013, focusing on the HA1 domain of the hemagglutinin gene. The national influenza centre is part of the Global influenza surveillance response tasked with the responsibility of monitoring circulating viruses and reporting to WHO. The isolates used in this study were collected from sites shown in figure 3.1.

3.2 Ethical considerations

The study did not involve human or animal subjects. Instead, it utilized nucleotide and protein sequence data previously deposited by Professor Wallace Bulimo's research group in GenBank and GISAID databases. Permission to use the Kenyan human influenza A virus gene sequences previously deposited in Gen Bank was sought and granted by the Principal Investigator of the USAMRD-K FLU surveillance program protocol (KEMRI SERU#981 WRAIR31267).

3.3 Sampling and Data collection

To understand the evolutionary changes occurring in the influenza virus strains that circulated in Kenya during the study period, the amino acid HA1 sequences of the Kenyan strains were compared to reference vaccine strains recommended by the WHO for the periods before, during, and just after the 2009 influenza pandemic. The Kenyan isolates collected from sites based on geographical location and demographics were

retrieved from the databases (figure3.1). Noteworthy all the HA sequences of the local A/H3N2, A/H1N1pdm09, and A/sH1N1strains that have been previously been sequenced from viruses were obtained from clinical specimens collected from hospitals constituting the human respiratory virus surveillance program, within the Kenya Medical Research Institute (KEMRI). HA sequences of less than 987 nucleotides in size including those not well-curated were left out. A total of fifty-two (n=52) representative HA gene nucleotide sequences of Kenyan human influenza A /H3N2, fifty-six (n=63) A/sH1N1, and ninety-seven (n=97) A/H1N1pdm09 virus isolates, previously deposited in GenBank (*http://www.ncbi.nlm.nih.gov/genbank*) and GSAID (*http://platform.gisaid.org/epi3/frontend*) genetic databases were retrieved including those of corresponding WHO influenza virus vaccine strain compositions for the respective years. In addition, seventy (n=70) A/H3N2, sixty-three (n=63) A/H1N1 and seventy-nine (n=79) A/H1N1pdm09 sequences of global reference strains were downloaded bringing the whole sequence dataset for the study to A/H3N2 (N=122); A/sH1N1 (N=126) and A/H1N1pdm09 (N=176). The accession numbers of HA sequences of the subtypes used in the study are given in (Appendix II).

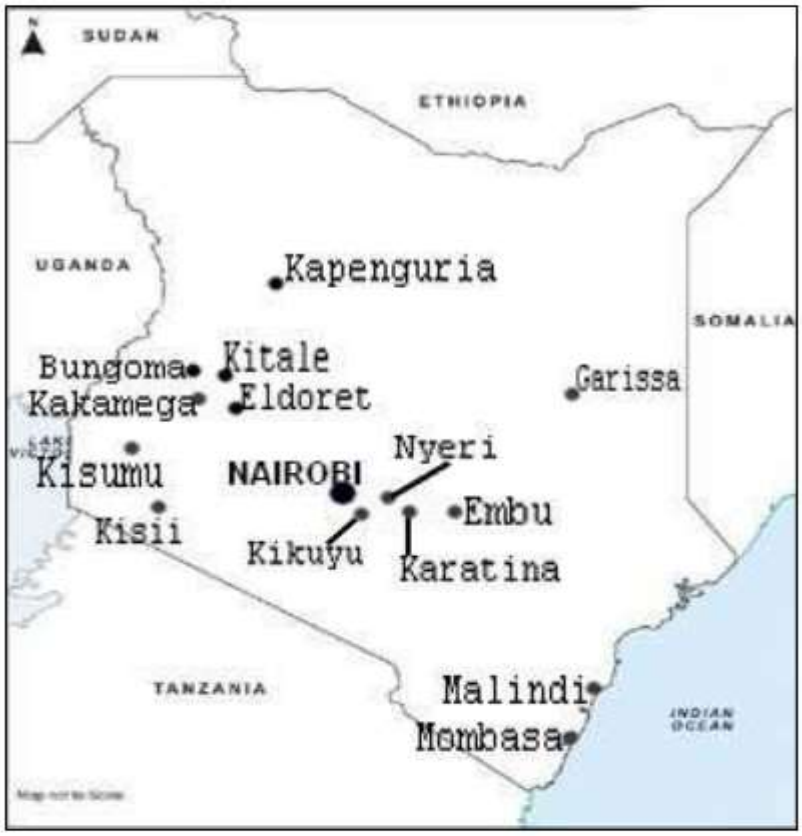

Figure 3.1: Map of Kenya displaying the sites where the study isolate used in the study originated from

3.4 Phylogenetic relationships

Phylogenetic and evolutionary analyses were carried out using Bayesian analysis approaches to determine the evolution of the HA1 gene in the period of study (J. Huelsenbeck & Ronquist, 2005; Ronquist, Huelsenbeck, & Teslenko, 2011).

The HA nucleotide sequences were aligned using the Multiple Sequence Comparison by Log-Expectation (MUSCLE) V3.8 (Edgar, 2004). The alignment sequences were then imported into Bioedit Sequence Alignment Editor software (Hall, Biosciences, & Carlsbad, 2011) and trimmed, leaving only the region encoding the HA1 gene domain. Translation of the HA1 domain nucleotide code into amino acid code was also carried out using Bioedit software. Phylogenetic clustering was estimated using MrBayes, a

Bayesian inference that uses Markov chain Monte Carlo (MCMC) methods to estimate the posterior distribution of model parameters (J. P. Huelsenbeck & Ronquist, 2001). The best-fit nucleotide substitution model for the tree reconstruction was predicted by the jModel Test (Posada & evolution, 2008; Ronquist et al., 2011) implemented in MEGA V. 6.0 (Kumar, Stecher, Li, Knyaz, & Tamura, 2018). The best-fit nucleotide substitution model was found to be K2 + Gamma.

3.5 Natural Selection pressure Prediction

Among other methods of inferring selection pressure, the single likelihood ancestral counting (SLAC) and fixed effects likelihood (FEL), have been mainly used to study the molecular evolution of viruses by estimation of dN and dS rates per site between the nucleotide sequence(Pond & Frost, 2005). The overall selection pressure underlying the evolution of the HA1 domain of the hemagglutinin gene of Kenyan influenza A virus strains, as well as that acting at individual codon sites encoding the HA1 region of the hemagglutinin protein was determined by the single likelihood ancestor counting (SLAC) together with the fixed effects likelihood (FEL) methods implemented in the online Datamonkey online tool (*http://www.datamonkey.org*). The online datamonkey tool has been a vital tool in studying the selection types and studying evolutions in influenza viruses. This tool employs the HyPhy package to compute the dN and dS at each codon site and determine the nature of selection. The ratio of the nonsynonymous substitution rate (d_N) to the synonymous rate (d_S) was read as follows: (d_N/d_S) >1= positive selection, while (d_N/d_S) <1= negative selection and (d_N/d_S) = 1 neutral selection. The selection analyses by both methods were assessed at a p-value <0.05.

3.6 N-glycosylation sites Prediction

The prediction of N-linked glycosylation sites (amino acids series: Asparagine-X-Serine/Threonine, where X stands for any amino acid except Aspartate or Proline) within the HA1 domain protein sequence was performed using the online NetNGlyc 1.0

server (*http://www.cbs.dtu.dk/services/NetNGlyc/)*. A score cut-off value of >0.5 was considered suggestive of glycosylation.

3.7 Evolutionary Analyses

Molecular evolutionary rates, time to the most recent common ancestor (TMRCA), and time-scaled phylogenetic estimation of the local strains was performed using the Bayesian statistical inference approach implemented in the Bayesian Evolutionary Analysis Sampling Trees (BEAST) v1.7.5 software package (Drummond & Rambaut, 2007). The analysis was performed using a relaxed molecular clock (uncorrelated lognormal) and a Hasegawa–Kishino–Yano (HKY) + Gamma model of nucleotide substitution. Aligned nucleotide sequence files were imported into the BEAUti (Bayesian Evolutionary Analysis Utility) program, incorporated in the BEAST package for calibration, to specify the evolutionary model and run options, generating an output XML file for BEAST analyses. The BEAST program employs a Bayesian Markov Chain Monte Carlo MCMC tree re-construction approach. Two independent chains were run for 100 million states and sampling was performed at every 100,000th generation. The successful convergence of the runs was assessed by the interpretation of generated .log txt files in Tracer v1.6 software (Drummond, Suchard, Xie, & Rambaut, 2012). Convergence was established at Effective Sample Size (ESS) values >200 on various statistical parameters estimated by the completed BEAST run. Subsequently, a maximum credibility tree (MCC) was generated using the Tree Annotator v1.8.2 software, incorporated in the BEAST package, and next visualized and annotated appropriately using the Fig tree v1.40 software (Rambaut, 2009)

3.8 Prediction of vaccine efficacy using the $P_{\text{-epitope}}$ model

The vaccine efficacy of the Kenyan human influenza A/H3N2, A/H1N1/pdm09, and s/H1N1 was estimated using the P-epitope method across the seven seasons of influenza viruses. The antigenic distance was calculated based on the fraction of amino acid substitutions in the dominant HA1 epitope. This method assumes the principle that

vaccine efficacy has a linear correlation with the antigenic distance between the vaccine strain and the dominant strains in circulation.

$$P_{-\text{epitope}} = \frac{\text{Number of substitutions in the dominant epitope}}{\text{Total number of amino acids in the dominant epitope}}$$

The association between the VE and the $P\text{-}_{epitope}$ for A/H3N2 is given by the equation $VE = -2.47 \times P_{epitope} + 0.47$, where VE is 47% when $P\text{-}_{epitope} = 0$, whereas $E = -1.19 \times P$ epitope + 0.53 for influenza A/H1N1/pdm09 and s/H1N1 viruses (Gupta et al., 2006). The influenza A(H3N2) vaccine efficacy with P epitope = 0 is 47% as a perfect match between vaccine and circulating viruses while for the influenza A/H1N1/pdm09 and s/H1N1 viruses, the vaccine efficacy is 53% when P epitope =0

CHAPTER FOUR

RESULTS

4.1 Amino Acid Sequence Substitution and Phylogenetic Analyses

4.1.1 H3N2 Viruses

For the year 2007, eight amino acid sequences of the HA1 of the A/H3N2 Kenyan isolates were analyzed in comparison to the WHO reference vaccine strains [A/Wisconsin/67/2005] A/H3N2-like virus, they revealed the following amino acid substitutions: G50E, D122N, S138A, V186G, I223V, and K140I relative to the vaccine strain (Table 4. I). Amino acid changes S138A and K140I affected antigenic site A while V186G was at antigenic site B. Those obtained in the 2008 influenza season differed from A/Brisbane/10/2007 at three amino acid residue positions V112I, N144S/K, and K173E/Q, with substitutions N144S/K and K173E/Q affecting antigenic sites A and D, respectively (Table I). Furthermore, the 2009 isolates contained five amino acid variations: K158N, K173Q, N189K, T212A, and I214S/T relative to A/Brisbane/10/2007. The amino acid changes K158N and N189K affected antigenic site B, and K173Q was at antigenic site D. The Kenyan 2010 isolates displayed five amino acid differences: S45N, K62E, K144N, T212A, and S214I relative to A/Perth/16/2009 (Table 4.1). Accordingly, amino acid changes S45N affected antigenic site C, K62E antigenic site E, while K144N was at antigenic site A. For the 2011 influenza season, no Kenyan A/H3N2 sequences were found in the genetic databases. However, five isolates obtained in 2012 contained six amino acid changes relative to A/Perth/16/2009 (Table 4.1). The S45N mutation affected antigenic site C, K62E antigenic site E, while K144N was at antigenic site A. Furthermore, the Kenyan 2013 isolates harboured eleven amino acid differences relative to A/Victoria/361/2011 vaccine virus. These included Q33R, G78D, T128A, I140R, R142G, N145S, R156H, V186G, E190D, Y219S, and N278K (Table I). The G78D change occurred at antigenic

site E; I140R, R140G, and N145S at antigenic site A; T128A, R156H, and V186G at antigenic site B; and N278K antigenic site C.

Table 4.1: Mutation at the antigenic sites of influenza A/H3N2 circulating in Kenya compared to the contemporaneous vaccine strain from 2007 to 2013

Influenza Subtype	Year	Vaccine strain	Mutations in circulating strains	Mutation at the antigenic sites	
				Site	Mutation
A/H3N2	2007	A/Wisconsin/67/2005	G50E, D122N, S138A, V186G, I223V, K140I	A	S138A, K140I
				B	V186G
	2008	A/Brisbane/10/2007	V112I, N144S/K, K173E/Q	A	N144S/K
				D	K173E/Q
				B	K158N, N189K
				D	K173Q
	2010	A/Perth/16/2009	S45N, K62E, K144N, T212A, S214I.	A	K144N
				C	S45N
				E	K62E
	2011	A/Perth/16/2009	NO Kenyan ISOLATES RETRIEVED		
	2012	A/Perth/16/2009	S45N, K62E, K144N, V224I, T212A, S214I	A	K144N
				C	S45N
				E	K62E
	2013	A/Victoria/361/2011	Q33R, G78D, T128A, I140R, R142G, N145S, R156H, V186G, E190D, Y219S, 278K.	A	I140R, R140G, N145S
				B	T128A, R156H, V186G
				C	N278K
				D	G78D

Phylogenetic inference showed that Kenyan A/H3N2 isolates clustered into three different clades/lineages, consistent with the year of isolation (Figure 4.1). Those isolated in 2007/2008 fell into the A/Brisbane/10/2007-like virus cluster, while those obtained in 2009/2012 clustered with A/Perth/16/2009-like viruses (Figure 4.1). The Kenyan 2013 A/H3N2 isolates belonged to clade 3C.3 of the A/Samara/73/2013-like viruses, divergent from clade 3C.1 of the A/Victoria/361/2011-like viruses (vaccine strain for 2013 in the Southern Hemisphere) (Figure 4.1). All the Kenyan isolates

contained potential N-linked glycosylation motifs at HA1 residue positions 8, 22, 63, 133, 165, and 285. However, these were all present in the vaccine virus strains.

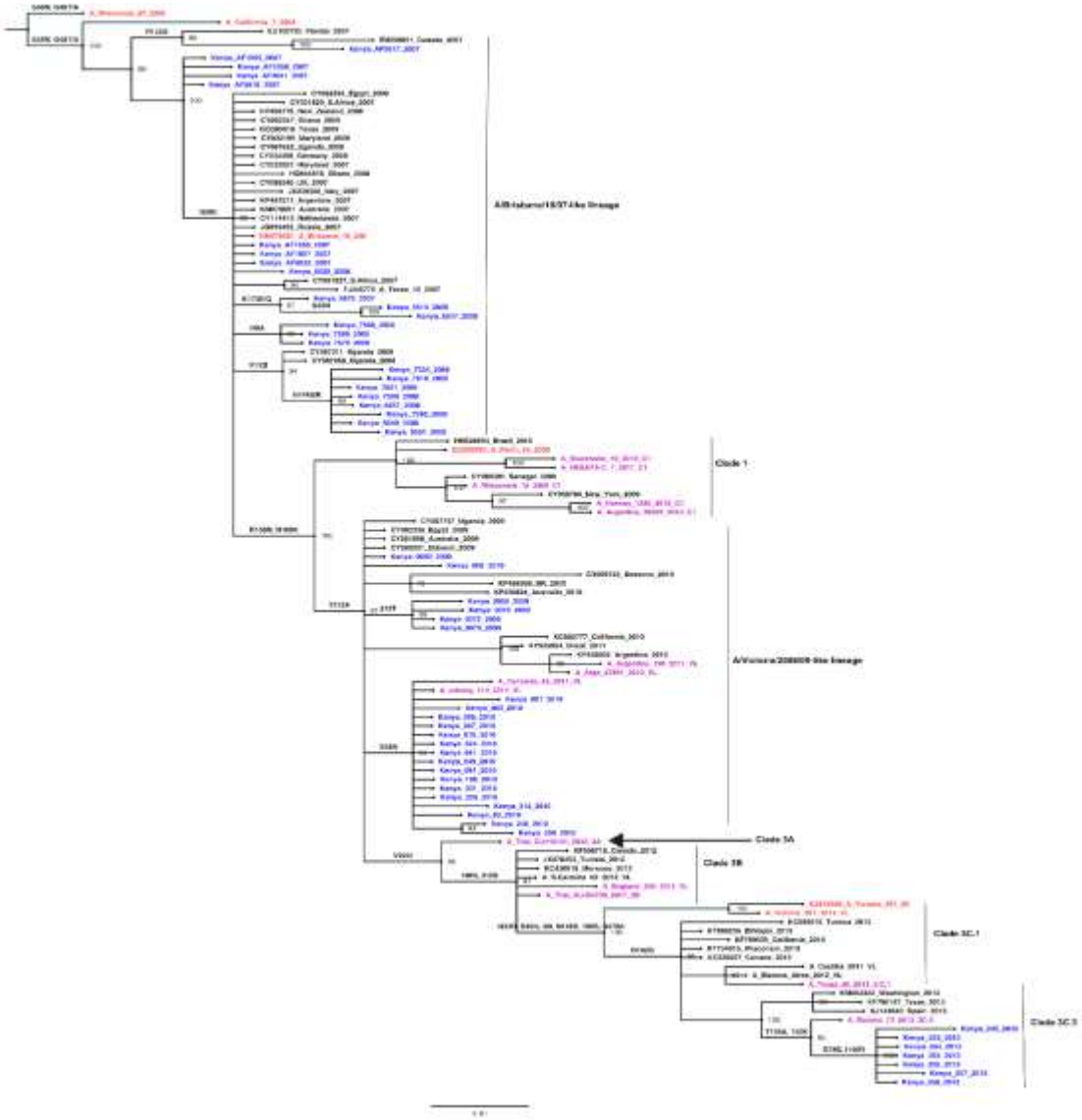

Figure 4.1: Phylogenetic analysis of influenza A/H3N2 virus HA1 amino acid sequences.

The Kenyan viruses are indicated in blue colour. Those in purple represent reference strains of known clades. Those in red represent WHO vaccine reference strains for the southern hemisphere. Those in Black represent the global reference strains.

4.1.2 A/H1N1pdm09

The Kenyan human influenza A/H1N1pdm09 isolates displayed amino acid variations P83S (all), D97E/N (2011-2013), V47I (among 2009 strains), S185T/R/K (2011-2012), S203T (all), K283E (2012-2013), V234I (2012-2013), D222E (among 2009 strains) and I321V compared to the reference WHO vaccine strain A/California/7/2009 (H1N1)-like virus. However, none of these mutations detected in the HA1 domain of the Kenyan A/H1N1pdm09 isolates occurred at the predicted antigenic sites (Table 4.2). Phylogenetic analysis revealed that the majority of A/H1N1pdm09 strains detected in Kenya between 2009-2011 belonged to clade 7 while a minority were in clades 5 and 6A (Figure4. 2). Those detected in 2013 belonged to clade 6C (Figure 4.2). The Kenyan A/H1N1pdm09 strains were interspersed among the global strains used in the analysis. All these viruses were characterized by P83S and S203T amino acid mutations compared to the A/California/7/2009 vaccine strain. Furthermore, a proportion of the Kenyan viruses isolated in 2009 had additional mutations that led to sub-clusters. Thus, the first sub-cluster was characterized by a D232E mutation and the next sub-cluster by three mutations, V47L, A48S, and N129D. Viruses isolated in 2012-2013 formed a sub-cluster characterized by two mutations, D97N and K293E. Therefore, the Kenyan viruses formed discrete clusters based on the collection/sampling year (Figure 4.2).

Table 4.2: Mutation at the antigenic sites of Influenza A/H1N1pdm2009 circulating in Kenya compared to the contemporaneous vaccine strain from 2009-2013

Influenza Subtype	Year	Vaccine strain	Mutations in circulating strains	Mutation at the antigenic sites	
seasonal A/H1N1 pdm2009	2009	A/California_07_2009	V30A, V47I, A482, P83S, N128D, S203T, D222E.	Sa	NONE
				Sb	NONE
				Ca_2	NONE
				Sa/Sbx	NONE
	2010	A/California_07_2009	P83S, D97E/N, S185T, S203T, I321V.	Sa	NONE
				Sb	NONE
				Ca_2	NONE
				Sa/Sbx	NONE
	2011	A/California_07_2009	P83S, D97N, S185T, S203T, I321V	Sa	NONE
				Sb	NONE
				Ca_2	NONE
				Sa/Sbx	NONE
	2012	A/California_07_2009	P83S, S84K, S85T, D86N, G88R, D97N, P182Q, S183N, T184K, S185K, D187E, S190T, L191P, S203T, H228D, V283E, I321V	Sa	NONE
				Sb	NONE
				Ca_2	NONE
				Sa/Sbx	NONE
	2013	A/California_07_2009	P83S, D97N, S185R/T, S203I, V234I, K283E, I321V	Sa	NONE
				Sb	NONE
				Ca_2	NONE
				Sa/Sbx	NONE

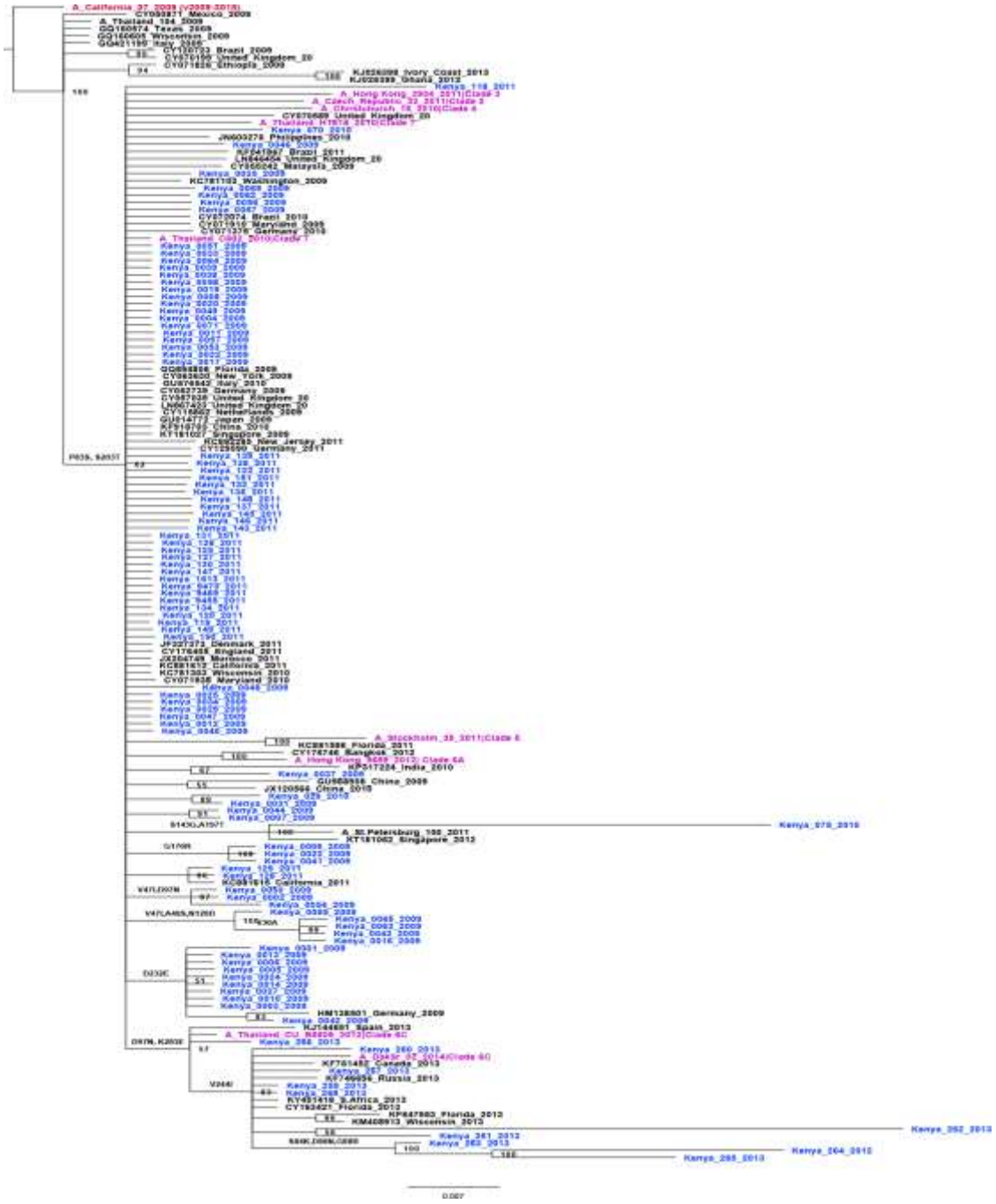

Figure 4.2: Phylogenetic analysis of influenza A/H1N1pdm09 virus HA1 amino acid sequences.

The Kenyan viruses are indicated in blue colour. Those in pink represent reference strains of known clades. The one in red represents the WHO vaccine reference strain for the southern hemisphere (2009-2013). Those in Black represent the global reference strains

4.1.3 Seasonal A/H1N1

The Kenyan A/sH1N1 isolate strains were compared to the WHO-recommended contemporaneous vaccine reference strains A/New Caledonia/20/99(H1N1)-like virus, A/Solomon Islands/3/2006 (H1N1)-like virus, and A/Brisbane/59/2007 (H1N1)-like virus (Table 4.3). When, compared to the 2007 reference strain (A/New Caledonia/20/99(H1N1)-like virus, the Kenyan viruses isolated in 2007 showed amino acid substitutions/mutations at fifteen loci amongst the 20 isolates that were retrieved from the GeneBank for the year of 2007. These mutations included; D35N, T82K, Y94H, Q104H, S121N/R, K140E, V165A, R188K, R208K, W251R, T266N, E273K, and D186N/G. Amongst these only five mutations occurred at antigenic sites. Thus, the T82K mutation occurred at the antigenic site Cb, K140E at predicted antigenic site Ca_2, V165A at the antigenic site Sa, R188K at the antigenic site Sb, and R208K at the antigenic site Ca_1 (Table 4. 3). Noticeably, the mutation W251R and D186N/G occurred close to the Sa and Sb antigenic sites respectively.

In the year 2008, A/Solomon Islands/3/2006 (H1N1)-like virus was used as the vaccine strain. Twenty-six viruses isolated in Kenya during this year were compared to the reference strain to predict the amino mutations. Ten mutations were predicted; D35N, R73K, T128V, K145R, N183S, G185N, R188K, A189T, R222Q, and E273K. The mutation T128V occurred at the predicted antigenic site SA, K145R at the antigenic site Ca_2 and R188K & A189T at the predicted antigenic site Sb.

In the year 2009 sixteen Kenyan isolates were retrieved from the database and compared against the vaccine A/Brisbane/59/2007 (H1N1)-like virus strain to check for the amino acid substitution sites. The following mutations were observed; N183S, G185S/N/A,

N186D/G, A189T, Q104H, S121N/R, and D272G (Table 4.3). Out of the eleven mutations observed, only one (A189T) was seen to be at a predicted antigenic site, *viz*. site Sb. However, although the mutations N183S, G185S, and N186D did not occur at antigenic sites, they are close to the antigenic site Sb and may affect antigenicity at this site. Phylogenetic analysis showed that the A/sH1N1 strains that circulated in Kenya between 2007-2009 belonged to clade B characterized by the E273K amino acid change (Figure 4.3). They were interspersed among the foreign strains used in the analysis. Overall, all mutations that occurred and were detected amongst Kenyan influenza A viruses were also observed in the global strains used in the analysis, except V47I (in A/H1N1pdm09), I140R (in A/H3N2), and Q104H (in seasonal A/sH1N1) (Appendix I, II & III).

Table 4.3: Mutation at the antigenic sites of Influenza A/sH1N1 circulating in Kenya compared to the contemporaneous vaccine strain from 2007-2013

Influenza Subtype	Year	Vaccine strain	Mutations in circulating strains	Mutation at the antigenic sites	
seasonal A/H1N1	2007	A/New Caledonia/20/99	D35N, T82K, Y94H, Q104H,	Cb	T82K
				Sb	R188K
seasonal A/H1N1			S121N/R, K140E, V165A,	Ca_2	K140E
				Ca_1	R208K
			R188K, 208K, W251R,	Sa	V165A
			T266N, E273K, and D186N/G.		
	2008	A/Solomon Islands/3/2006	D35N, R73K, T128V, K145R, N183S, G185N, R188K, A189T, R222Q, E273K	Sa	T128V
				Sb	R188K, A189T
				Ca_2	K145R
				Sb	A189T

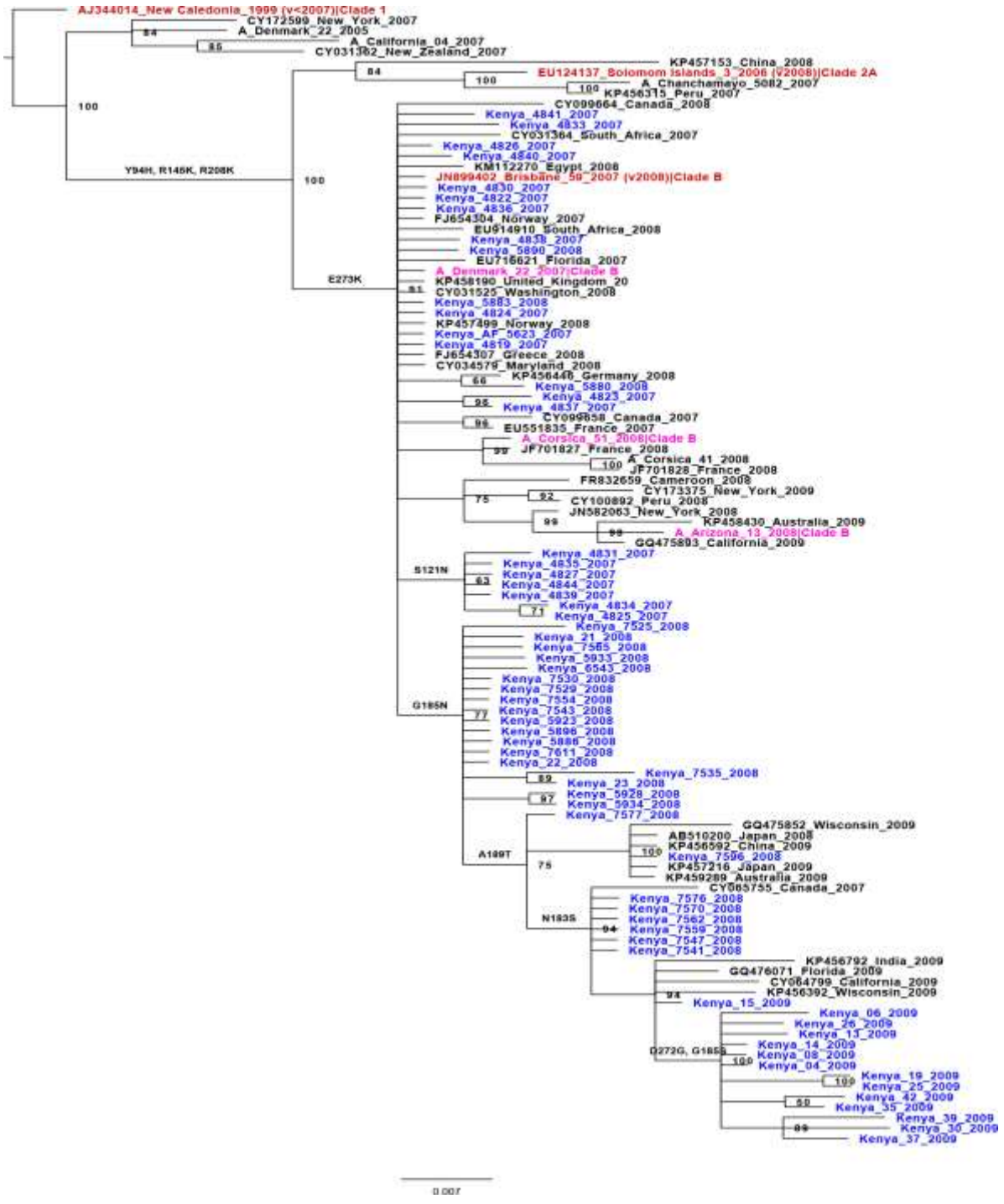

Figure 4.3: Phylogenetic analysis of influenza A/sH1N1virus HA1 amino acid sequences.

The Kenyan viruses are indicated in blue colour. Those in pink represent reference strains of known clades. The one in red represents the WHO vaccine reference strain for the southern hemisphere. Those in Black represent global reference global reference strains.

4.2 Prediction of vaccine efficacy using the P-epitope model

4.2.1 H3N2 vaccine efficacy estimation

The estimated vaccine efficacies of the 2007 - 2013 vaccine strains against the Kenyan A/H3N2 isolates are summarized in Table 4.4. The $P\text{-}_{epitope}$ between A/Wisconsin/67/2005 vaccine strain and Kenyan 2007 isolates was 0.1579 (dominant epitope A; amino acid changes D122N, S138A, and K140I), suggesting a vaccine efficacy (VE) of 17% (E=8% of 47%). The $P\text{-}_{epitope}$ between A/Brisbane/10/2007 and Kenyan 2008/2009 isolates was 0.0526 (dominant epitope A; mutation N144S/K) and 0.0952 (dominant epitope B; mutation K158N and N189K) suggesting a VE of 72.36% and 49.98%, respectively. A/Perth/16/2009 was the recommended WHO vaccine strain for the 2010, 2011, and 2012 influenza seasons. The $P\text{-}_{epitope}$ value between A/Perth/16/2009 and Kenyan 2010 A/H3N2 isolates was 0.0526 (dominant epitope A; mutation N144K), suggesting vaccine efficacy of 72.36%. No sequences for A/H3N2 viruses that circulated in Kenya during the 2011 influenza season were available in GenBank/GSAID databases; thus, no analysis was carried out for this period. For the 2012 influenza season, 40% (2/5) of the analysed A/H3N2 isolates possessed I140R, R142G, K144N, N145S, and G186S amino acid changes at site A of the HA1 domain relative to A/Perth/16/2009. These resulted in negative vaccine efficacy (Table 4.4). All the Kenyan 2013 isolates contained three amino acid substitutions (T128A, R156H, and V186G) affecting site A of the HA1domain. The estimated $P_{\text{-}epitope}$ value between these isolates and the vaccine strain A/Victoria/361/2011 was 0.1579 (Table 4.4), signifying a worst-case (8%) vaccine efficacy.

Table 4.4: Efficacy estimation among A/H3N2 vaccine strains and mutations found on the dominant epitope of Influenza A(H3N2) viruses that circulated in Kenya (2007-2013).

Year	Vaccine strain	No. of strains	Dominant epitope	No. of mutations	Residue variations	P-epitope	Efficacy (47%)
2007 (N=8)	A/Wisconsin/67/ 2005	7	A	3	D122N, S138A, K140I	**0.1579**	8
		1	B	4	T128A, I157S, V186G, I192T	0.1905	0
		8	A	1	N144S	**0.0526**	34
2008 (N=14)	A/Brisbane/10/ 2007	1	B	1	K158R	0.0476	35
		2	C	1	T48A	0.037	38
		2	D	1	K173C	0.0488	35
		1	E	1	C57R	0.0455	36
2009 (N=5)		4	B	2	K158N, N189K	**0.0952**	24
		1	D	2	K173C, R208K	0.0976	23
2010 (N= 15)	A/Perth/16/ 2009	11	A	1	K144N	**0.0526**	34
		2	D	1	D175E	0.0732	29
		2	E	2	K62E, G78D	0.091	25
2012 (N=4)		2	A	5	I140R, R142G, K144N, N145S, G186S	**0.2105**	-5
		1	C	2	S45N, D53N	0.0741	29
		1	D	1	D175E	0.073	29
2013 (N=6)	A/Victoria/361/ 2011	6	B	3	T128A, R156H, V186G	**0.1905**	0
2013 (N=6)	A/Texas/50/ 2012	6	A	3	I140R,R142G,N145S	**0.1579**	8

4.2.2 A/H1N1 pdm09 vaccine efficacy estimation

In studying the effect of accumulated mutations in the HA1 gene the estimated vaccine efficacy was determined using the P epitope method (Table 4.5). When the A/H1N1 pdm09 was compared to the WHO-recommended vaccine A/California/07/2009, a P-

$_{epitope}$ value of 0.0882 was yielded. This is attributed to P83S, A48S, and V47I amino acid changes in the dominant epitope E, this suggested a vaccine efficacy of 80% (42.5 % of 53%) of that of a perfect match (Table 4.5). In 2010, the vaccine efficacy using the P-$_{epitope}$ model was 87% (46% of 53%), characterized by mutations P83S, and S84R, in the dominant E epitope. The year 2011 accumulated four mutations in epitopes B and E, yielding a P-$_{epitope}$ value of 0.090909 and 0.05882 respectively translating to 42% and 46% vaccine efficacies respectively. The year 2012, was characterized by a shift of epitope from E to B, and a significant drop in vaccine efficacy, estimating a worst-case vaccine efficacy of 39% (20.55% of 53%). In all, 87% of the strains obtained in the study period between 2009-2013, contained a dominant mutation at the E epitope at position 83. This gave a worst-case vaccine efficacy of 39%% (20.55% of 53%) for the years 2012 and 2013 (Table 4.5).

Table4.5: Vaccine efficacy estimation using the P-epitope model and the number of mutations of human influenza A/pdm2009 circulating in Kenya compared with the A/California/07/2009 vaccine strain.

Year	Vaccine Strain	No of Strains	epitope	No.of Mutations	Residue Differences	P-$_{epitope}$	Vaccine Efficacy (53%)
2009 N=68	A/California /07/2009	67	**E***	**3**	**P83S, A48S, V47I**	**0.0882**	**42.5**
		1	A	2	T120A, K142R	0.08333	42.5
		14	D	1	D222E	0.020833	52.975
		1	C	2	D35N, T278I	0.030303	49.394
2010 N=31		2	**E***	**3**	**P83S, S84R**	**0.05882**	**46**
		1	A	3	N129K, S143G, R252K	0.125	38.125
		6	A	1	P137S, D127E, S120P, S128P	0.041667	48.042
2011 N=29		27	**E***	**4**	**P83S, S71T, N260K, S75P**	**0.05882**	**46**
		26	**B***	**4**	**S183T, A186G, S185T, S190G**	**0.090909**	**42.18**
		2	D	2	S204T, S164F, A215G	0.041666	48.04
2012 N=11		1	**B***	**6**	**S183N, T184K, S185K, D187E, S190T, L191P**	**0.272727**	**20.55**
		1	E	4	P83S, S84K, S85T, D86N	0.11764	30
		6	**B***	**5**	**S183N, T184S, S185R, A186G, D187H**	**0.272727**	**20.55**
2013 N=9		5	E	5	P83S, P84K, A48V, S85T, D86N	0.147058	35.5
		1	C	1	K283E	0.030303	49.394
		1	D	1	M227I	0.020833	50.52

4.2.3 s/H1N1 vaccine efficacy estimation

The estimated vaccine efficacies of the A/sH1N1 human influenza 2007-2009 vaccine strain against the Kenyan A/sH1N1 isolates are summarized in Table 4.6. The P-$_{epitope}$ between A/New Caledonia /20/1999 vaccine strain and Kenyan 2007 isolates was 0.25 (dominant epitope A; amino acid changes S121N, K140E, V165A), suggesting a vaccine

efficacy (VE) of 44% (E=23.25% of 53%) (Table 4.6). The $P\text{-}_{epitope}$ between the vaccine strain A/Solomon Islands /3/2006 and Kenyan 2008 and 2009 isolates was 0.090909 (dominant epitope C; mutation D35N, R73K, E273K) and 0.272727 (dominant epitope B; mutation G155C, N183S, I184V, G185S, N186D, A189T) suggesting a VE of 80% and 39%, respectively.

Table 4.6: Vaccine efficacy estimation using the P epitope model and the number of mutations of human influenza A/sH1N1 circulating in Kenya compared with WHO-recommended vaccine strain for the respective years.

Year	Vaccine Strains	No of Strains	Epitope	No. of Mutations	Residue differences	P-epitope	Vaccine efficacy
2007 N=20	A/New/Caledonia /20/1999	13	D	2	Y94H, R208K	0.041666	48.04
		12	C	2	D35N, E273K	0.060606	45.79
		19	**A**	**3**	**S121N,K140E, V165A**	**0.25**	**23.25**
		23	**C**	**3**	**D35N, R73K, E273K**	**0.090909**	**42.18**
		8	B	3	N183S,G185N, A189T	0.136363	36.77
2008 N=26	A/Solomon Islands/3/2006	2	D	3	E169G,R222C, T210N	0.0625	45.56
		1	E	3	V57I, R73K, G259S	0.08823	42.5
2009 N=16	A/Brisbane/59/2007	16	**B***	**6**	**G155C,N183S, I184V, G185S,N186D, A189T**	**0.272727**	**20.5**

4.3 Glycosylation analyses of the HA1 sequences

All the Kenyan isolates contained potential N-linked glycosylation motifs (HA1 residue positions: 8, 22, 63, 133, 165, and 285). All the Kenyan isolates in the three subtypes (i.e. A/H1N1pdm09, seasonal A/sH1N1 & A/H3N2) had retained all the potential N-linked glycosylation sites in the HA1 domain of the hemagglutinin protein, except

seasonal A/sH1N1 strain designated A_Kenya_4841_2007(Table 4.7, Table 4.8 and Table 4.9). The Kenyan isolate (A_Kenya_4841_2007) exhibited a loss of N-linked glycosylation site at amino acid residue position 159 of the HA1domain (Table 4.9). The amino acid 159 is located at the antigenic site Sa and was expected to result in an antigenically altered strain.

Table 4.7: N-linked glycosylation sites analyses in the HA1 domain of Kenyan A/H3N2 isolate strains

	Predicted N-linked glycosylation sites in the HA1 domain					
Strains	8	22	63	133	165	285
A/Victoria/361/2011 (2013) *	□	□	□	□	□	□
A/Perth/16/2009 (2010-2012) *	□	□	□	□	□	□
A/Brisbane/10/2007 (2008-2009) *	□	□	□	□	□	□
A/Wisconsin/67/2005 (2007) *	□	□	□	□	□	□
All Kenyan isolates (2007-2013)	□	□	□	□	□	□

(*) = WHO vaccine reference strains; □ = present

Table 4.8: N-linked glycosylation sites analyses in the HA1 domain of Kenyan A/H1N1pdm09 isolate strains

	Predicted N-linked glycosylation sites in the HA1 domain		
Strains	11	23	287
A/California*	□	□	□
All Kenyan isolates (2007-2013)	□	□	□

(*) = WHO vaccine reference strain; □ = present

Table 4.9; N-linked glycosylation sites analyses in the HA1 domain of Kenyan seasonal A/H1N1 isolate strains

	Predicted N-linked glycosylation sites in the HA1 domain				
Strains	11	23	54	125	159
A/New Caledonia/20/99 (2007) *	□	□	□	□	□
A/Solomon Islands/3/2006 (2008) *	□	□	□	□	□
A/Brisbane/59/2007 (2009) *	□	□	□	□	□
All Kenyan isolates 2007-2008 except	□	□	□	□	□
A_Kenya_4841_2007	□	□	□	□	×

(*) = WHO vaccine reference strains; □= present; X = absent

4.4 Selection Pressure and Molecular Evolutionary analyses

Overall selection pressures in the HA1 domain of Kenyan human influenza A/H3N2, A/H1N1pdm09, and seasonal A/H1N1 were estimated to be 0.56, 0.44, and 0.41, respectively. Both the SLAC and FEL methods detected a different number of amino acid residue sites in the HA1 domain of influenza A/H1N1pdm09 and A/sH1N1 virus isolates as negatively selected (Table 4.10). The FEL method, however, detected a single amino acid residue at codon position 183 as evolving under positive selection ($p<0.05$) in the Kenyan A/H1N1pdm09 isolates (Table 4.10). Codon 183 is close to the Sa antigenic site. The proximity to a major antigenic site could explain the positive selection pressure exhibited at this site. Interestingly, no negatively or positively selected sites were detected in the Kenyan A/H3N2 isolates by either the SLAC or FEL methods.

Table 4.10: Natural selection analyses at specific codons of the HA1 region of Kenyan human influenza A viruses

			Residue Changes						
			From		To				
Subtype	Analysis Method	Residue positions	Codon	Amino acid	Codon	Amino acid	Normalized dN-dS	P-values	Selection
A/H1N1pdm09	SLAC	175	GTG	Val	GTA	Val	-19.3791	0.037	Negative
		276	AAT	Asn	AAC ACT	Asn Thr	-44.79	0.0047	
	FEL	50	TTG	Leu	TTA	Leu	-35.6734	0.024	
		83	TCT	Ser	TCG	Ser	-32.9274	0.033	
		155	GGA	Gly	GGG	Gly	-38.5833	0.02	
		175	GTG	Val	GTA	Val	-36.075	0.013	
		183	TCT	Ser	AAT ACT	Asn Thr	46.4956	0.05	Positive
		226	AGA AGG	Arg	AGG AGA	Arg	-54.8623	0.011	Negative
		235	GAG	Glu	GAA	Glu	-33.9819	0.025	
		276	AAT	Asn	AAC ACT	Asn Thr	-167.857	0.001	
		295	ATA	Ile	ATT	Ile	-40.0151	0.045	
		301	GGA	Gly	GGG	Gly	-39.1535	0.02	
A/H1N1 (seasonal)	FEL	18	ACA	Thr	ACG	Thr	-154.59	0.021	
		64	AAC	Asn	AAT	Asn	-162.925	0.019	
		314	GTT	Val	GTC	Val	-117.96	0.03	

4.5 Bayesian Time-scaled phylogenetic analyses

Time-scaled phylogenetic analyses of the local human influenza A virus strains based on HA1 domain nucleotide sequences of the hemagglutinin gene showed that the Kenyan A/H3N2, A/H1N1pdm09 & seasonal A/sH1N1 isolates shared the most recent common ancestor (tMRCA) at 7.5 (95% HPD = 6.4-8.9), 5.9 (95% HPD = 4.7-7.1) and 2.7 (95% HPD = 2.3-3.2) years ago from present respectively. The inferred maximum clade credibility (MCC) phylogenies are shown in Figure 4.6, Figure 4.7 & Figure 4.8. Besides, evolutionary analyses revealed that the mean evolutionary rate of the Kenyan A/H3N2, A/H1N1pdm09 and seasonal A/H1N1 isolates was $4.6x10^{-3}$ (95% HPD = $3.0x10^{-3}$ to $6.3x10^{-3}$), $5.4x10^{-3}$ (95% HPD = $4.0x10^{-3}$ to $7.2x10^{-3}$) and $5.1x10^{-3}$ (95% HPD = $3.3x10^{-3}$ to $7.0x10^{-3}$) nucleotide substitutions per site per year, respectively (Table 4.11).

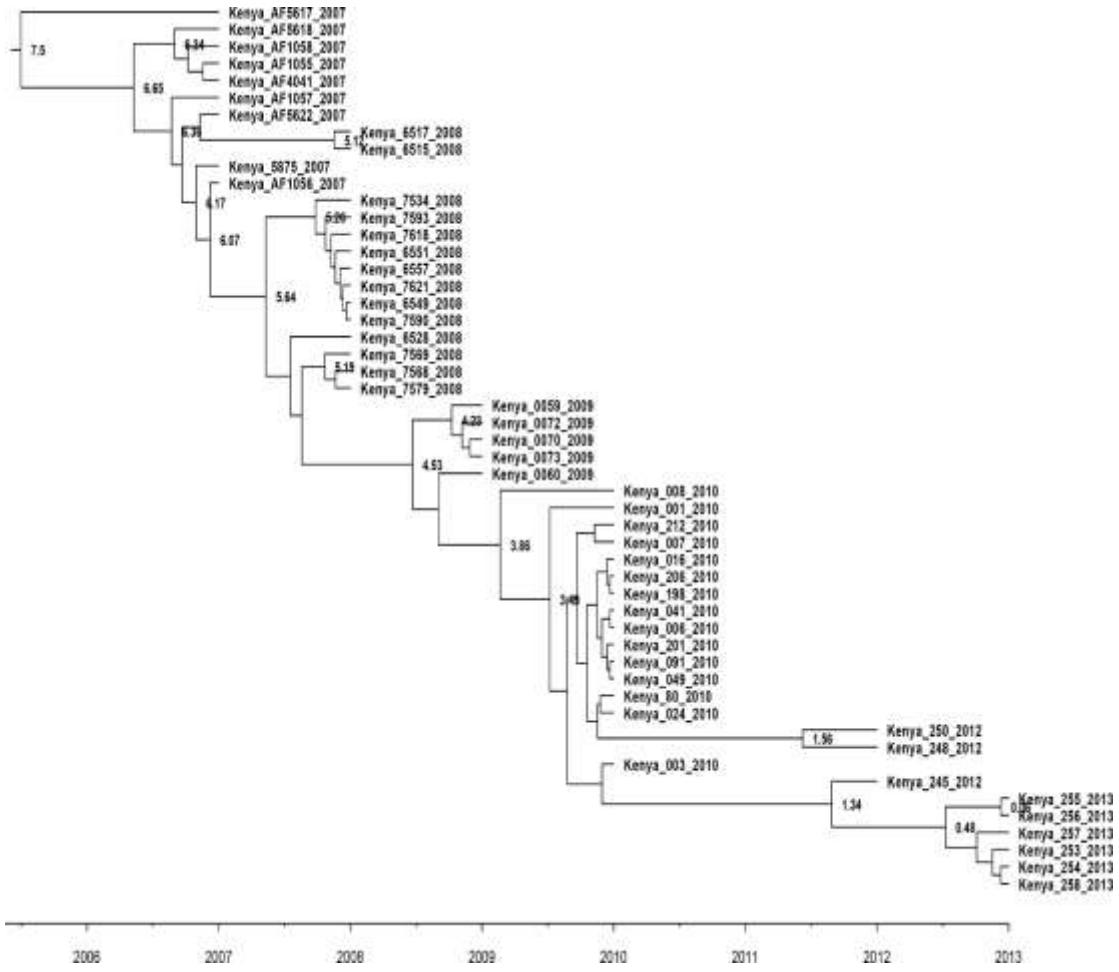

Figure 4.4: Maximum clade credibility tree from Bayesian analysis of Kenyan human influenza A/H3N2 virus isolate strains. The numbers at the nodes represent heights

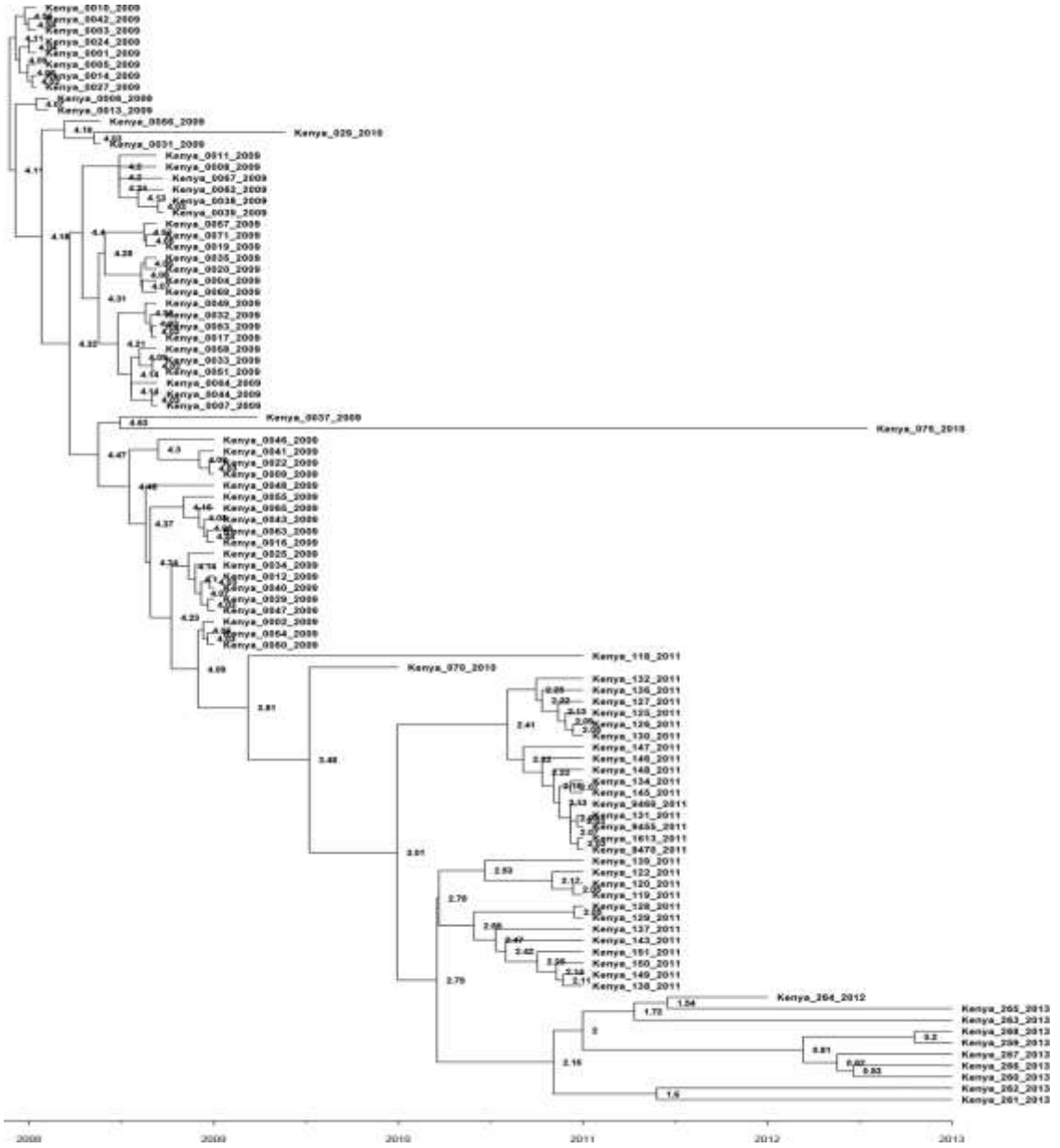

Figure 4.5: Maximum clade credibility tree from Bayesian analysis of Kenyan human influenza A/H1N1pdm09 virus isolate strains. The numbers at the nodes represent heights.

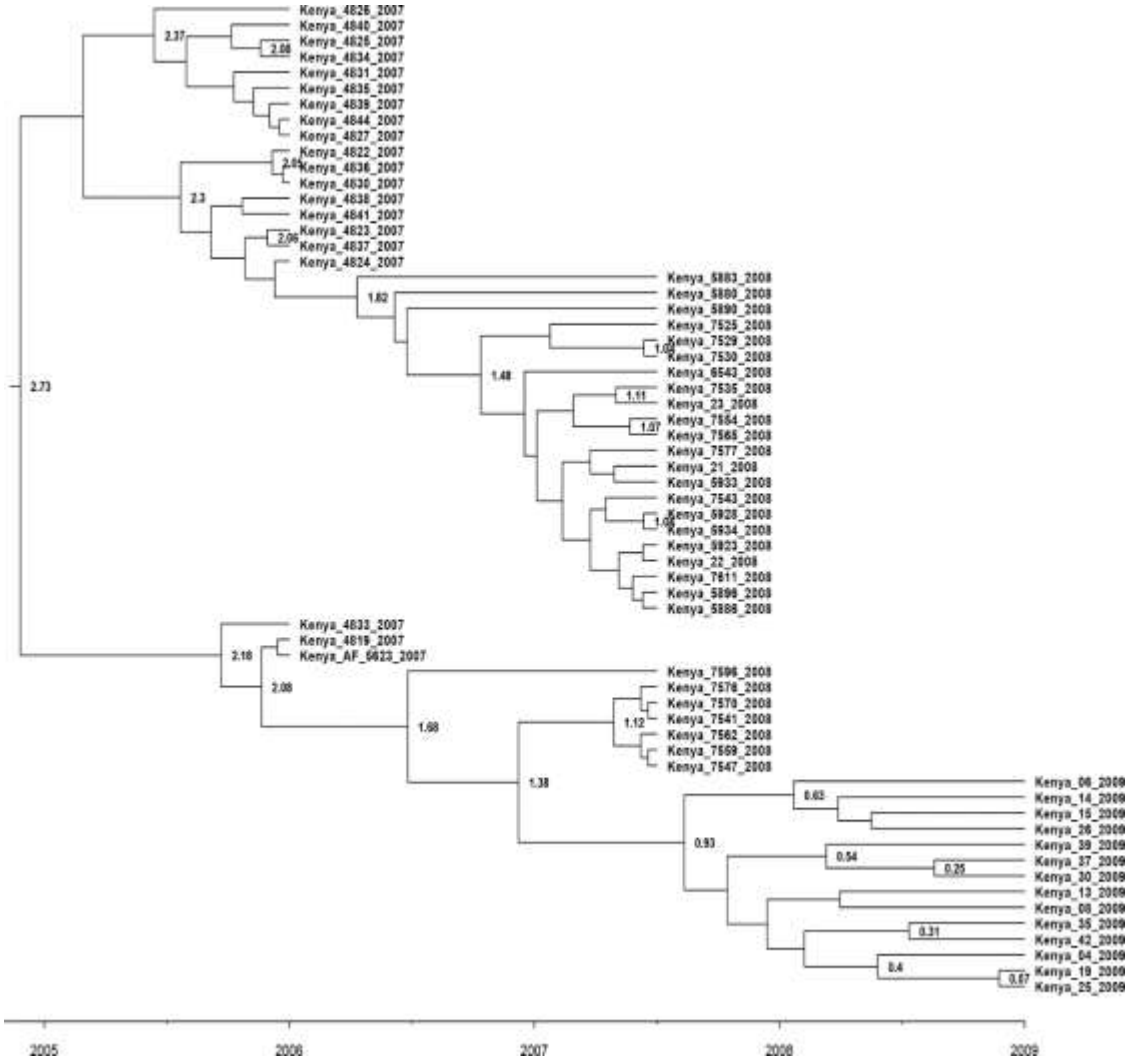

Figure 4.6: Maximum clade credibility tree from Bayesian analysis of Kenyan human influenza A seasonal H1N1 virus isolate strains. The numbers at the nodes represent heights

Table 4.11: Rates of nucleotide substitutions per site in the HA1 domain of Kenyan human influenza A virus isolates

Influenza A type	mean rate of nucleotide substitutions/site/year	95% Highest Posterior Density (HPD)	
		Lower	Upper
A/H3N2	4.637×10^{-3}	3.0436×10^{-3}	6.3679×10^{-3}
A/H1N1pdm09	5.4×10^{-3}	4.7×10^{-3}	7.2×10^{-3}
A/H1N1 (seasonal)	5.088×10^{-3}	3.2724×10^{-3}	7.0447×10^{-3}

CHAPTER FIVE

DISCUSSION

This study sought to characterize the genetic, antigenic, and evolutionary patterns of human influenza A viruses isolated in Kenya between 2007 and 2013 relative to WHO vaccine reference strains and those detected elsewhere, focusing specifically on the HA1 protein. Genetic variations observed among influenza viruses are often attributed to the virus-encoded non-proofreading, error-prone RNA-dependent RNA polymerase (RdRP), an enzyme involved in transcription and virus genome replication mechanisms (Manley, 2013; Samji, 2008). The overall effect of the low-fidelity RdRP is the emergence of influenza virus strains with the potential to evade host immunity due to accumulated point mutations at the antigenic sites that are also the protective immunological epitopes (Michael W. Deem & Pan, 2009; Gupta *et al*., 2006; Manley, 2013). The first characterized human influenza A virus strains were detected in Kenya in 2006 (A/H3N2), 2007 (A/sH1N1), and 2009 (A/H1N1pdm09) (Bulimo *et al*., 2008; Bulimo, Mukunzi, *et al*., 2012; Gachara, Symekher, Otieno, Magana, Opot, Bulimo, *et al*., 2016). The period of 2007-2013 represents a unique time in history when once three subtypes of influenza A virus co-circulated in the human population in Kenya as a result of the introduction and eventual domestication of a zoonotic influenza A virus that caused a pandemic between 2009-2010 (Majanja *et al*., 2013; Waiboci *et al*., 2011). Remarkably, the emergence of the A/H1N1/pdm09 virus marked the displacement of s/H1N1 where to date it continues to co-circulate with A/H3N2 in Kenya and the rest of the world (Majanja *et al*., 2013)

Phylogenetic inference revealed that the Kenyan A/H3N2 virus strains clustered into three genetic clades clustering together according to the three vaccine strains. Those isolated in 2007 to 2008 influenza seasons clustered within the A/Brisbane/10/2007-like virus clade; the 2009 to 2012 isolates clustered within the A/Victoria/361/2011-like virus clade, whereas those obtained in 2013 belonged to clade 3C.3 of the of

A/Samara/73/2013(H3N2)-like virus. Overall, these results show that the A/H3N2 Kenyan viruses evolved along the same trajectories as global strains. Findings in this study are compatible with those of other studies that reported circulation of A/Brisbane/10/2007, A/Victoria/361/2011, and clade 3C.3 of the A/Samara/73/2013(H3N2)-like viruses during 2008 - 2009 and 2013 seasons, respectively (Dawood *et al*., 2014; Tewawong *et al*., 2015). Consistent with the findings of a separate study (Agustiningsih *et al*., 2018), the majority of the virus strains clustered based on the isolation year. Conversely, as indicated by Suwannakarn *et al*., 2010, the Kenyan 2007 A/H3N2 viruses were drift variants of the vaccine strain. This was due to additional amino acid changes in the HA1 absent in A/Wisconsin/67/2005 (Southern Hemisphere 2007 and Northern Hemisphere 2007–2008) vaccine strain but present in A/Brisbane/10/2007 (Southern Hemisphere 2008 and Northern Hemisphere and 2008–2010 vaccine strain) and 2008 A/H3N2 isolates strains. Contrary to findings by Falchi *et al (Falchi et al., 2011).* the A/H3N2 viruses circulating in Corsica highlands in 2007 were closely related to A/Wisconsin/67/2005. The Kenyan 2008 and 2009 isolates shared T212A and N144S & K158N amino acid substitutions respectively, with those that circulated in a neighbouring country in the same period (Dawood *et al*., 2014). Consistent with findings by Tewawong *et al (Tewawong et al., 2015).* all the Kenyan isolates obtained in 2013 belonged to clade 3C.3 and were characterized by T128A and R142G amino acid substitutions among other mutations, relative to the vaccine strain A/Victoria/361/2011.

Furthermore, apart from the Kenyan A/sH1N1 strain designated A_Kenya_4841_2007, which displayed a loss of N-linked glycosylation at amino acid residue position 159 of the HA1 domain, the majority of the Kenyan influenza A viruses did not exhibit any loss or gain of N-linked glycosylation despite the amino acid variations in the HA1 protein. This supports the previous findings (Falchi *et al*., 2011; Skowronski et al., 2014) whereby all potential N-glycosylation sites detected among the Kenyan A/H3N2 strains were conserved among the vaccine strains. The gain or loss of N-linked glycosylation can affect the antigenicity of the virus HA glycoprotein (Falchi *et al*., 2011; Korsun *et*

al., 2017; Tewawong et al., 2015). Regarding natural selection, the estimated mean d_N/d_S value of 0.56 revealed that the evolution of the HA1 domain of the Kenyan influenza A virus was largely driven by purifying selection, signifying that a majority of the amino acid changes in the HA1 domain did not favor virus adaptability to the host. These findings are in agreement with those of previous studies (Nelson *et al*., 2014; Tewawong *et al*., 2015). The mean evolutionary rate for the Kenyan A/H3N2 viruses was estimated as $4.17x10^{-3}$ (95% HPD = $3.09x10^{-3}$ to $5.31x10^{-3}$) (for A/H3N2); $5.4x10^{-3}$ [95% HPD = $4.0x10^{-3}$ to $7.2x10^{-3}$] (for A/H1N1pdm09) and $5.1x10^{-3}$ [95% HPD = $3.3x10^{-3}$ to $7.0x10^{-3}$] (for A/sH1N1); nucleotide substitutions per site per year. The estimate is similar to the global nucleotide substitution rate for A/H3N2 viruses in the HA1 [4.84×10^{-3} (95% HPD = 4.32×10^{-3} to 5.38×10^{-3})] for the period between 1968 and 2011 (Westgeest *et al*., 2014). Besides, coalescent analyses showed that the time of circulation of the most recent common ancestor (tMRCA) for A/H3N2 virus strains analysed in this study was September 2001 (95% HPD = September 1998 to October 2003), for A/H1N1/pdm09, 25th October 2008 with intervals of credibility between 3rd January 2006 and 12th August 2009; while for A/sH1N1, 19th March 2007 with intervals of credibility between 12th February 2006 and 12th August 2007. The estimation of October 2008 as tMRCA for the Kenyan A/H1N1pdm09 viruses is not surprising. The apparent inconsistency with the findings of previous studies (Gachara, Symekher, Otieno, Magana, Opot, & Bulimo, 2016) may be explained by the fact that different data sets were used in the calculations. Whereas Gachara et al used full genomes for their estimation of tMRCA, this study utilized a partial sequence of only one of the eight genes, namely the HA1 domain. Nevertheless, similar findings of the tMRCA to those of this study have been reported elsewhere when the HA1 domain is used to calculate this parameter (Mugosa *et al*., 2016; Smith *et al*., 2009).

A significant proportion of amino acid substitutions detected among the Kenyan A/H3N2 isolates affected the antigenic sites. Accordingly, vaccine efficacies (antigenic distance) against the A/H3N2 strains assessed for respective years, utilizing the in-silico $P\text{-}_{epitope}$ model (Gupta *et al*., 2006) revealed sub-optimal vaccine efficaciousness against

the A/H3N2 strains that circulated in Kenya during 2007, 2009, 2012, and 2013 influenza seasons. The amino acid variations S138A, K140I (2007 isolates relative to A/Wisconsin/67/2005); K158R, N189K (2009 isolates relative to A/Brisbane/10/2007); K144N (2012 isolates relative to A/Perth/16/2009) and K140R, R140G, N145S (2013 isolates relative to A/Victoria/361/2011) on A epitopes of the HA1 domain of the hemagglutinin gene were plausibly attributable for the decline in vaccine efficacy against the virus strains. The N144D substitution was not unique to the strains that circulated in Kenya, it was also seen in Thailand and Europe (Tewawong *et al*., 2015). Conversely, the A/Brisbane/10/2007 and A/Perth/16/2009 2008 vaccine strains exhibited modest vaccine efficacy against the Kenyan A/H3N2 strains that circulated in the 2008 and 2010 influenza seasons, respectively. Overall, these results are compatible with findings by Tewawong *et al (Tewawong et al., 2015),* Dawood *et al (Dawood et al., 2014),* Eick-Cost *et al* (Eick-Cost *et al*., 2012), and Kittikraisak *et al* (Kittikraisak *et al*., 2015) who in their separate works reported modest effectiveness of A/H3N2 vaccine component against the circulating strains and influenza-associated illnesses in the same period in different parts of the world. Moreover, as reported by Tewawong *et al* (Tewawong *et al*., 2015) the change in the influenza virus vaccine component for A/H3N2 to A/Victoria/361/2011-like strain for the Southern hemisphere in the 2013 season did not offer sufficient protection against the circulating A/H3N2 strains as the latter had evolved away from A/Victoria/361/2011-like viruses belonging to genetic sub-clade 3C.1 to 3C.3 of A/Samara/73/2013-like viruses.

Regarding the A/H1N1pdm09 strains, disparate genetic divergence was displayed compared to the reference vaccine strain A/California/07/2009 (H1N1)-like virus, none of the amino acid variations affected antigenic receptor binding sites, suggesting antigenic match. Mutations within epitopes of the HA1 globular domain can affect its structure leading to a change in antigenic properties (Korsun *et al*., 2017). Nonetheless, all the Kenyan A/H1N1pdm09 strains contained S203T and V321I amino acid changes, with post-pandemic strains exhibiting slightly higher genetic differences. However, none of these mutations detected in the HA1 domain of the Kenyan A/H1N1pdm09 isolates

occurred at the predicted antigenic sites. Hence, the A/H1N1pdm09 remained antigenically stable throughout the study period and as such there was no change in vaccine antigen formulation for this strain recommended by WHO. Moreover, the A/H1N1pdm09 viruses that circulated between 2009-2012 belonged to varied global phylogenetic clades namely; clades 7, 5, and 6A, with the former being predominant. The Kenyan A/H1N1pdm09 strains that circulated in 2013 belonged to clade 6C. These findings mirror those of a previous study, reported elsewhere (Grudinin *et al*., 2015). The estimated vaccine efficacy of A/California/07/2009 (H1N1) was between, 21% and 46% against the A/H1N1 pdm09 Kenyan circulating strains displaying a vaccine efficacy estimation of below 50%. This efficacy range has been reported in Cameroon and Thailand, in the range of 24.55 and 35.77%, (Monamele *et al*., 2019). The $P\text{-}_{epitope}$ model, estimates have displayed how close the Kenyan circulating strains are close to the vaccine strain and emphasize the significance of considering genetic data from the globe for the selection and formulation of flu vaccine. The vaccine's perfect match for the A/H1N1pdm09 ranges from 38.77% to 86.79%, indicating a higher efficaciousness when compared with A/H3N2 Kenyan strains.

The antigenic distance of seasonal H1N1 Kenyan strains was estimated *in silico* against the vaccine strains A/New Caledonia/20/1999, A/Solomon Islands/3/2006, and A/Brisbane/59/2007 for the period 2007-2009 respectively. The year 2007 was characterized by mutations S121N, K140E, and V165A (dominant epitope A), R73K, and E273K for 2008 (dominant epitope B). In 2009, six mutations were observed in the dominant epitope B, suggesting a worst-case VE of 38.68% (E=20.5% of 53%). The few mutations in the A/sH1N1 during 2007- 2009 differentiating them from the WHO-recommended vaccines indicate that non-synonymous changes in the HA1 gene especially at the antigenic region, have been more frequent in A/H3N2, than the A/sH1N1. These findings are in agreement with the previous studies in France (Falchi *et al*., 2011).

This study had a few shortcomings. First, most of the HA sequences available were of partial lengths, necessitating the use of only the HA1 region of the HA gene for the

analyses. Some of the sequence data lacked full information regarding collection dates, limiting temporal- special analysis. Furthermore, since serologic assays more particularly the hemagglutination inhibition assay (HAI) were not performed, it was not possible to definitively determine that indeed this mutation resulted in an antigenically distinct strain. The serological data would have served to confirm the net effect of genetic variations noticed in the HA1 domain on vaccine efficacy calculated by the P-$_{epitope}$ method. Cognisant of the fact that the antigenic properties of the Kenya influenza A subtypes and vaccine efficacy data reported in the present study were generated based solely on genetic and *in-silico* P-$_{epitope}$ method, the findings should be interpreted with caution since vaccine efficacy studies in the context of the antigenic distance between the vaccine and circulating strains can be inaccurate due to mutations introduced to the vaccinating virus during propagation in eggs (Skowronski *et al*., 2014). Despite the limitations, the present study has outlined the genetic and evolutionary aspects of human influenza A viruses that circulated in Kenya from 2007 to 2013 influenza seasons and highlighted the discrepancy in vaccine efficaciousness.

CHAPTER SIX

CONCLUSION AND RECOMMENDATIONS

6.1 Conclusion

A. Based on the observation of this study, the study concludes that the influenza A viruses that circulated in Kenya during the study period were antigenically similar to global strains but genetically distinct. The evolution patterns were in the same trajectories as the global strains.
B. The A/H3N2 isolates clustered into three lineages consistent with the year of isolation, while the sH1N1 were in clade B characterized mainly by E273K amino acid change. The majority of A/H1N1pdm09 belonged to clade 7 while a minority in clade 5 and 6A
C. The estimated dN/dS value estimates revealed a purifying selection among the Kenyan isolates signifying the amino acid changes across the period of the study did not favor the virus adaptability
D. Likewise, the study has shown that in the seven years from 2007-2013, the WHO recommended influenza A vaccines for Kenya and the southern region just before, during, and post 2009 influenza pandemic were variably effective. The amino acid variations at the hemagglutinin gene are attributable to dominant epitope shifts and vaccine efficacy decline

6.2 Recommendations

I. The study findings underscore the importance of and need for consistent surveillance and advanced molecular characterization of influenza viruses globally. Information from surveillance programs such as the one instituted in Kenya by DoD GEIS inform decision-making across the world and enhance

global health security by guiding decisions regarding the appropriate annual influenza vaccine formulations.

II. The evolution of the A/H3N2 virus in the country was observed to be largely via progressive genetic drift. This may not have been clear if the analysis of HA1 was not performed. This finding, therefore, underscores the need for intensified surveillance to monitor the diversity of influenza A viruses.

III. Further studies should be conducted to compare the clade 7 dominance and adaptation to transmissibility among Kenyan and global strains

IV. Regular vaccine studies are recommended using the P- epitope model and other models available to determine the viruses' genetic and antigenic changes and to be well prepared in the event of shifts and drifts that trigger human epidemics and pandemics.

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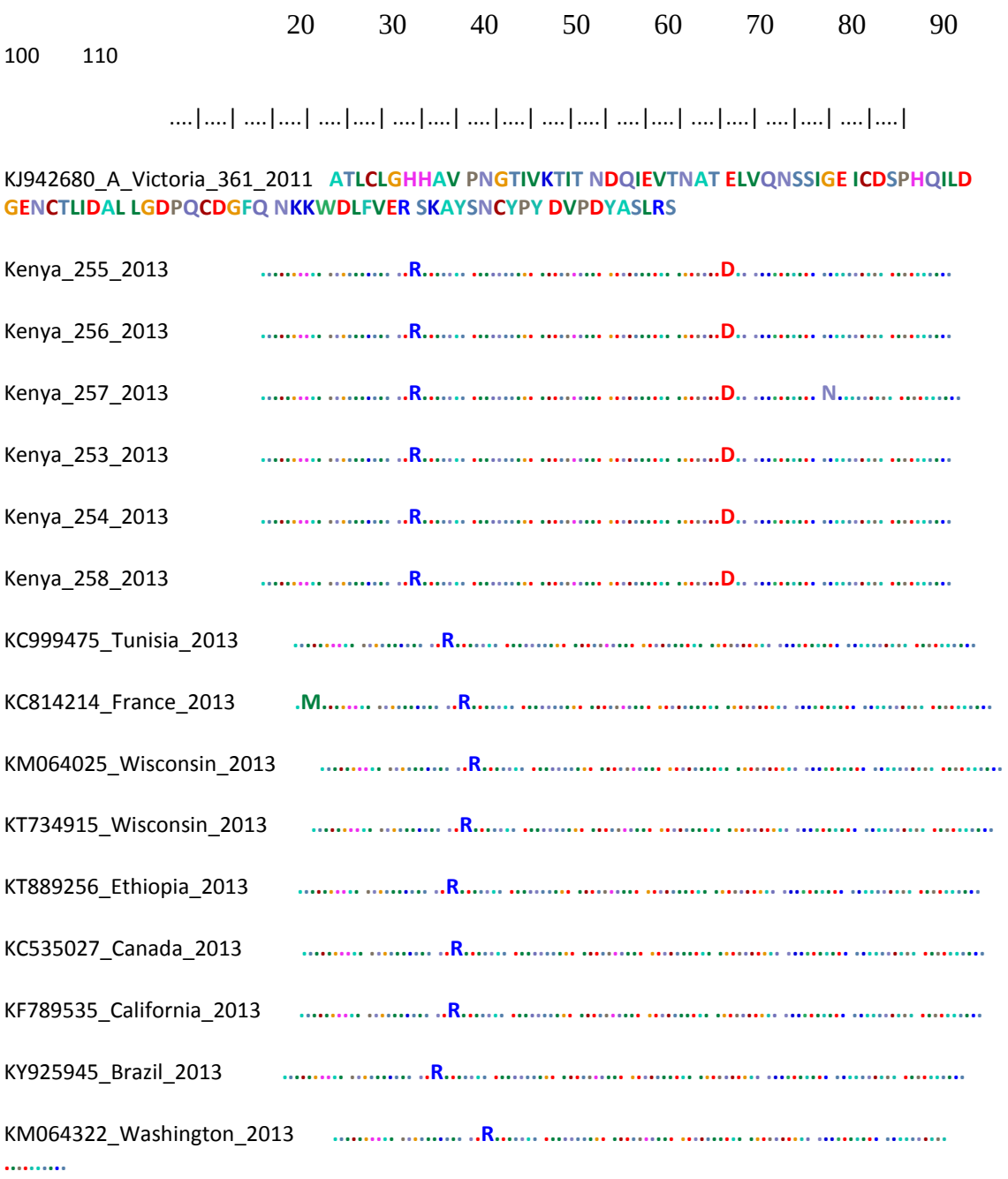
APPENDICES

Appendix I: A/H3N2 HA1 domain amino acid sequence comparison



KJ144640_Spain_2013 .....R.....

KF790147_Texas_2013 .....R.....

KY451443_S.Africa_2013 .....R.....

KY451452_S.Africa_2013 .....R.....

KY451445_S.Africa_2013 .....R.....

KF790482_Florida_2013 .....R.....

CY163426_New York_2013 .....R.....

GQ293081_A_Perth_16_2009 .....S..T.. .....K.....

Kenya_248_2012 .....T..

Kenya_245_2012 .....R..... .....E..... .....D..

Kenya_250_2012 .....T.. ..N.....

JX978755_Russia_2012 .....S..T..

KF598718_Canada_2012 .....S..T..

JX262049_Italy_2012 .M..... .....N.....

KC430918_Morocco_2012 .....S..T..

JX978453_Tunisia_2012 .....S..T..

KT889235_Russia_2012

KY925954_Brazil_2011 .....S..T.. ..N..... .....H.....

KC883387_Wisconsin_2011 .....S..T.. ..N..... .....H.....

JX978462_Tunisia_2011 .....S..T..

KM821347_Australia_2011

Kenya_008_2010 .....S..T..

Kenya_001_2010 .....A ....T.. ....D..

Kenya_212_2010 .....T..

Kenya_003_2010 ..K.....T..

Kenya_007_2010 .....T..

Kenya_024_2010 .....T..

Kenya_80_2010 .....T..

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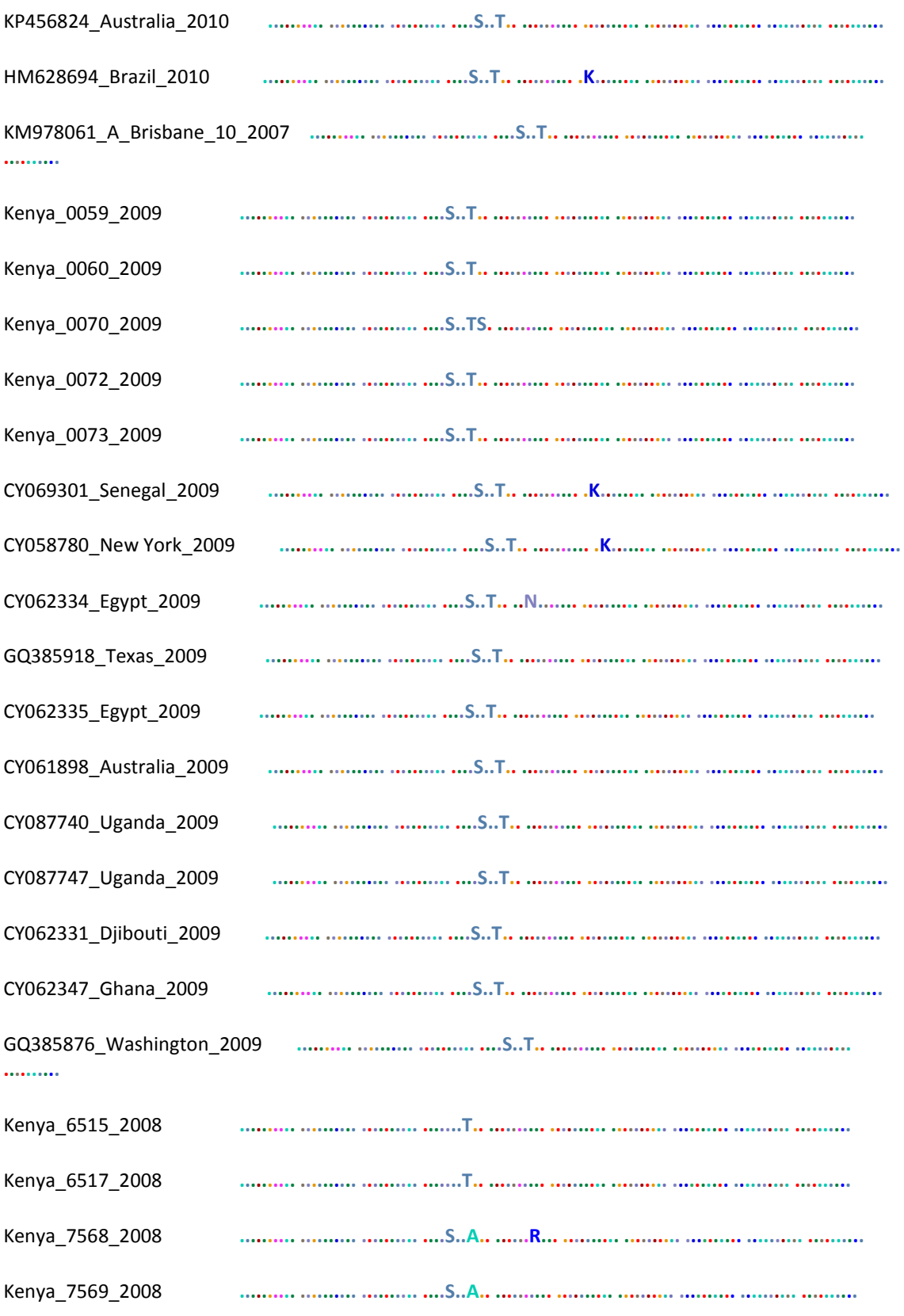
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Kenya_0073_2009
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CY058780_New York_2009
CY062334_Egypt_2009
GQ385918_Texas_2009
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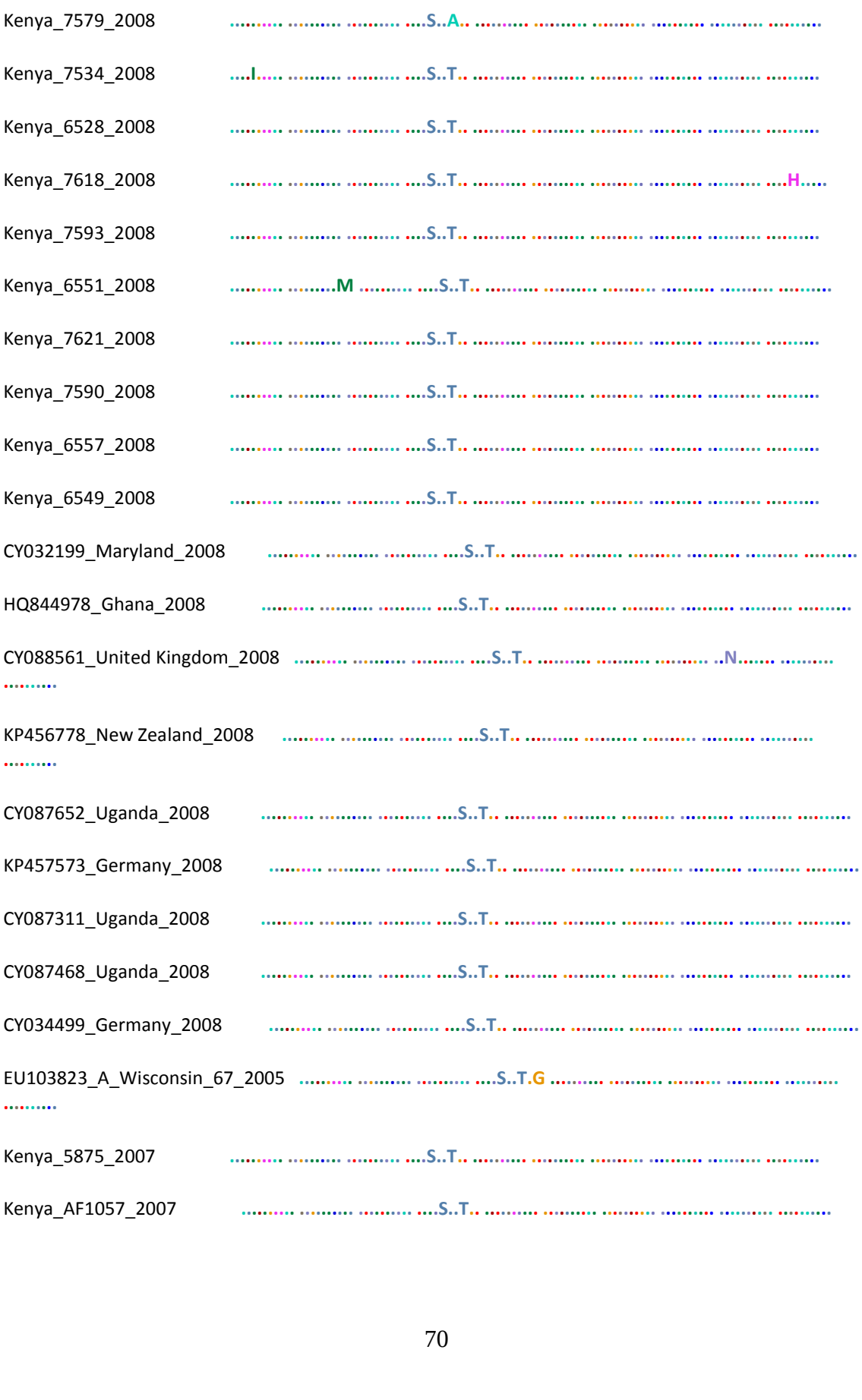
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Kenya_7590_2008
Kenya_6557_2008
Kenya_6549_2008
CY032199_Maryland_2008
HQ844978_Ghana_2008
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CY087652_Uganda_2008
KP457573_Germany_2008
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Kenya_5875_2007
Kenya_AF1057_2007

Kenya_AF1058_2007 ....S..T.G ....

Kenya_AF5618_2007 ....S..T.G ....

Kenya_AF5622_2007 ....S..T.. ....

Kenya_AF4041_2007 ....S..T.G ....

Kenya_AF1055_2007 ....S..T.G ....

Kenya_AF1056_2007 ....S..T.. ....

Kenya_AF5617_2007 ....S..T.G ....

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CY088540_United Kingdom_2007 ....S..T.. ....
.....

CY031829_South Africa_2007 ....S..T.. ....

CY031827_South Africa_2007 ....S..T.. ....

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CY025843_New York_2007 ....S..T.G ....

CY114413_Netherlands_2007 ....S..T.. ....

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Kenya_257_2013 ....A.. .....R .G..S..... .....H.... ....G...D ....

Kenya_253_2013 ....A.. .....R .G..S..... .....H.... ....G...D ....

Kenya_254_2013 ....A.. .....R .G..S..... .....H.... ....G...D ....

Kenya_258_2013 ....A.. .....R .G..S..... .....H.... ....G...D ....

KC999475_Tunisia_2013 ....N..... ....S..... .....H.... ....G..RD .....
.....

KC814214_France_2013 ....S..... .....H.... ....G...D ....

KM064025_Wisconsin_2013 ....T ....S..... .....H.... ....G...D .....
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KT889256_Ethiopia_2013 ....S..... .....H.... ....G...D ....

KC535027_Canada_2013 ....S..... .....H.... ....G...D ....

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KY925945_Brazil_2013 ....A.. ..... .G..S..... .....H.... ....G...D ....

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KY451443_S.Africa_2013 ....N...A.. ..... .G..S..... .....H.... ....G...D .....
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KY451452_S.Africa_2013 ....N...A.. ..... .G..S..... .....H.... ....G...D .....
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JX978453_Tunisia_2012 .....S..... .....H..... .....G...D .....

KT889235_Russia_2012 .....M ....S..... .....H..... .....G...D .....

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KM821347_Australia_2011 .....H..... .....G...D .....

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Kenya_001_2010 .....H..... .....G...D .....A.. .....

Kenya_212_2010 .....H..... .....E..... .....G...D .....A.. .....

Kenya_003_2010 .....H..... .....G...D .....A.. .....

Kenya_007_2010 .....H..... .....G...D .....A.. .....

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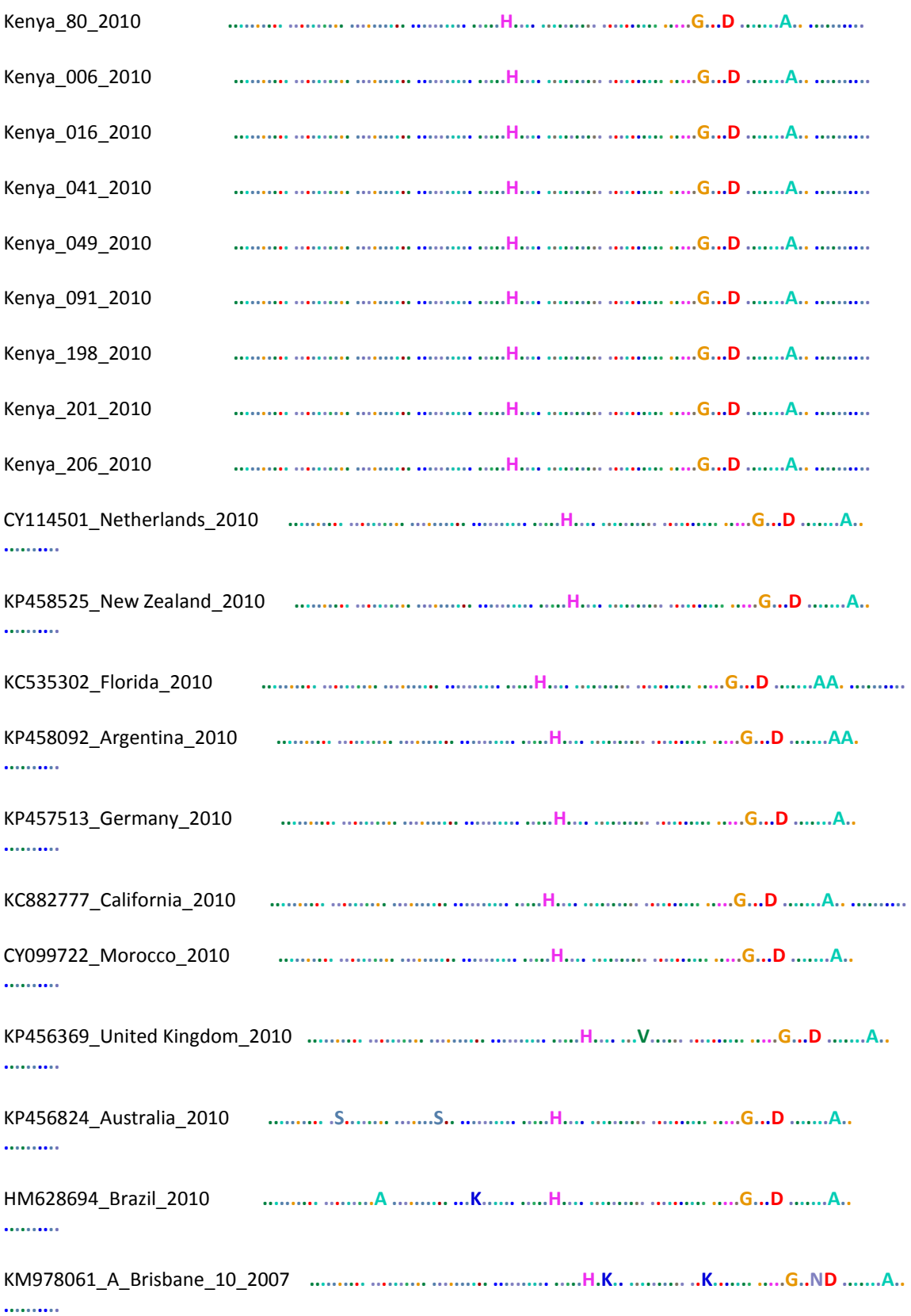
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Kenya_6515_2008
Kenya_6517_2008
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Kenya_7569_2008

Kenya_7579_2008 ....H.K.. ....G..ND .....A.. .....

Kenya_7534_2008 .I..... ....S..... .....H.K.. ....G..ND .....A.. .....

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KP457573_Germany_2008 ....H.K.. ....G..ND .....A.. .....R...

CY087311_Uganda_2008 .I..... ....H.K.. ....G..ND .....A.. .....

CY087468_Uganda_2008 .I..... ....H.K.. ....G..ND .....A.. .....

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Kenya_AF5618_2007 ....H.K.. ....K..... .....G..ND .....A..
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Kenya_AF5622_2007 ....H.K.. ....K..... .....G..ND .....A..
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EU100720_Florida_2007 ....K .G.D..... .....H.K.. ....K..... .....G..ND .....A..
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220 230 240 250 260 270 280 290 300 310

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Kenya_254_2013 .....S. .....K..

Kenya_258_2013 .....S. .....K..

KC999475_Tunisia_2013 .....S. .....K.. .....R.....

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KM064025_Wisconsin_2013 .....S. .....K..

KT734915_Wisconsin_2013 .....S. .....K..

KT889256_Ethiopia_2013 .....S. .....K..

KC535027_Canada_2013 .....S. .....K..

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KM064322_Washington_2013 .....S. .....X. .....K..
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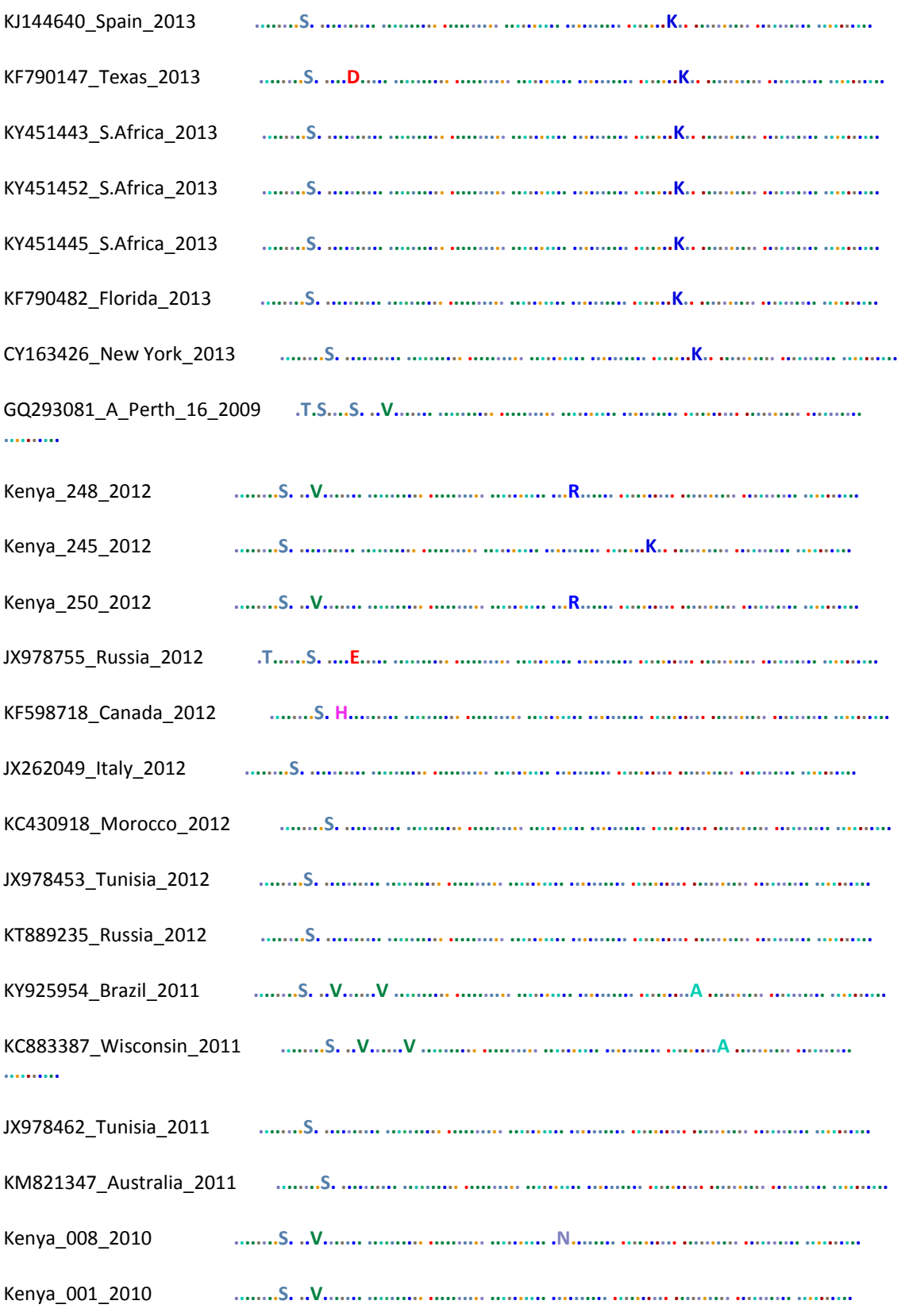
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Kenya_245_2012
Kenya_250_2012
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Kenya_206_2010 .....S. ..V..... ....

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KC535302_Florida_2010 .....S. ..V.....V ....A ....

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Kenya_7534_2008 .T.....S. ..V.....

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CY032199_Maryland_2008 .T.....S. ..V.....

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CY031829_South Africa_2007 .T.....S. ..V..... ..W.....
.....

CY031827_South Africa_2007 .T.....S. ..V.....

EU659851_Canada_2007 .T.....S. ..V..... ....D.....

EU100720_Florida_2007 .T.....S. ..V.....

CY022874_Germany_2007 .T.....S. ..V..... ....S.....
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CY114413_Netherlands_2007 .T.....S. ..V.....
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....|....| ....|

KJ942680_A_Victoria_361_2011 QSTLKLATGM RNVPE

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Kenya_254_2013 ....

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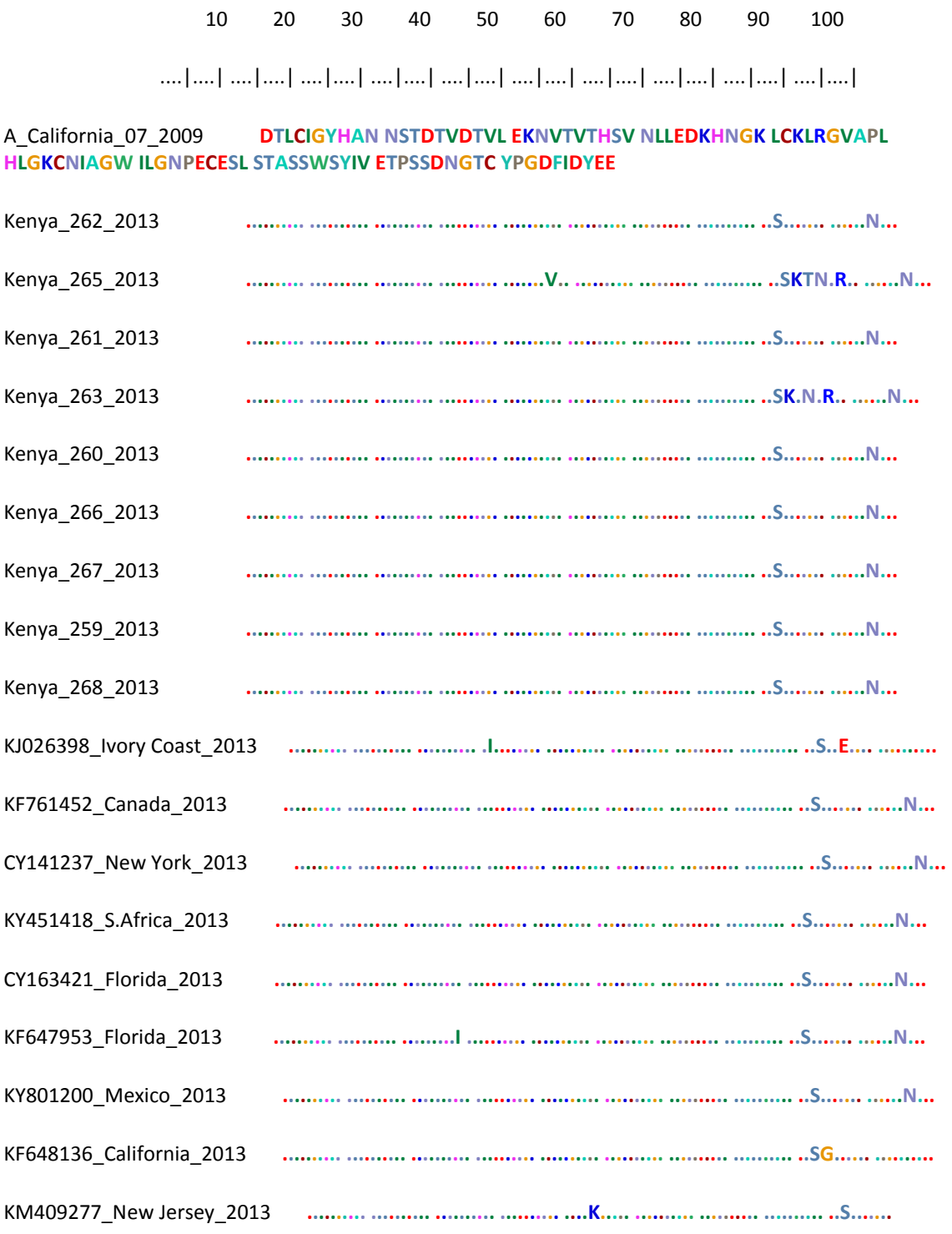
Appendix II: A/H1N1pdm09 HA1 domain amino acid sequence comparison



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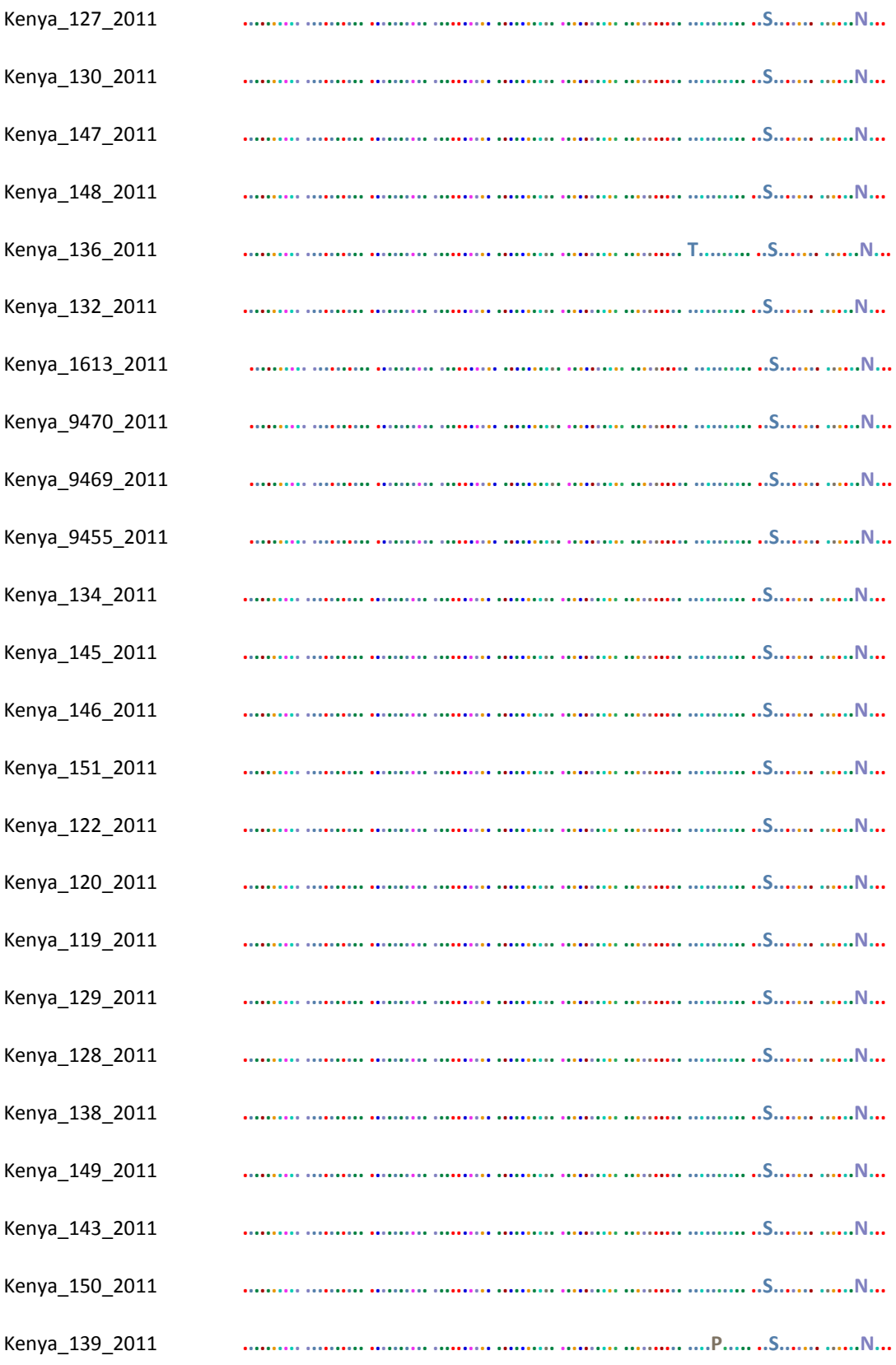
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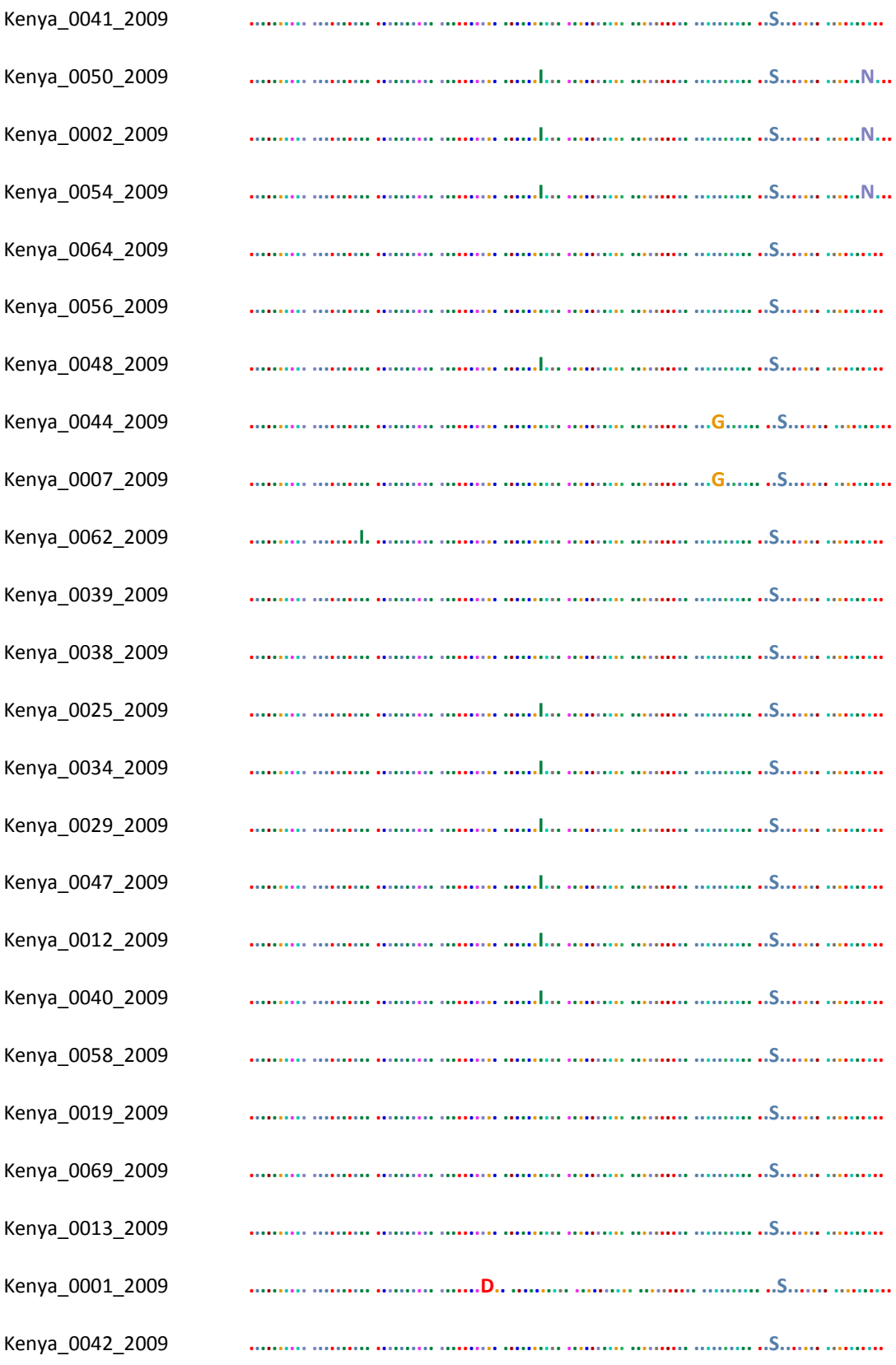
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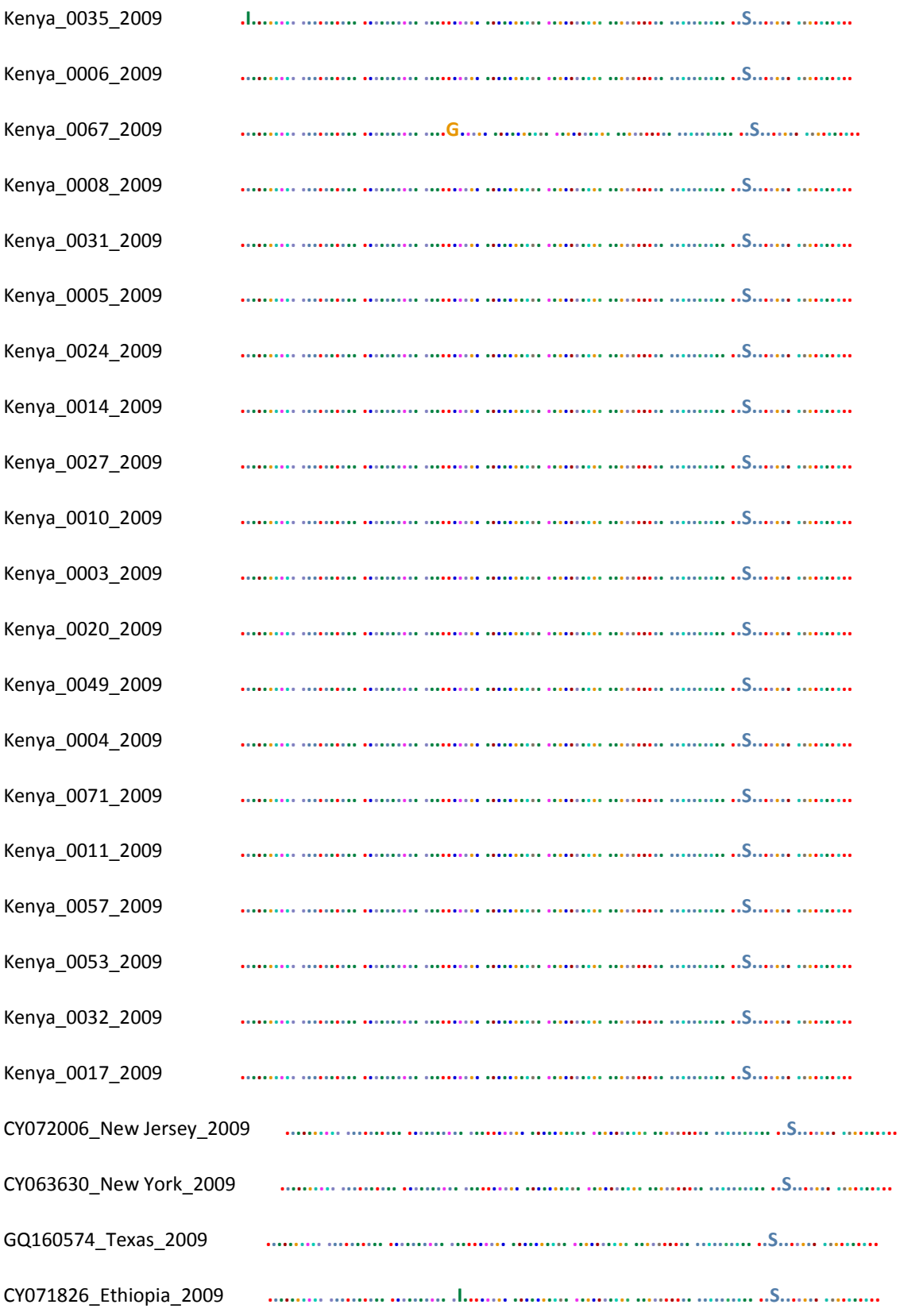
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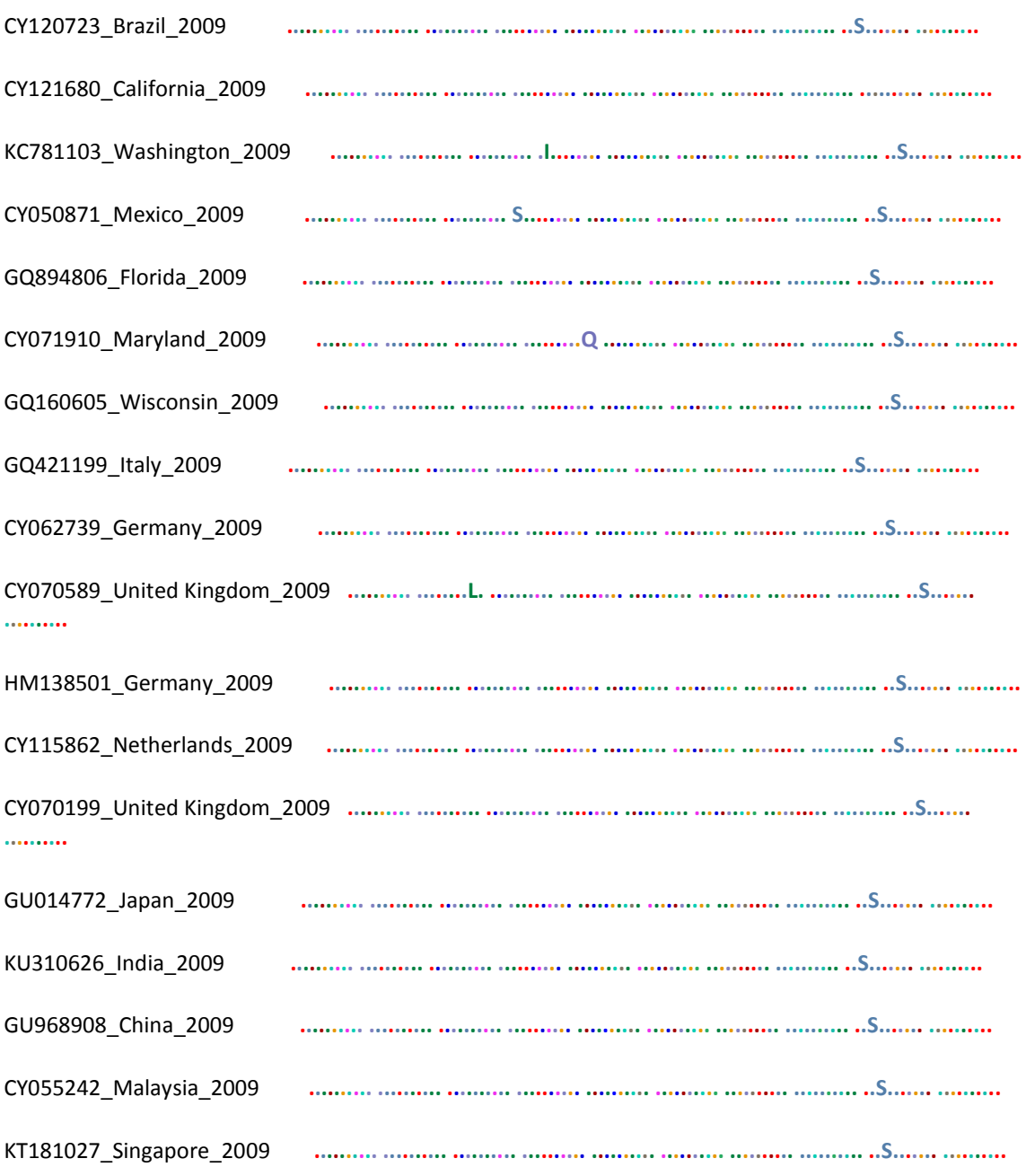
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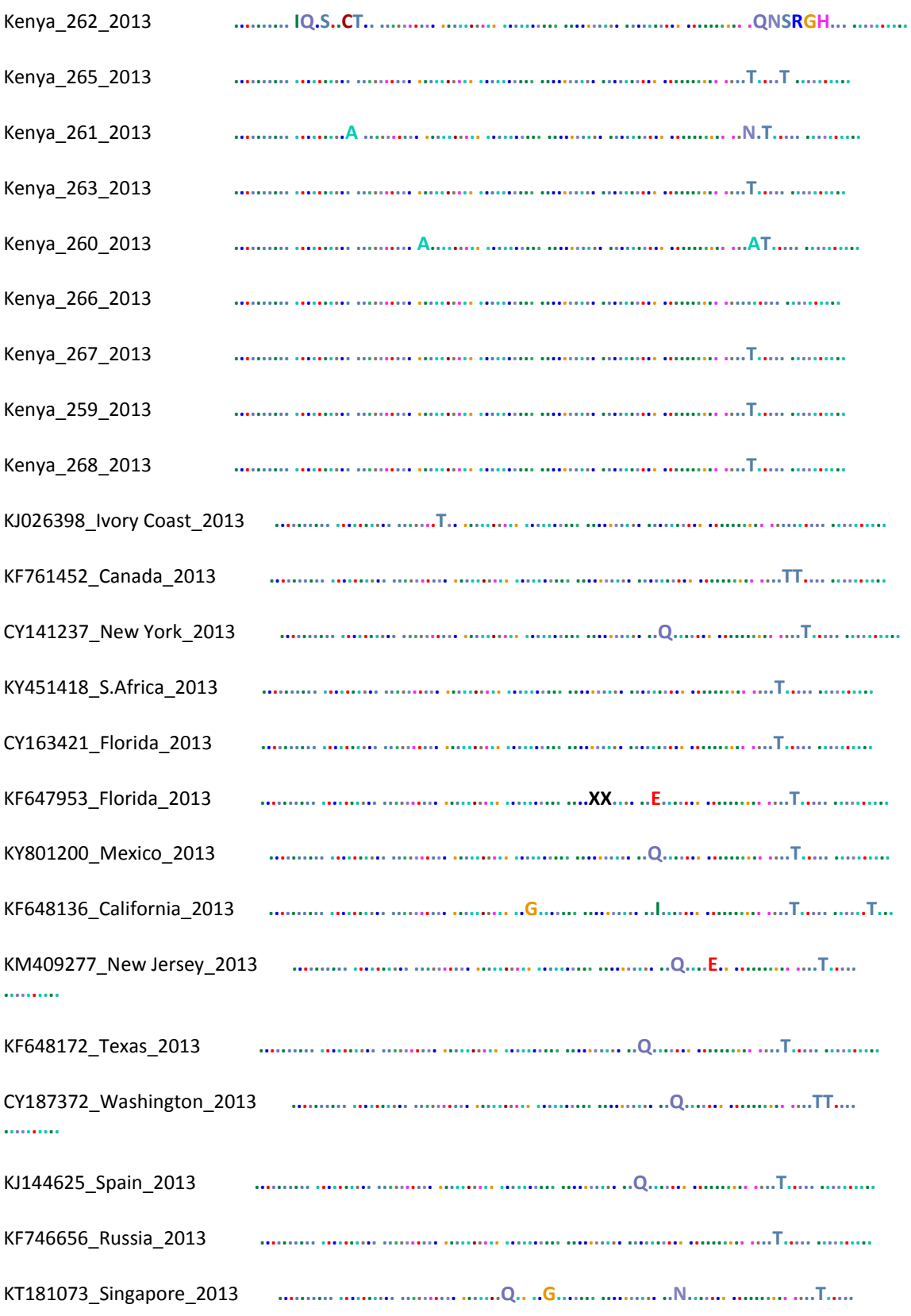
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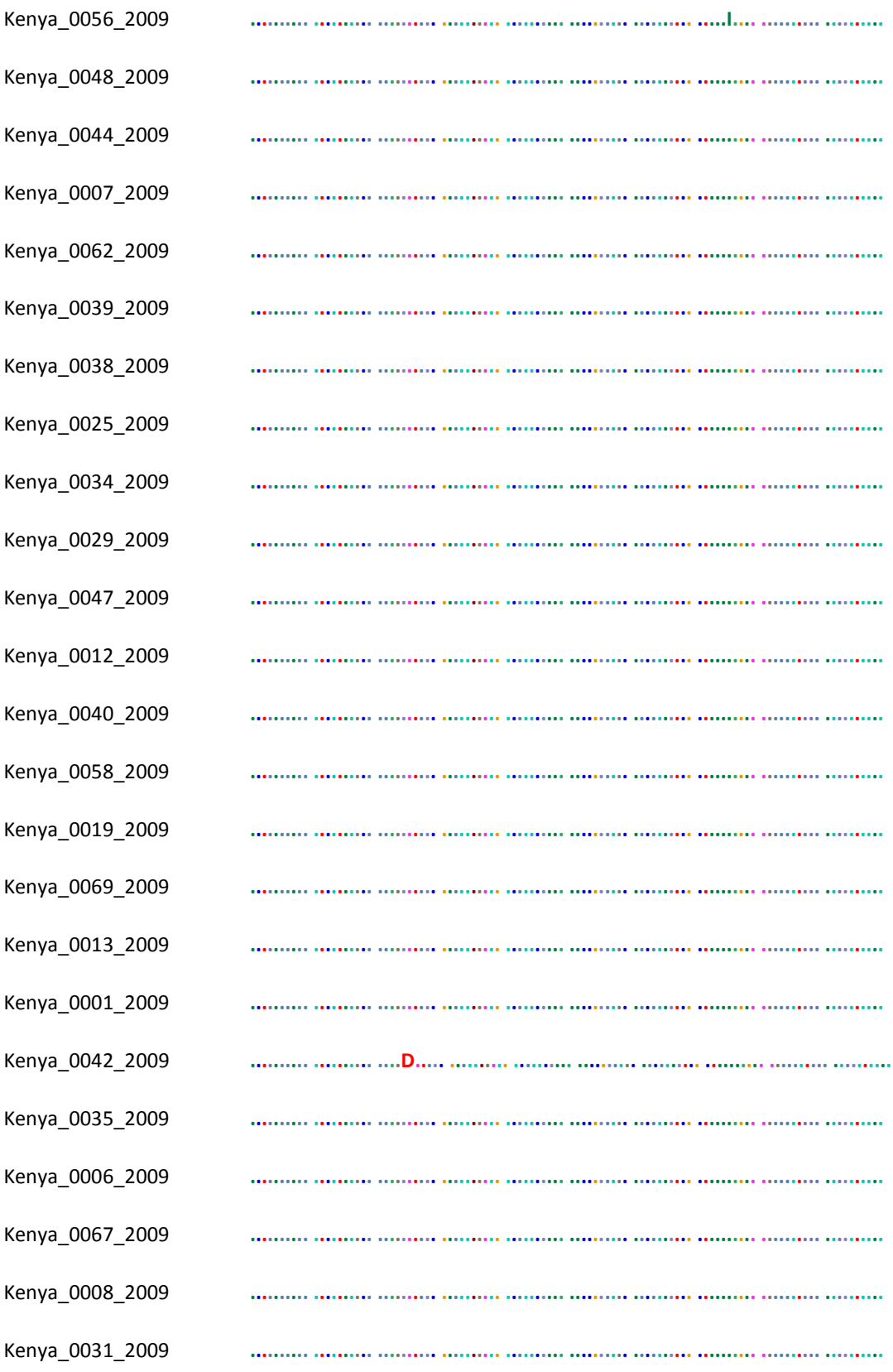
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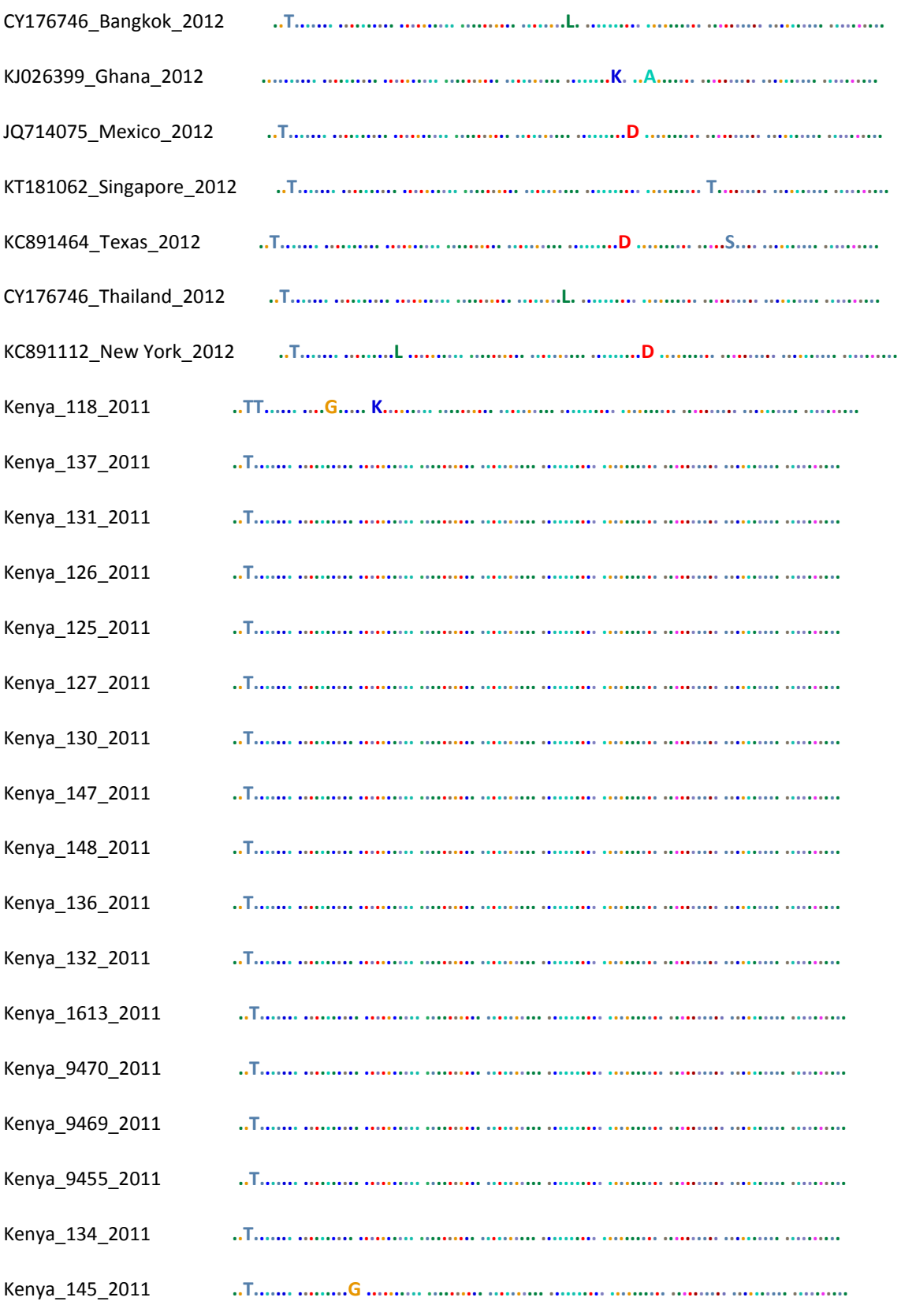
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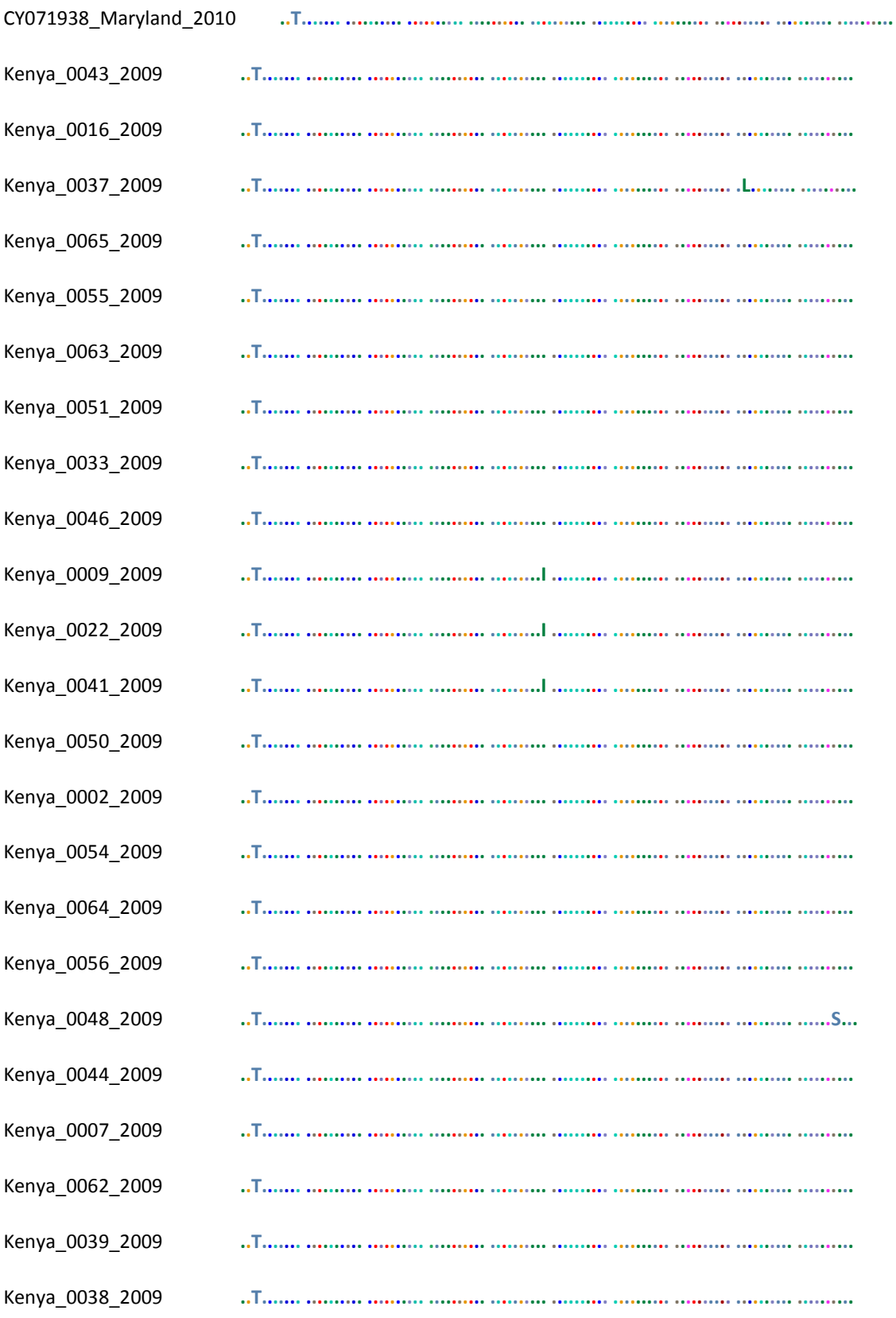
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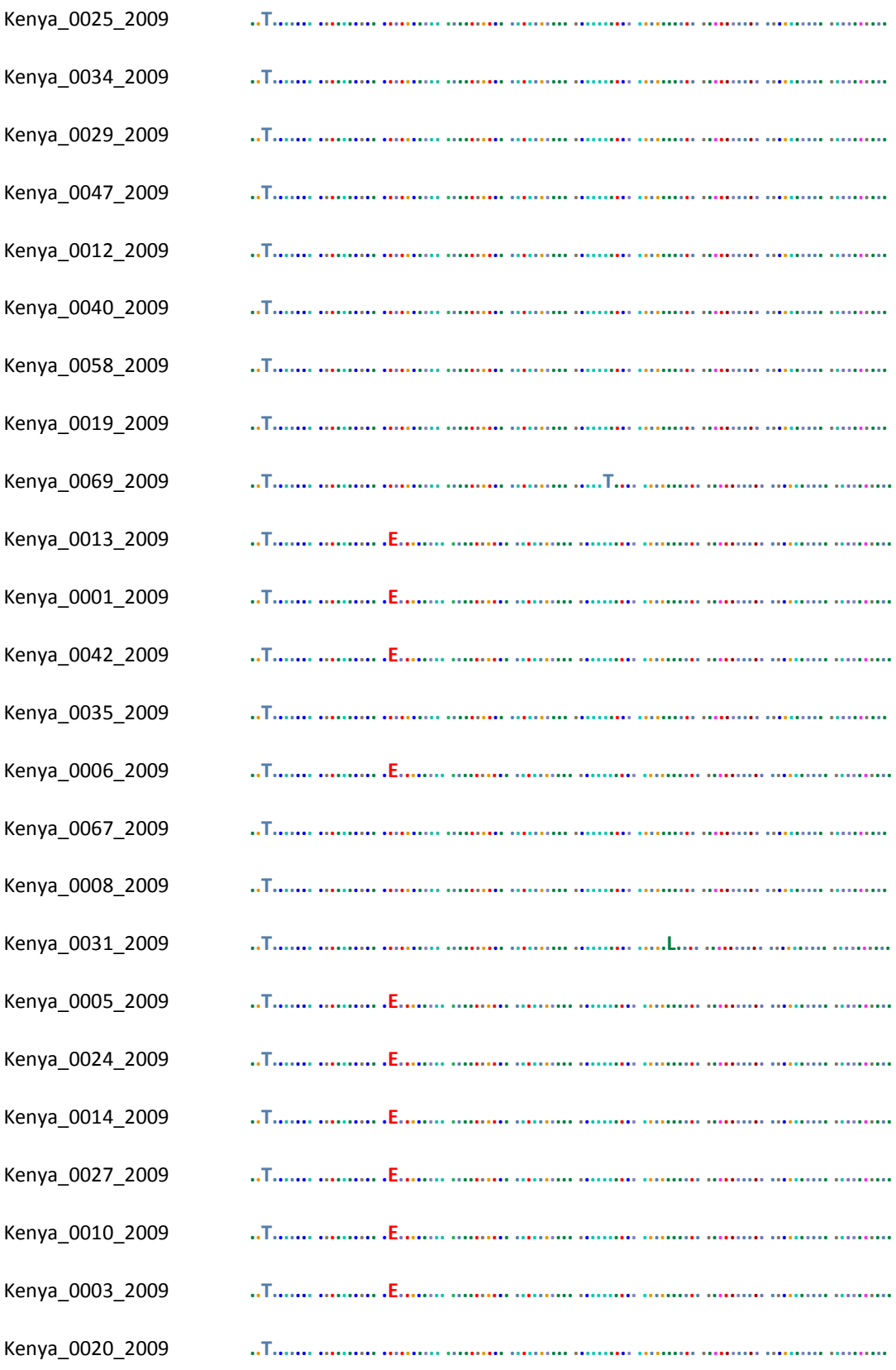
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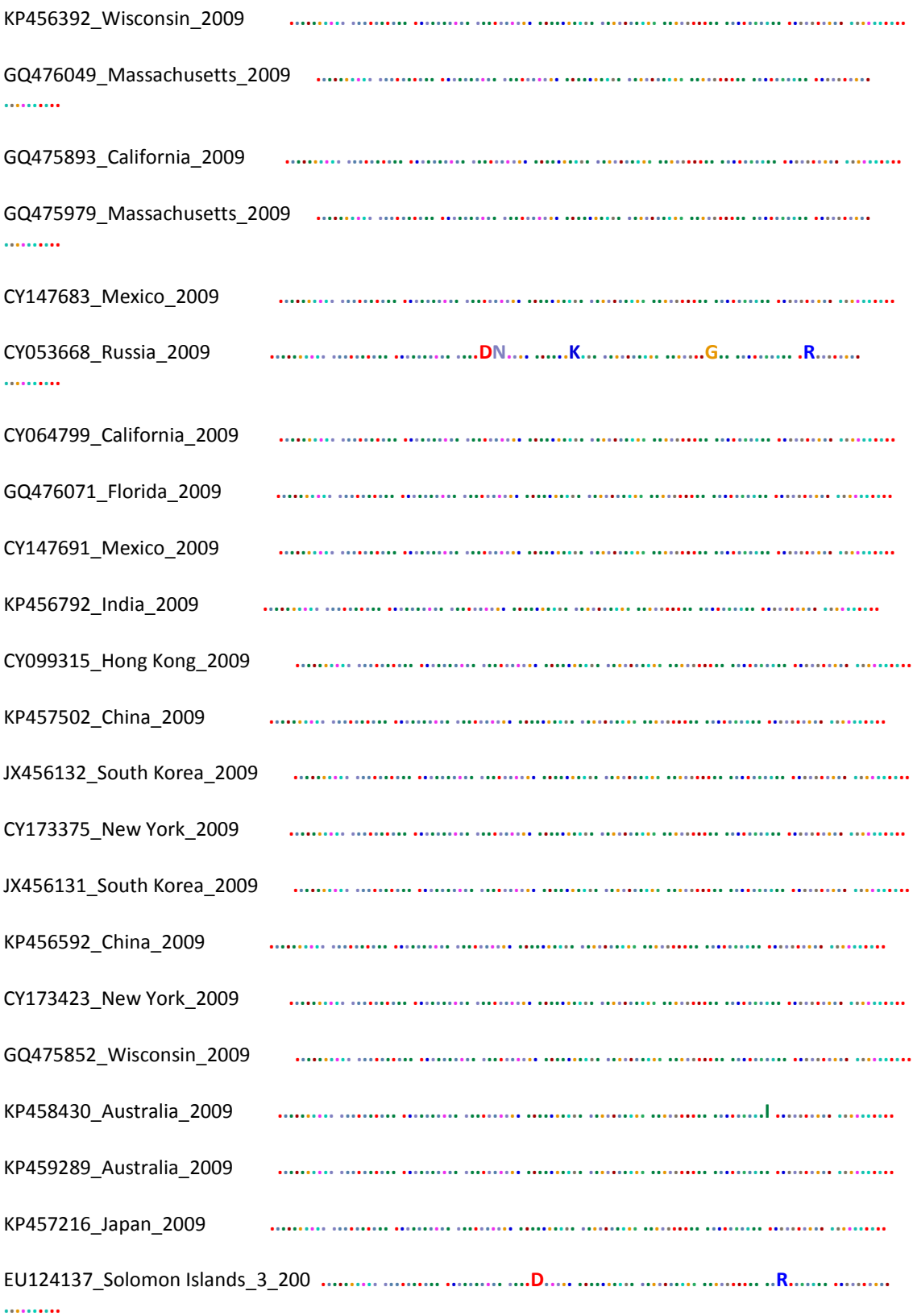
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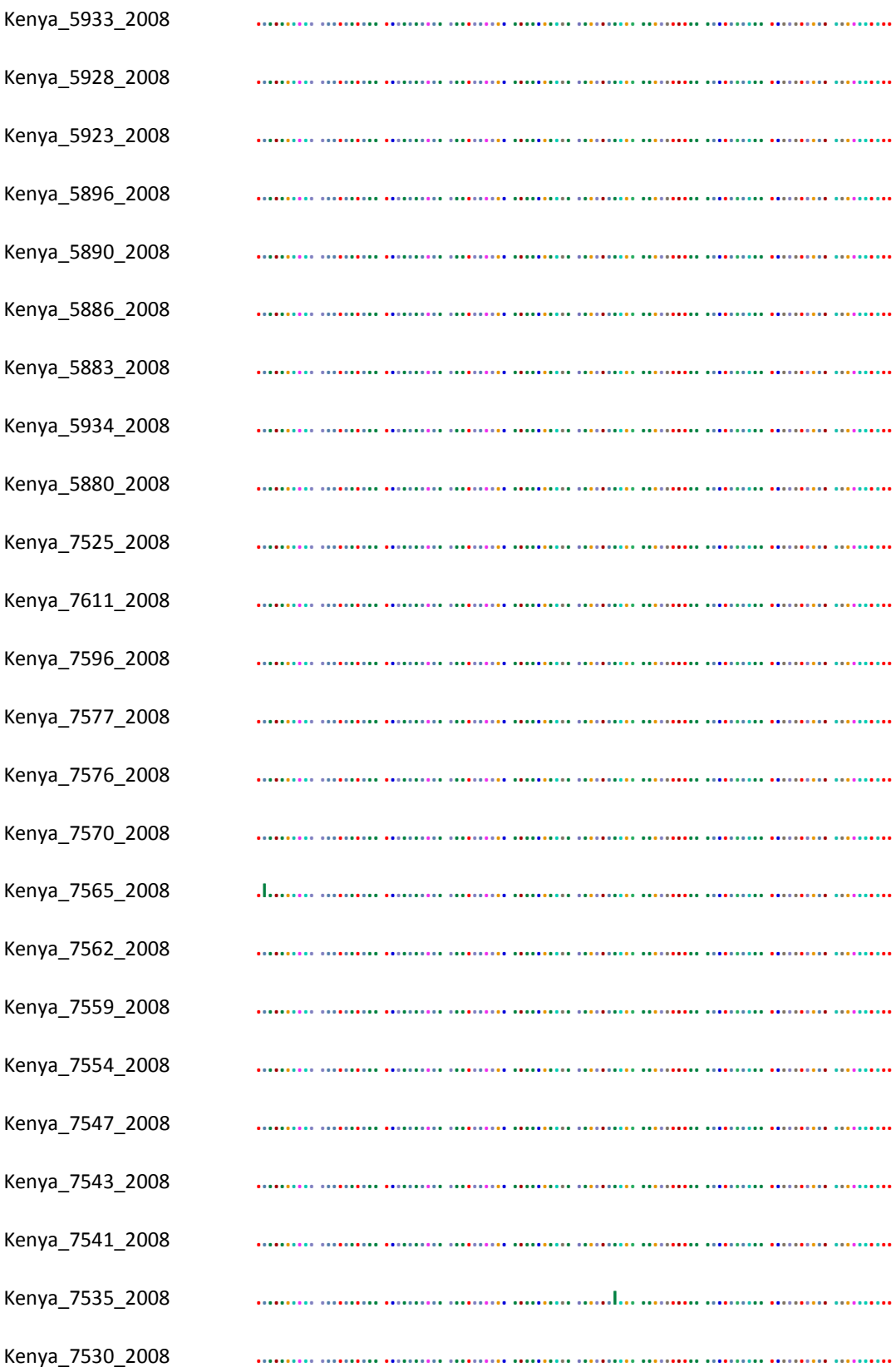
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AB510200_Japan_2008
KP457153_China_2008


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CY031364_South Africa_2007 ....

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KP457880_France_2007 .... K..... .....

AB451164_Japan_2007 ....DN.... ....

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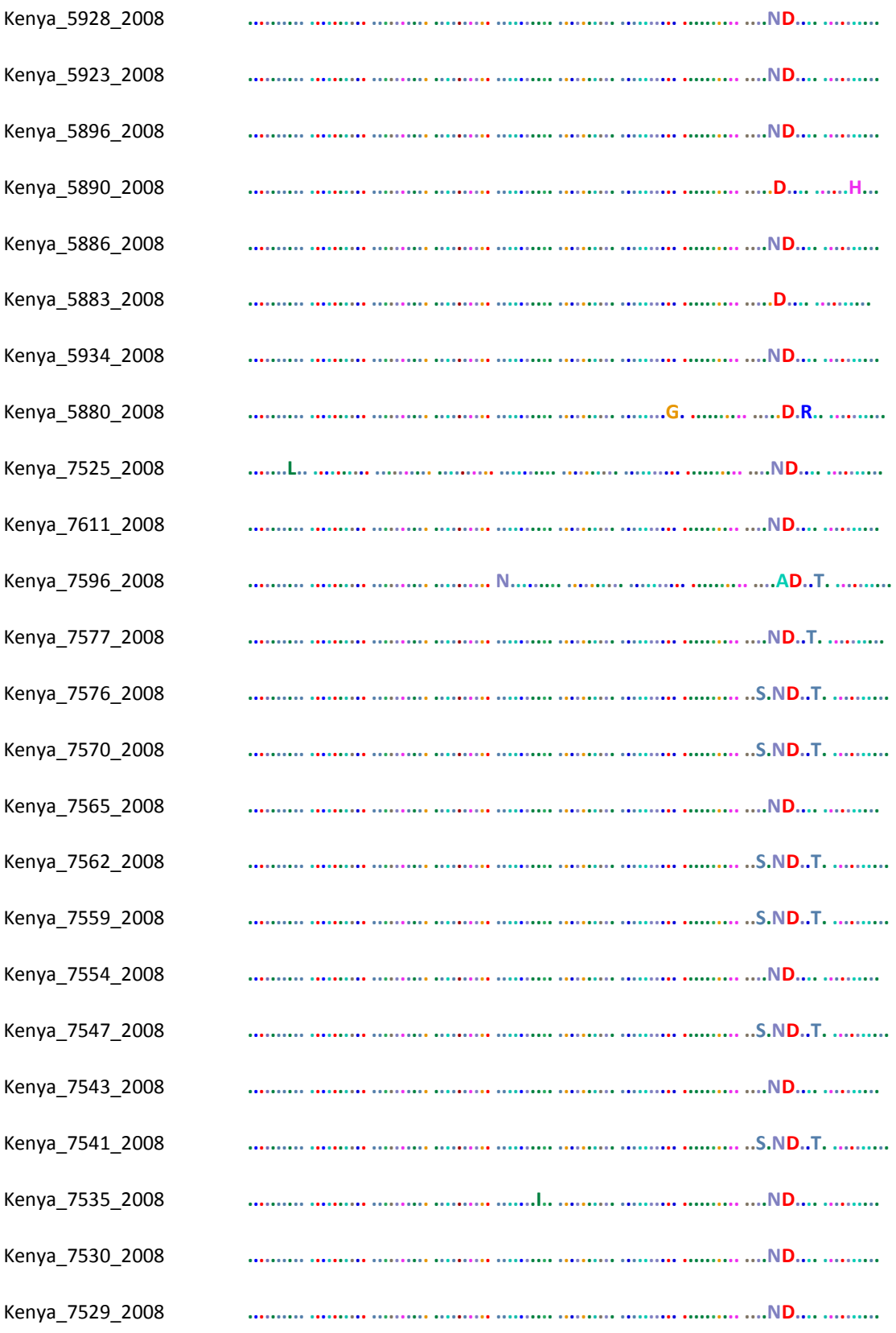
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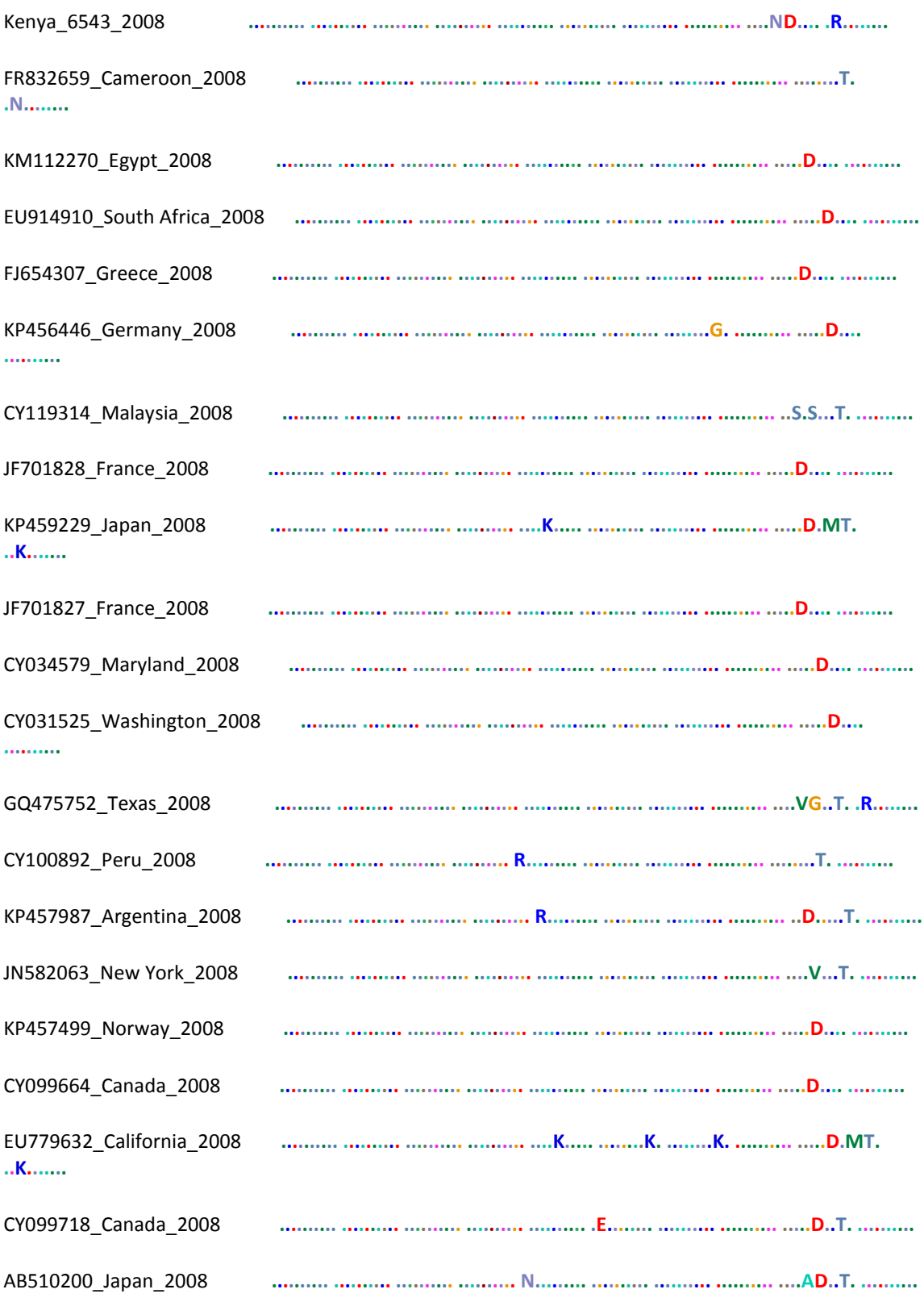
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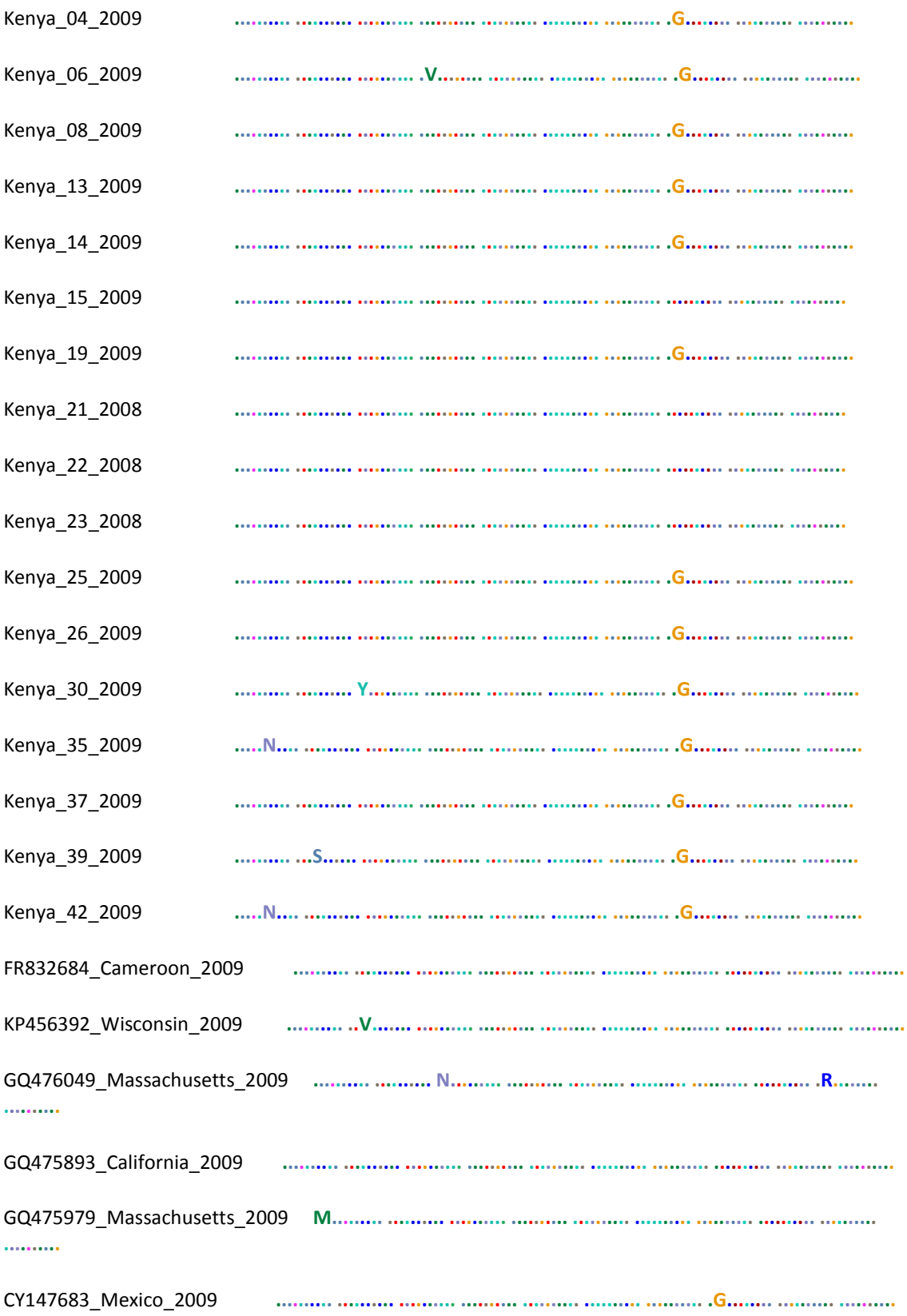
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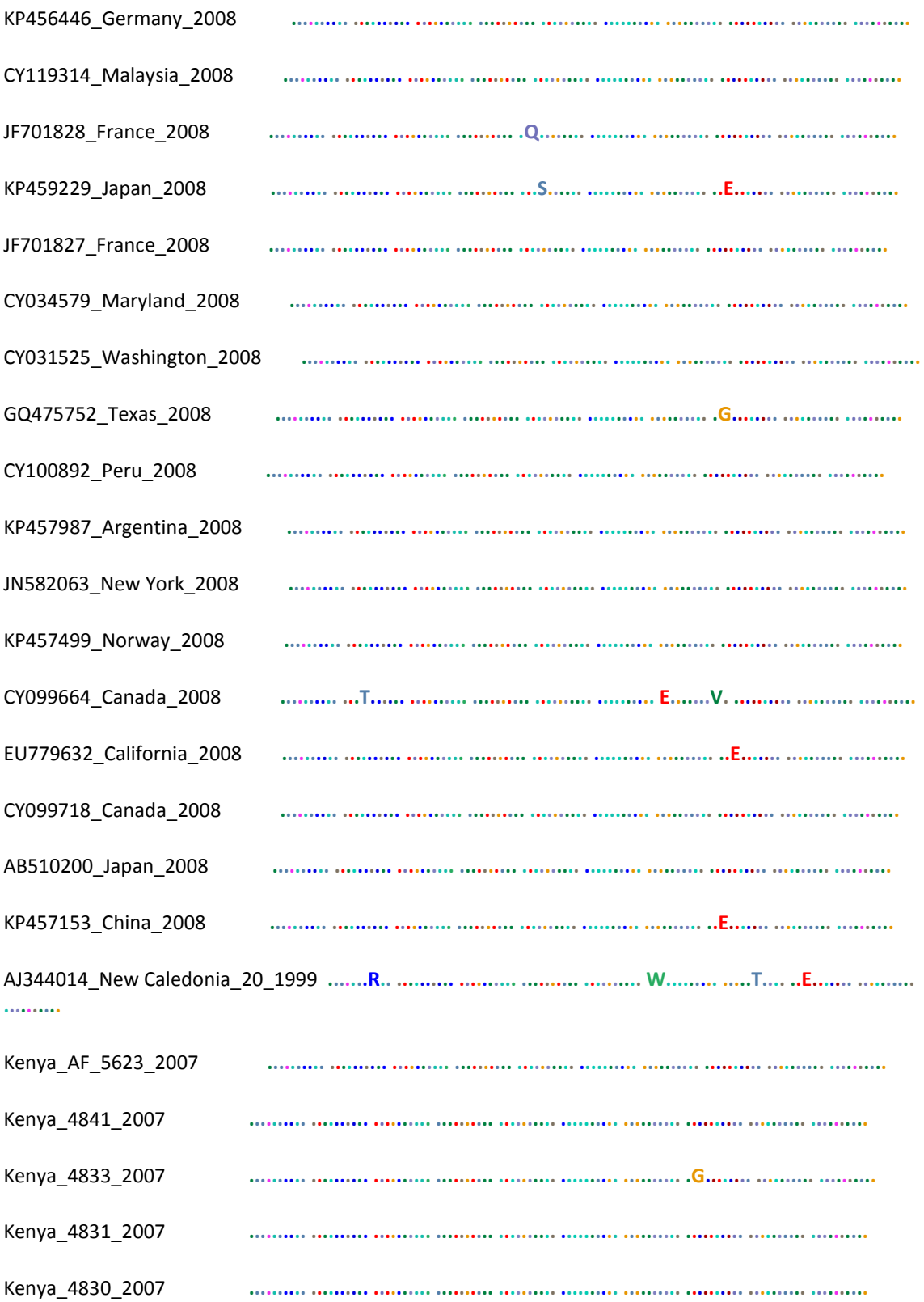
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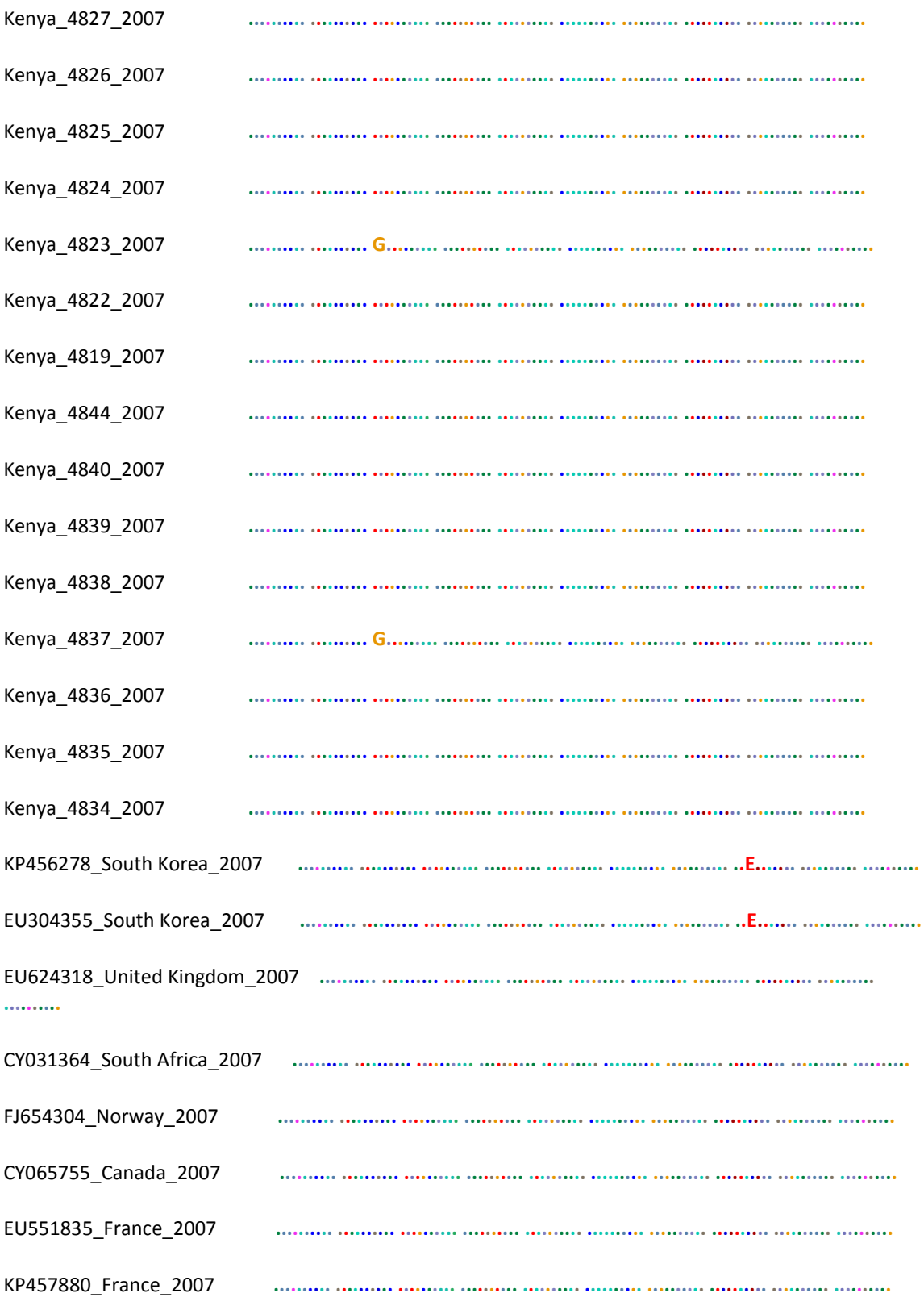
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Kenya_7565_2008

Kenya_7562_2008

Kenya_7559_2008

Kenya_7554_2008

Kenya_7547_2008

Kenya_7543_2008

Kenya_7541_2008

Kenya_7535_2008

Kenya_7530_2008

Kenya_7529_2008

Kenya_6543_2008

FR832659_Cameroon_2008

KM112270_Egypt_2008

EU914910_South Africa_2008

FJ654307_Greece_2008

KP456446_Germany_2008

CY119314_Malaysia_2008

JF701828_France_2008

KP459229_Japan_2008

JF701827_France_2008

CY034579_Maryland_2008

CY031525_Washington_2008

GQ475752_Texas_2008

CY100892_Peru_2008

KP457987_Argentina_2008

JN582063_New York_2008

KP457499_Norway_2008 ..……. ……. …..

CY099664_Canada_2008 ..……. ……. …..

EU779632_California_2008 ..……. ……. …..

CY099718_Canada_2008 ..……. .M..…… ..T..

AB510200_Japan_2008 ..……. ……. …..

KP457153_China_2008 ..……. ……. …..

AJ344014_New Caledonia_20_1999 ..……. ……. …..

Kenya_AF_5623_2007 ..……. ……. …..

Kenya_4841_2007 ..……. ……. …..

Kenya_4833_2007 ..……. ……. …..

Kenya_4831_2007 ..……. ……. …..

Kenya_4830_2007 ..……. ……. …..

Kenya_4827_2007 ..……. ……. …..

Kenya_4826_2007 ..……. ……. …..

Kenya_4825_2007 ..……. ……. …..

Kenya_4824_2007 ..……. ……. …..

Kenya_4823_2007 ..……. ……. …..

Kenya_4822_2007 ..……. ……. …..

Kenya_4819_2007 ..……. ……. …..

Kenya_4844_2007 ..……. ……. …..

Kenya_4840_2007 ..……. ……. …..

Kenya_4839_2007 ..……. ……. …..

Kenya_4838_2007 ..……. ……. …..

Kenya_4837_2007 ..……. ……. …..

Kenya_4836_2007 ..a.....t. .a.....t. .....

Kenya_4835_2007 ..a.....t. .a.....t. .....

Kenya_4834_2007 ..a.....t. .a.....t. .....

KP456278_South Korea_2007 ..a.....t. .a.....t. .....

EU304355_South Korea_2007 ..a.....t. .a.....t. .....

EU624318_United Kingdom_2007 ..a.....t. .a.....t. .....

CY031364_South Africa_2007 ..a.....t. .a.....t. .....

FJ654304_Norway_2007 ..a.....t. .a.....t. .....

CY065755_Canada_2007 ..a.....t. .a.....t. .....

EU551835_France_2007 ..a.....t. .a.....t. .....

KP457880_France_2007 ..a.....t. .a.....t. .....

AB451164_Japan_2007 ..a.....t. .a.....t. .....

KP458190_United Kingdom_2007 ..a.....t. .a.....t. .....

CY099658_Canada_2007 ..a.....t. .a.....t. .....

CY026331_Texas_2007 ..a.....T. .a.....t. .....

EU716621_Florida_2007 ..a.....t. .a.....t. .....

KP458508_California_2007 ..a.....T. .a.....t. .....

CY172599_New York_2007 ..a.....t. .a.....t. .....

CY031362_New Zealand_2007 ..a.....t. .a.....t. .....

KP456315_Peru_2007 ..a.....t. .a.....t. .....

Appendix IV: Publication

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Genetic analysis of HA1 domain of influenza A/H3N2 viruses isolated in Kenya during the 2007–2013 seasons reveal significant divergence from WHO-recommended vaccine strains

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ABSTRACT

Background: Influenza viruses evolve rapidly and cause regular seasonal epidemics in humans challenging effective vaccination. The virus surface HA glycoprotein is the primary target for the host immune response. Here, we investigated the vaccine efficacy and evolution patterns of human influenza A/H3N2 viruses that circulated in Kenyan in the period before and after the 2009 A/H1N1 pandemic, targeting the HA1 domain.

Materials and methods: A hundred and fifteen HA sequences of Kenyan virus viruses were analyzed relative to the corresponding WHO vaccine reference strains using bioinformatics approaches.

Results: Our analyses revealed varied amino acid substitutions at all the five antigenic sites (A–E) of the HA1 domain, with a majority the changes occurring at sites A and B. The Kenyan A/H3N2 viruses isolated during 2007/2008 seasons belonged to A/Brisbane/10/2007-like viruses lineage, while those circulating in 2009–2012 belonged to the lineage of A/Victoria/361/2011-like viruses. The 2013 viruses clustered in clade 3C.3 of the A/Samara/73/2013-like viruses. The mean evolutionary rate of the A/H3N2 viruses analyzed in the study was at 4.17×10^{-3} (95% HPD = 3.09×10^{-3}–5.31×10^{-3}) nucleotide substitutions per site per year, whereas the TMRCA was estimated at 11.18 (95% HPD = 9.00–14.12) years ago from 2013. The prediction of vaccine efficacy revealed modest vaccine efficaciousness during 2008, and 2010 influenza seasons, whilst sub-optimal effectiveness was registered in 2007, 2009, 2012 and 2013. Further, the overall selective pressure acting on the HA1 domain was estimated at 0.56 ($\omega < 1$), suggesting that a majority of codon sites in the HA1 epitopes were evolving under purifying selection.

Conclusions: Generally, our results highlight the genetic plasticity of A/H3N2 viruses and reveal considerable disparity in vaccine efficaciousness against the A/H3N2 viruses that circulated in Kenya, specifically during 2007, 2009, 2012, and 2013 influenza seasons. Our findings underscore the importance and need for consistent surveillance and molecular characterization of influenza viruses, to inform decision making and enhance early of detection of strains with epidemic/pandemic potential as well as benefit in guiding decisions regarding the appropriate annual influenza vaccine formulations.

Appendix V: Accession numbers in GenBank and GISAID of HA influenza A/H3N2, s/H1N1, and A/H1N1pdm09 gene sequences used in the study.

Name	Accession No.
A/H3N2-Kenyan strains (2007-20113)	EU204965, EU204967-68, EU221476, FJ265663, FJ662957, FJ662960, FJ662961 -64, FJ662966, FJ662972, FJ662988 -93, FJ663007-8, HM347425 -29, HQ185195, HQ185200 -01, HQ185204, HQ185207, HQ185211, HQ229614, HQ229626, HQ229636, JQ396182 -3, KF451872, KF451875, KF451877, KF451880 -85, KX431187 -88, KX431191, KX431195, CY022909 -12
A/H1N1-Kenyan strains (2007-2009)	AJ344014, ABW34452, ACE81955-73, EU124137, ACI41211-19, ACM45837-53, JN899402, ADH59464, ADH59508, ADH59494, ADH59522, ADH59530, ADH59529, ADH59467, ADH59496, ADH59513, ADH59469, DH59524, ADH59479, ADH59518, ADH59491, ADH59499,
A/H1N1pdm09-Kenyan strains (2009-2013)	ADH59491-95, DH59466-79, ADH59513-29, ADN23950, ADH59480-511, ADH59530-31, AFB77616-20, AFB77623-27, AFB77628-33, KJ680526-29, AFB77634-43, AGT18734-35, AGT18718-25,
A/H3N2 reference and vaccine strains	KF598718, KF789535, KF790147, KJ144640, KJ942680, KM064322, KM978061, KM978061, KP456778,KP456824, KP456369, KP457611, KP458092, KT734915, KT889256, JQ655463, JX239588, JX978453, KC430918, KC535027, KC882777, KC999475, HM628694, GQ293081, GQ385918, FJ445770, EU659851, KY925954, CY031827, CY031829, CY032199, CY032557, CY034499, CY061898, CY062331, CY062334, CY062335, CY062347, CY062347, CY069301, CY087311, CY087468, CY087652, CY087747, CY088540, CY099722, CY114413, EPI301374, EPI305337, EPI316191, EPI335734, EPI335854, EPI346607, EPI352716, EPI356601, EPI381901, EPI407060, EPI460558, EPI556816, EPI606448, EPI607276, EPI684747, EPI684771, EU100720, EU103823, HQ844978, CY0558780, KT889256, KF789535, KT735915
A/H1N1 reference and vaccine strains	AJ344014, CY172599, EPI139098, CY031362, KP457153, EU124137, EU521840, KP456315, CY099664, CY031364, KM112270, JN899402, FJ654304, EU914910, EU716621, EPI171440, KP458190, CY031525, KP457499, FJ654307,

	CY034579, KP456446, CY099658, EU551835, EPI314471, JF701827, EPI314484, JF701828, FR832659, CY173375, CY100892, JN582063, KP458430, GQ475893, GQ475852, AB510200, KP456952, KP457216, KP459289, CY065755, KP456792, GQ476071, CY064799, KP456392
A/H1N1pdm09-referenceand vaccine strains (2009-2013)	CY064799, CY050871, GQ169382, GQ160574, GQ160605, GQ421199, CY120723, CYO70199, CY071826, KJ026398-99, EPI319447, EPI280343, CY070589, CY080331, JN603276, KF041867, LN846454, CY055242, KC781103, CY072074, CY071910, CY071375, EPI302574, GQ894806, CY063630, GU576542, CY062739, CY057039, LN867423, CY115862, GU014772, KF918703, KT181027, KC882285, CY129590, JF327373, CY176405, JX204749, KC881612, KC781303, CY071938, EPI352290, KC881596, CY176746, KP317224, GU968908, JX120566, EPI320141, KT181062, KC881615, HM138501, KJ144651, KP941689, EPI539470, KF761452, KF746656, KY451418, CY163421, KF647953, KM408913