Infection, Genetics and Evolution 9 (2009) 840–846

Contents lists available at ScienceDirect

Infection, Genetics and Evolution

journal homepage: www.elsevier.com/locate/meegid

Molecular characterization of non-subtype C and recombinant HIV-1 viruses from Cape Town, South Africa☆

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ARTICLE INFO

Article history:
Received 30 December 2008
Received in revised form 30 April 2009
Accepted 3 May 2009
Available online 14 May 2009

Keywords:
HIV-1
Non-subtype C
Phylogenetics
Recombinants
Cape Town
South Africa

ABSTRACT

HIV was first diagnosed within South Africa in 1982. Homosexual transmission of HIV-1 dominated the epidemic within the country in the early stages of the 1980s. Currently heterosexual transmission of HIV-1 is responsible for the majority of HIV cases in South Africa with subtype C HIV-1 being responsible for an estimated 95% of infections. Only a few papers have been published on non-subtype C HIV-1 detection within South Africa. This study characterized subgenomic and near full-length sequences of non-subtype C HIV-1 viruses from the Cape Town area. Amplification and direct sequencing characterized partial gene fragments of 11 samples. Phylogenetic analysis of the sequenced data, with online subtyping tools (REGA and jpHMM) and the drawing of NJ-trees revealed the presence of subtypes A1, B, and F1 as well as recombinant viral forms such as AD, AG and AC. Near full-length genome characterization of 4 of the 11 samples was performed. Analysis of sequenced data with the use of subtyping, recombination identification, and tree drawing tools revealed one subtype B and one A1 isolate. The other two isolates were identified as AC and AD recombinants. The data that was gathered will greatly improve our knowledge of non-subtype C isolates circulating within South Africa.

1. Introduction

The human immunodeficiency virus (HIV) is characterized by a high degree of genetic variation. The virus exists in two distinct forms, HIV-type 1 (HIV-1) and HIV-type 2 (HIV-2) (Peeters, 2001). HIV-1 has spread all over the world, whereas HIV-2 is mostly confined to areas of West Africa (Essex and Mboup, 2002). HIV-1 can be divided into three groups: group M (major), group N (non-M or non-O) and group O (outlier) (Peeters, 2001). Group M HIV-1 is responsible for the majority of infections worldwide and can be subdivided into nine subtypes and at least 43 circulating recombinant forms (CRFs) (http://www.hiv.lanl.gov). Subtype C HIV-1 is responsible for nearly 52% of HIV infections worldwide and is found in parts of the Indian subcontinent and eastern and southern Africa (Ariën et al., 2007).

In South Africa the HIV-1 epidemic was initially associated with homosexual transmission (Sher, 1989). These viruses were later identified as HIV-1 subtype B and D (Becker et al., 1995; Engelbrecht et al., 1995). By the late 1980s a second subtype C HIV-1 epidemic was recognized amongst heterosexual individuals and has since then became the major method of transmission (van Harmelen et al., 1997, 1999). Today the HIV-1 epidemic has spread widely with as many as 6.2 million infected people in South Africa alone (UNAIDS, 2008). HIV-1 subtype C has been extensively studied in South Africa (Engelbrecht et al., 1995, 2001; Becker et al., 1995; van Harmelen et al., 1997, 1999, 2001; Hunt et al., 2001, 2003; Treurnicht et al., 2002; Gordon et al., 2003; Bessong et al., 2005; Bell et al., 2007; Jacobs et al., 2008a,b; Rousseau et al., 2006; Papathanasopoulos et al., 2002; zur Megede et al., 2002).

Although HIV-1 subtype C still maintains a dominant position within southern Africa, HIV-1 diversity may impact on a wide spectrum of fields, e.g. antiretroviral treatment (Spira et al., 2003), diagnostics and the development of an effective vaccine (Buonaguro et al., 2007; Peeters et al., 2003). To date only a few reports have been published on non-subtype C HIV-1 isolates from South Africa (van Harmelen et al., 1997; Engelbrecht et al., 1995; Loxton et al., 2005; Papathanasopoulos et al., 2001; Bredell et al., 2002; Rousseau et al., 2006).

During a previous study we have characterized the V3 region of 410 virus isolates and detected non-subtype C isolates in and around the Cape Town area (GenBank accession numbers EF547460–EF547511). In this study the aim was to characterize

☆ Nucleotide sequence data reported in this paper are available in GenBank database under the accession numbers: FJ647145–FJ647168.

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doi:10.1016/j.meegid.2009.05.001

partial gene fragments and near full-length genomes (NFLGs) of some of these isolates.

2. Materials and methods

2.1. Specimens and DNA isolation

HIV-1 positive samples are routinely collected for molecular characterization at Tygerberg Academic Hospital and surrounding clinics in the Cape Town area. Four hundred and ten samples were previously sequenced in the V3 region. Nine suspected non-subtype C were chosen for analysis based on their V3 sequencing data. One subtype C isolate and one subtype B isolate were also included (Table 1). DNA was extracted from uncultured peripheral blood mononuclear cells (PBMC) by using the QIAamp DNA mini Kit (QIAGEN, Hilden, Germany) according to the manufacturer's protocol.

2.2. DNA amplification

Prenested and nested, *gag* p24 (485 bp), *pol-integrase* (944 bp) and *env* gp41 (438 bp), amplification assays were performed on all 11 samples with GoTaq DNA polymerase (Promega, Madison, Wisconsin, USA). The PCR methods and primers were adapted from Swanson et al., 2003. Briefly, each amplification reaction was carried out in a final volume of 50 μl containing; 0.2 mM of each dNTP, 20 μM of forward and reverse primers, 1.5 mM of $MgCl_2$, and 1 U of *Taq* polymerase. Samples were denatured at 95 °C for 1 min, followed by 40 cycles of 94 °C for 30 s, 45 °C or 50 °C primer annealing for 30 s, 68 °C extension for 60 s, and a final extension step of 68 °C for 10 min. For the *pol-integrase* assays the 68 °C extension time was increased to 90 s. For each reaction 2.5 μl of product was carried over to the nested reactions which was performed with the same conditions and cycling parameters as the prenested PCRs.

Four (R84, TV329, TV314 and TV412) of the original 11 samples were chosen for near full-length genome (NFLG) amplification which was achieved in four overlapping fragments; LTR-*gag* (1.09 kbp), *gag-pol* (3.89 kbp), *pol-env* (3.94 kbp), and *env*-LTR (1.64 kbp) with the use of GoTaq DNA polymerase (Promega, Madison, WI, USA). A NFLG sequence is considered to be a sequence of more than 8000 bp, but shorter than the full-length HIV-1 genome of 9719 bp.

For the LTR-*gag* fragment the primer pairs MSF12 (Rodenburg et al., 2001) and p24-7 (Swanson et al., 2003) were used for the prenested reaction and Up1A (Mwaengo and Novembre, 1998) and p24-6 (Swanson et al., 2003) for the nested amplification reaction. For the *gag-pol* fragment, p24-1 and poli8 primers were used for the prenested PCR and p24-2 and poli6 for the nested PCR (Swanson et al., 2003). Poli7 and Menv19R were used for the amplification of the prenested *pol-env* products and PPF17 and LP7728 for the nested *pol-env* products (Swanson et al., 2003). The prenested env-LTR products were obtained with the use of primers 7496F and 9131R-2. Primers 7542F and 9110R-2 were used in the nested PCR assay.

Reaction and cycling conditions were adapted from the previously mentioned GoTaq PCR cycling conditions according to annealing temperature and fragment size. For the LTR-*gag* and *env*-LTR fragments an extension time of 90 s was used and an annealing temperature of 50 °C for both the prenested and nested amplification assays. Similarly an extension time of 4 min was used for the *gag-pol* and *pol-env* fragments with annealing temperatures of 45 and 50 °C for the prenested and nested amplification assays, respectively. Agarose gel electrophoresis and ethidium bromide staining was used to detect all nested PCR fragments.

Table 1
Demographic data of patient samples with the results of the phylogenetic analysis of the partial gene fragments.

Sample	Race and gender	Age	Country of infection	CD4	ARV treatment[a]	*gag* p24			*pol-integrase*			*env* gp41			Subtype of sample
						REGA data	jpHMM data	NJ-data	REGA data	jpHMM data	NJ-data	REGA data	jpHMM data	NJ-data	
R84	Caucasian male	72	South Africa	NA	No	B	B	B	B	B	B	B	B	B	B
TV86[b]	African male	35	South Africa	207	No	C	C	C	C	C	C	C	C	C	C
TV101	African female	23	South Africa	2000	No	A1	A1	A1	A1D	A1[c]	Unclass[d]	A1	A1	A1	A1 recombinant
TV218	African female	25	South Africa	NA	ND[e]	C	C	C	C	C	C	A1	A1[b]	A1	CA recombinant
TV239	African male	27	South Africa	64	No	A1	A1	A1	A1/H	A1[c]	A1	A1	A1	A1	A1 recombinant
TV314	African male	36	South Africa	229	No	A1	A1	A1	A1	A1[c]	A1	A1	A1[e]	A1	A1
TV340	African male	38	DRC[f]	3	No	–	–	–	G/B	G[c]	G	A1	A1	A1	AG recombinant
TV412	African male	46	Kenya	71	No	A1	A1	A1	A1/D	A1[c]	A1	A1	A1	A1	A1 recombinant
TV441	African female	25	South Africa	178	Yes	C	C	C	C	C[c]	C	A1	A1[e]	A1	CA recombinant
TV480	Coloured female	33	South Africa	NA	No	C	C	C	CJ	C	C	–	–	–	C recombinant
TV515	Coloured female	31	South Africa	NA	No	F1	F1	F1	F1	F1	F1	F1	F1	F1	F1

[a] ARV—antiretroviral.
[b] TV—Tygerberg virology.
[c] Bootstrap values were not supportive.
[d] Unclass—unclassifiable.
[e] ND, not determined.
[f] DRC—Democratic Republic of the Congo.

2.3. DNA sequencing

PCR products of *gag*, *integrase* and *env* gene fragments were prepared for sequencing with the PCR Product Pre-Sequencing Kit (USB Corporation, Cleveland, OH, USA). Near full-length genome amplified products; LTR-*gag* (1.09 kbp), *gag-pol* (3.89 kbp), *pol-env* (3.94 kbp), and *env*-LTR (1.64 kbp) were prepared for sequencing with the Wizard SV gel and PCR clean-up Kit (Promega, Madison, WI, USA). Both strands of each complete PCR product, were sequenced using the BigDye™ Terminator cycle sequencing ready reaction Kit (Applied Biosystems, Foster City, CA, USA) and appropriately chosen sequencing primers. Every sequencing reaction contained: 50 ng of the purified PCR product, 5 pmol of sequencing primer, 1.3 μl of Big Dye terminator enzyme mix, and 2.7 μl of Half Dye (Bioline, London, United Kingdom). Each sequencing reaction was performed under the following conditions: 25 cycles of denaturation at 96 °C for 10 s, primer annealing for 5 s and an elongation step at 60 °C for 4 min. Sequences were run on the ABI 3130xl automated DNA sequencer (Applied Biosystems, Foster City, California, USA).

Sequenced data were assembled into contiguous fragments in Sequencher v 4.8 (Gene Codes Corporation, Ann Arbor, MI, USA) and the assembled sequences were analyzed with GeneCutter to detect possible stop codons (http://www.hiv.lanl.gov/content/sequence/GENE_CUTTER/cutter.html).

2.4. Phylogenetic analysis

Partial *gag* (p24), *pol-integrase*, and *env* (*gp41*) fragments as well as assembled NFLG sequences were aligned with HIV-1 group M reference sequences (http://www.hiv.lanl.gov/content/sequence/NEWALIGN/align.html) using Clustal X (Thompson et al., 1997) and were manually edited with BioEdit v 7.0.9 (Hall, 2007).

Initial subtype determinations were performed on all sequenced data with the online viral subtyping tools: jpHMM at GOBICS (http://jphmm.gobics.de) (Zhang et al., 2006) and REGA v 2.0 (http://dbpartners.stanford.edu/RegaSubtyping) (de Oliveira et al., 2005). These results were confirmed with manual, detailed phylogenetic analysis. Neighbor-joining trees (Saitou and Nei, 1987) of the partial *gag*, *integrase* and *env* gene fragments as well as the NFLG fragments were constructed with MEGA v 4.1 (Tamura et al., 2007) with the use of the Kimura 2-parameter model of nucleotide substitution (Kimura, 1980). One thousand bootstrap replicates (Felsenstein, 1985) were performed on each dataset.

Sequenced fragments were also analyzed with the recombination identification programs; RIP (Siepel et al., 1995) and Simplot v 3.5.1 (Lole et al., 1999). The consensus alignment (excluding CRF01_AE) of the LANL database and a window size of 300 bp were used for the RIP analysis. Bootscan analysis was implemented in Simplot to identify recombinants, using a window and step size of 350 and 50 bp respectively with the multiple alignments obtained with Clustal X (Thompson et al., 1997) and BioEdit (Hall, 2007) as described.

After the recombinant identification was completed with Simplot and RIP, neighbor-joining trees of the different indicated recombinant sections were drawn. Briefly, multiple alignments of recombinant segments, containing pure viral subtypes, were constructed in Clustal X (Thompson et al., 1997), before neighbor-joining trees (Saitou and Nei, 1987) were constructed as described before.

2.5. Nucleotide sequence accession numbers

The sequences have been deposited in GenBank. Accession numbers are as follows: partial *gag*: FJ647150–FJ647155, partial

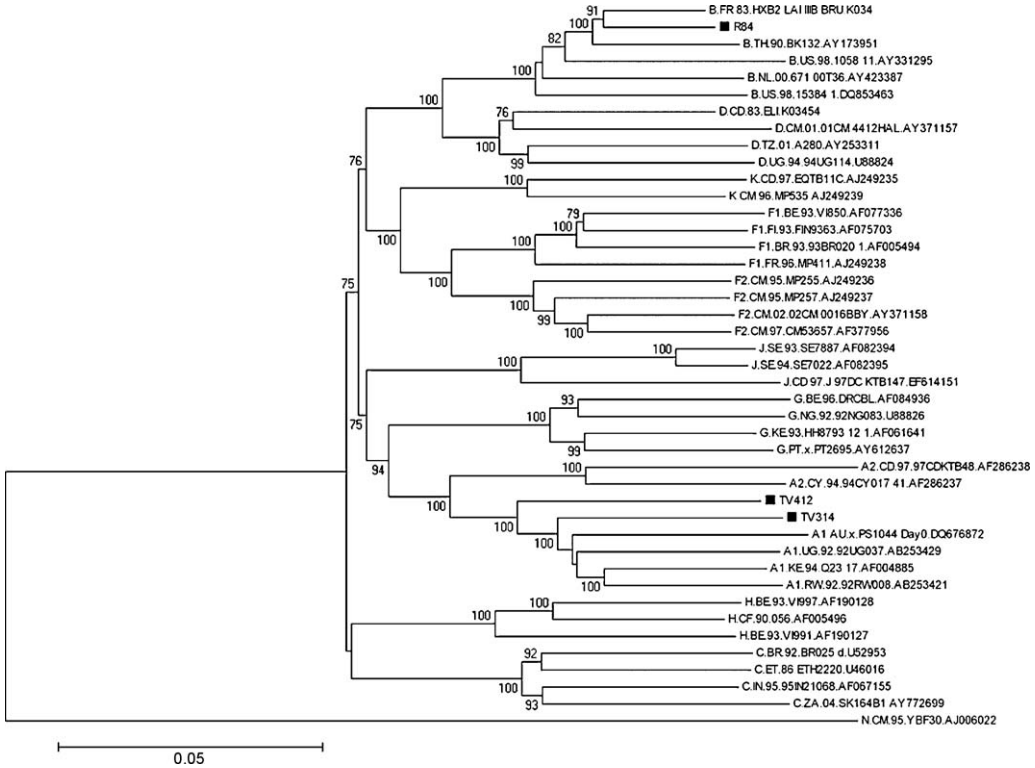

Fig. 1. Neighbor-joining tree (Saitou and Nei, 1987) of near full-length sequences (1230–8700 bp relative to HXB2) implemented in MEGA v 4.1 (Tamura et al., 2007). Bootstrap values greater than 70% are indicated (Felsenstein, 1985). The genetic distance corresponding to the length of the branches is shown in the scale in the bottom left hand corner. The evolutionary distance was computed using the Kimura 2-parameter method of nucleotide substitution (Kimura, 1980). All codon positions were included and all positions containing gaps were eliminated from the dataset.

pol: FJ647156–FJ647162, partial *env*: FJ647163–FJ647168, and NFLG: FJ647145–FJ647149.

3. Results

3.1. Study population

Most of the patients were infected via heterosexual transmission of the virus, except for R84 who was infected via homosexual transmission. All the patients became infected within South Africa except for TV340 and TV412 who became infected in the Democratic Republic of the Congo and Kenya, respectively. Only TV441 was receiving antiretroviral therapy at the time of sample collection. The age of the cohort ranged from 23 to 72 years with a medium age of 35.5 years. The cohort comprised all major demographic groups (African, Coloured and Caucasian) within South Africa.

3.2. Amplification and sequencing of PCR products

The amplification and sequencing of the partial gene fragments were all successful with the exception of the *env* gp41 amplification assay of TV480 and the sequencing of the *gag* p24 PCR product of TV340. The amplification of the longer fragments was also successful with the exception of the LTR-*gag* fragment of TV239,

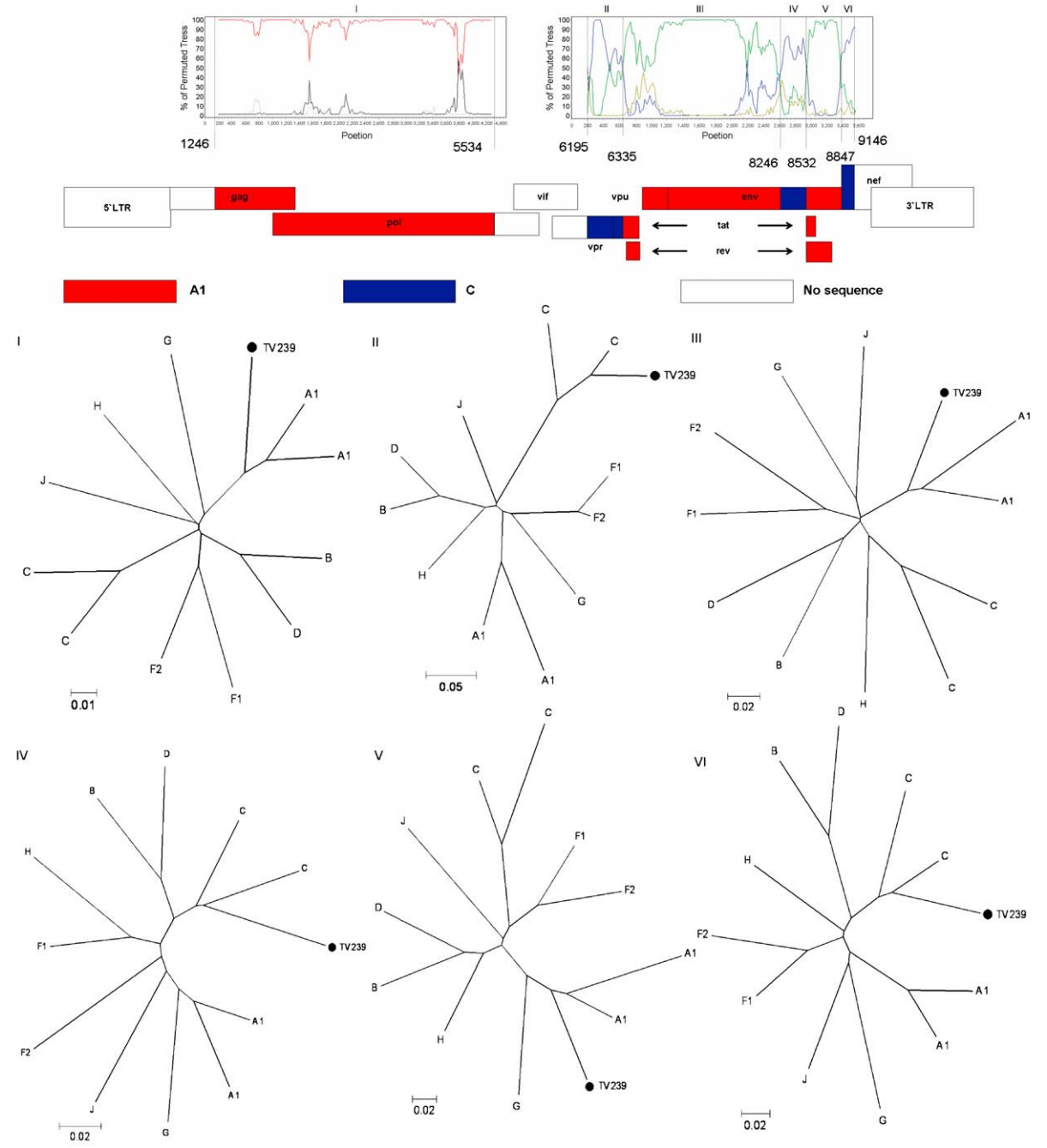

Fig. 2. Schematic diagram of recombinant segments of TV239 env-nef fragment. Radiation neighbor-joining tree (Saitou and Nei, 1987) of TV239 *env-nef* fragments with complimentary reference sequences (all coordinates relative to HXB2) implemented in MEGA v 4.1 (Tamura et al., 2007). The evolutionary distance was computed using the Kimura 2-parameter method of nucleotide substitution (Kimura, 1980). All codon positions were included and all positions containing gaps were eliminated from the dataset. Each Roman numeral corresponds with the same numeral in the schematic at the bottom.

TV314 and TV412. Sequencing of the four overlapping fragments produced contiguous fragments for R84 (601–9514 bp relative to HXB2), TV314 (1235–9551 bp relative to HXB2) and TV412 (1246–8254 bp relative to HXB2). The sequencing of TV239's products produced two fragments, stretching from 1245–5534 bp to 6195–9146 bp relative to HXB2, with a gap of 661 bp in the middle.

3.3. Phylogenetic analysis of sequenced data

The phylogenetic analysis of the partial gene fragments (Table 1) from 11 patients identified subtypes F1 (TV515), A1 (TV314), C (TV86) and B (R84). Seven potential recombinant forms were identified including AD recombinants (TV101 and TV412) and AC recombinants (TV218 and TV441). Other recombinants included AG (TV340), a possible C recombinant (TV480) and a possible A1 recombinant form (TV239).

Initial subtyping analysis obtained with REGA and jpHMM was confirmed with the detailed NJ-tree analysis. R84 and TV314 were initially identified as subtype B and A1 isolates, respectively. TV412 was initially identified as an AD recombinant and TV239 was identified as an AC recombinant with the REGA and jpHMM methods.

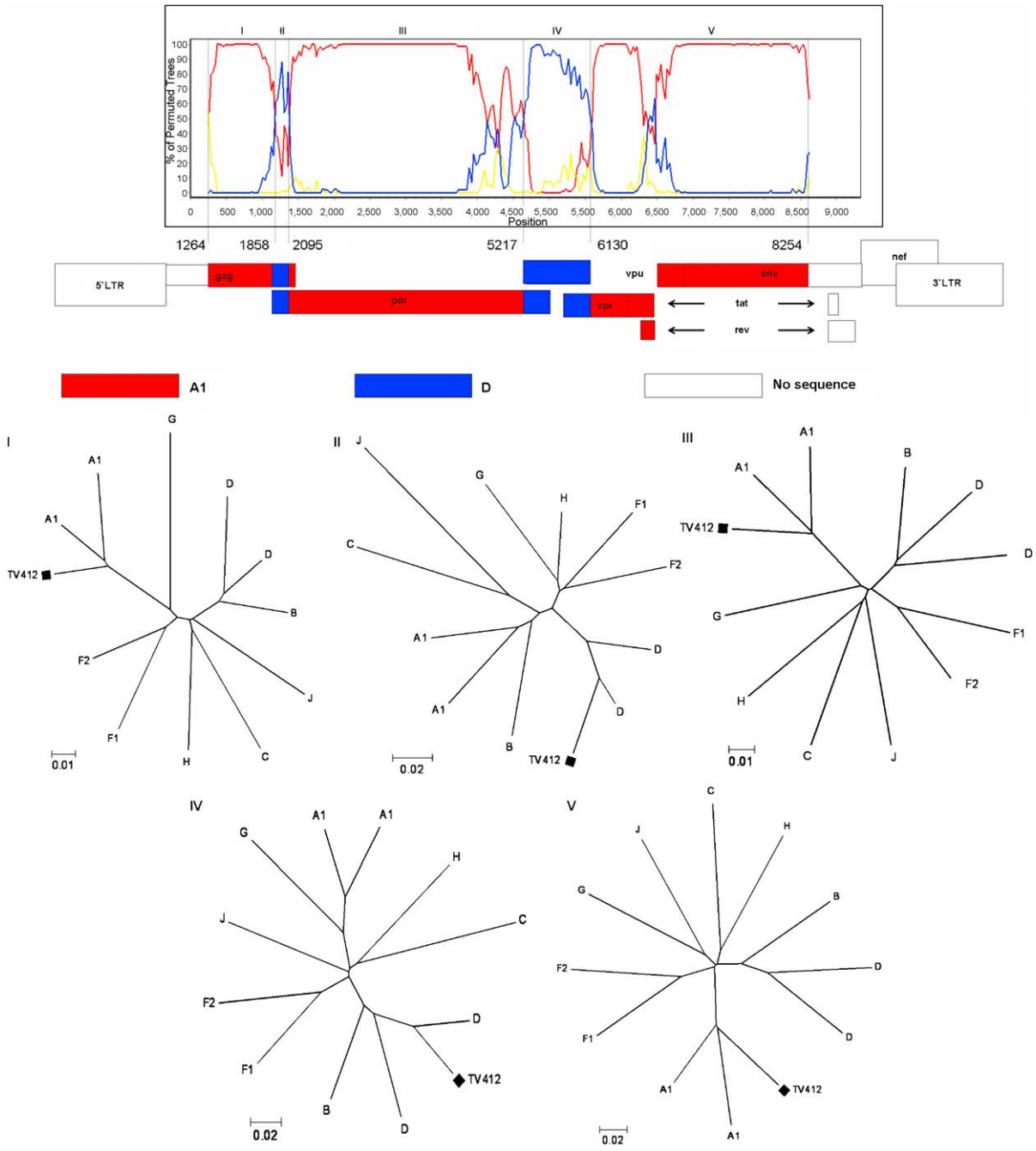

Fig. 3. Schematic diagram of recombinant segments of TV412. Radiation neighbor-joining tree (Saitou and Nei, 1987) of TV412 fragments with complimentary reference sequences (all coordinates relative to HXB2) implemented in MEGA v 4.1 (Tamura et al., 2007). The evolutionary distance was computed using the Kimura 2-parameter method of nucleotide substitution (Kimura, 1980). All codon positions were included and all positions containing gaps were eliminated from the dataset. Each Roman numeral corresponds with the same numeral in the schematic at the bottom.

Phylogenetic analysis of the NFLG sequenced data, through tree construction revealed one pure subtype B (R84) and one pure subtype A1 (TV314) isolate. In the neighbor-joining tree (Fig. 1), which contains the NFLG sequences from R84, TV314 and TV412 as well as reference sequences from the LANL database, R84 clustered with subtype B isolates and TV314 clustered with subtype A1 isolates. TV412 did not cluster within a group but was an outlier to the cluster containing other A1 sequences that indicates possible viral recombination. Separate trees were also constructed for the *gag-pol* and *env-nef* fragments of TV239 with reference HIV-1 sequences (data not shown). The *gag-pol* TV239 fragment clustered with other A1 sequences and the *env-nef* fragment was an outlier of a cluster containing A1 reference sequences.

3.4. Recombination detection

Near full-length sequence fragments were also analyzed with two recombination identification programs: RIP and Simplot v 3.5. Analysis with both programs revealed that R84 and TV314 had high sequence similarities with subtype B and A1 sequences respectively throughout the entire genome. The *gag-pol* fragment of TV239 showed high similarity with subtype A1 throughout the fragment. The *env-nef* fragment of TV239 showed signs of viral recombination breaking repeatedly throughout the fragments between subtype C and A1. TV412 also showed signs of recombination breaking repeatedly throughout the genome between subtype A1 and D. Neighbor-joining tree analysis of each of the recombinant fragments of TV239 *env-nef* (Fig. 2) and TV412 (Fig. 3), revealed the same recombination pattern as was observed with the RIP, Simplot and jpHMM analysis.

4. Discussion

The method of characterizing small subgenomic regions throughout the HIV genome to subtype an isolate or identify possible recombinants has been widely used in the past. In the present study subtypes A1, B, C and F1 were identified with this method of genome characterization. Several possible recombinant HIV viruses were also identified, including AD, CA, AG and other possible A1 and C recombinant forms. Some of the samples were previously classified through characterization of the V3 region alone. With the present study some of these isolates were reclassified as recombinant forms. TV340 previously identified as subtype A1 (EF547511) in the V3 region, was classified as an AG recombinant in this study. Similarly TV441 previously identified as subtype A1 (EF547489) in the V3 region, was classified as an AC recombinant form. Although characterization of partial genome fragments may give an indication of the viral subtype of a particular isolate; only full genome analysis of samples will allow one to make an accurate assumption on the subtype and recombination of a particular isolate.

Phylogenetic analysis of the four NFLG fragments has revealed the presence of a subtype B, one subtype C, one AD recombinant and one AC recombinant. Sample R84 represents only the second near full-length subtype B sequence from South Africa. This subtype B isolate, along with five subtype D viruses, which were characterized in two separate studies (Loxton et al., 2005; Jacobs et al., 2007), represent the only NFLG sequences of the first initial homosexual HIV epidemic within South Africa. The AC recombinant form that was characterized is the fourth AC recombinant form to date to be described within South Africa. Two AC recombinant forms have been described by Rousseau et al. (2006) and one by Papathanasopoulos et al. (2002). The recombination events in all four of the AC recombinant fragments differ and thus each one represents unique recombinant forms (URFs).

The A1 sequence that was obtained from the characterization of sample TV314 is only the second near full-length subtype A1 sequence to be described to date. Subtype A1 is the third most commonly found viral subtype within the country and is circulating in low numbers amongst demographically unlinked areas and is almost exclusively spread by heterosexual transmission.

TV412 is the first AD recombinant form to be identified and characterized within South Africa. The sample was isolated from a patient which became infected in Kenya. Subtypes A and D are circulating in large numbers in the East African region with a growing number of AD recombinant forms being detected within the region (Songok et al., 2004). As unrest in other African nations prevails one can expect an increase of different viral subtypes and recombinant forms being introduced into South Africa by political and economic refugees which will lead to an even greater increase in the number of non-subtype C HIV-1 infections.

5. Conclusion

This work represents partial *gag*, *pol* and *env* and near full-length sequences of non-subtype C HIV-1 sequences from Cape Town, South Africa. Phylogenetic analysis of the sequenced data revealed the presence of subtypes A, B, F1 as well as AC and AD recombinant viruses within the region. The data that was gathered in this study will greatly improve our knowledge of subtype distribution within the country. The study also indicated the need of analyzing larger gene fragments or near full-length sequences for subtyping and recombination identification. Due to the impact that HIV genetic diversity might have on vaccine design and development, as well as HIV diagnosis and the treatment of patients with antiretroviral therapeutic drugs, ongoing research into the epidemiology and spread of HIV subtypes and recombinants within South Africa is needed.

Acknowledgements

The study was made possible through funding from the South African National Research Foundation (NRF), and the Poliomyelitis Research Foundation (PRF). We thank Annette Laten for her help with the sequencing of samples. The authors are grateful for the training received at the 14th International Bioinformatics Workshop on Virus Evolution and Molecular Epidemiology, September 2008, Cape Town (http://www.rega.kuleuven.be/cev/workshop/).

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