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First Detection of a Cluster Novel HIV-1 Second-Generation Recombinant (CRF01_AE/CRF07_BC) among Men Who Have Sex with Men in Nanjing, Eastern China

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Abstract
Introduction: A large number of unique recombinant forms have been found in China in recent years. This study aimed to report on a cluster of novel HIV-1 recombinants. ***Methods:*** We constructed phylogenetic trees using the maximum likelihood (ML) method with 1,000 bootstrap replicates in IQ-TREE 1.6.8 software and determined recombination break points using SimPlot 3.5.1. ***Results:*** Overall, 9 near-full-length genome (NFLG) sequences were reported in this study, including 1 circulation recombinant form (CRF)01_AE NFLG sequence and 8 highly similar novel HIV-1 second-generation recombinants composed of CRF01_AE and CRF07_BC (CRF105_0107) isolated from a cluster HIV-positive male subjects infected among men who have sex with men (MSM) in Nanjing, eastern China. The phylogenetic analysis of NFLG showed 1 sequence named "nj16" to have at least 11 breakpoints inner virus and 7 other sequences to have at least 10 breakpoints inner virus. Our findings further showed as follows: first, this is the first time that a cluster of novel CRF105_0107 HIV-1 strains were identified among MSM in Nanjing, Jiangsu. Second, the Chinese "4a" cluster of CRF01_AE which mainly circulating in northern China has spread in Jiangsu for more than 15 years. Third, HIV-1 recombination events were active in Nanjing city, and novel recombinants could spread rapidly through some small-scale transmission networks. ***Conclusion:*** The continued emergence of novel recombinant HIV-1 strains in Nanjing suggests dynamics and complexity in the HIV epidemic among MSM in Jiangsu province. Further investigations and molecular epidemiological research should be taken to monitor and understand transmission networks among MSM.

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Introduction

HIV possesses extremely high genetic variability and high-speed replication ability, which leads to the formation of new circulation recombinant forms (CRFs) and unique recombinant forms (URFs) constantly. Globally, HIV-1 genetics have become quite complex with time. The most widely epidemic HIV-1 group known as the "M group" has been reported to consist of 9 subtypes (A, B, C, D, F, G, H, J, and K) and 104 CRFs by the Los Alamos National Laboratory HIV Sequence Database (https://www.hiv.lanl.gov/content/index). Similarly for China, the distribution of HIV-1 subtypes has changed dramatically over time [1, 2] with 2 CRFs of CRF01_AE and CRF07_BC playing an increasingly important role in the national or regional HIV pandemic in recent years [3]. So far, 24 CRFs have been reported to originate from China (https://www.hiv.lanl.gov/content/sequence/HIV/CRFs/CRFs.html), and a large number of URFs have been found in recent years [4–6], which suggests that HIV-1 recombination is active in China.

Sexual transmission remains the major means of HIV transmission in China. By the third quarter of 2018, China reported 849,602 HIV/AIDS survivors of whom 93.8% were infected through sexual behaviors [7]. In many metropolises, more than half of newly diagnosed HIV-1 infected clients were men who have sex with men (MSM). Therefore, MSM has become the most concerned group among newly diagnosed HIV-1 infected patients in many developed cities.

Jiangsu province is one of the most developed regions in eastern China, where the prevalence and incidence of HIV-1 among MSM have been rising for the last 10 years. The previous studies in Jiangsu showed the incidence of HIV-1 among MSM to have increased from 5.1% in 2011 to 6.6% in 2015 [8], with an estimated HIV prevalence of 10% among MSM in Nanjing alone (the capital of Jiangsu) [9].

A recent phylogenetic analysis using samples obtained from MSM in Jiangsu showed the main HIV-1 subtypes to be CRF01_AE (60.1%) and CRF07_BC (22.3%), which were introduced into Jiangsu resided MSM population in 2001 and 2004, respectively [10]. In 2009, the first URFs of 0107 (novel HIV-1 recombinants composed of CRF01_AE and CRF07_BC) were found among inject drug users in Jiangsu province [11]. Within 10 years, novel 0107 may be constantly found and transmitted in local areas. Till now, 0107 has been reported in many regions and different populations, like MSM in Xi'an [4], heterosexual male in Guangxi province [5], and MSM in Anhui province [6]. However, most studies reported no more than 3 0107 sequences, and only a few sequences had highly similar recombination structure. In this study, we characterized a group of highly similar novel recombinant CRF105_0107 strains (including 8 novel recombinants), which were different from all the previous studies.

Materials and Methods

Blood samples were collected from newly diagnosed HIV-1 infected patients in Nanjing city from January to December in 2017. A total of 233 pol sequences were obtained from 267 individuals, and the molecular epidemiology-based study showed that 9.4% (22/233) sample sequences were close to the reference sequence 01_BC/0107. It is noteworthy that 10 sequences were clustered in 1 branch with homosexual transmission routes (see online suppl. Fig. 1; see www.karger.com/doi/10.1159/000512135 for all online suppl. material). In order to clear their subtypes, we further obtained their HIV near-full-length genome (NFLG). Viral RNA was extracted from responding plasma samples using QIAamp Viral

Table 1. Basic information of 9 NFLG HIV-1 samples

ID	Accession number	Sequence length (based on HXB2)	Age	CD4	Samples collecting date	Gender	Risk factor	Subtype	Sex partners, *n*
nj16	MT347591	8,391 (778–9,180)	27	771	6 Mar 2017	male	MSM	0107	5
nj32	MT347594	8,380 (777–9,180)	25	283	20 Mar 2017	male	MSM	0107	10
nj70	MT347595	8,422 (779–9,182)	21	344	24 Apr 2017	male	MSM	0107	1
nj82	MT347596	8,383 (779–9,182)	29	633	24 Apr 2017	male	MSM	0107	2
nj95	MT347597	8,385 (779–9,181)	20	422	22 May 2017	male	MSM	0107	1
nj111	MT347589	8,393 (779–9,181)	19	430	22 May 2017	male	MSM	0107	15
nj135	MT347590	8,395 (779–9,182)	58	455	14 Aug 2017	male	MSM	01AE	10
nj271	MT347592	8,389 (771–9,180)	21	384	20 Nov 2017	male	MSM	0107	6
nj274	MT347593	8,450 (768–9,177)	22	200	20 Nov 2017	male	MSM	0107	2

NFLG, near-full-length genome; MSM, men who have sex with men.

RNA Mini Kit (Qiagen, GmbH, Hilden, Germany) and reverse transcribed into cDNA using SuperScript III Reverse Transcriptase (Invitrogen, Carlsbad, CA, USA). The process of NFLG amplification was executed following the protocol as described previously [12]. In order to eliminate the possibility of contamination, each sample has a negative reference in the process of genome extraction and amplification. Positive PCR products were purified and sequenced by BBI Life Sciences Corporation (Shanghai, China). DNA fragments from ABI files were assembled and adjusted manually using ChromasPro 1.6. Multiple alignments using fast Fourier transform (https://www.ebi.ac.uk/Tools/msa/mafft/) were used to align sequence database. Phylogenetic trees were constructed by the maximum likelihood (ML) method with 1,000 bootstrap replicates in IQ-TREE 1.6.8 software [13]. Recombina-

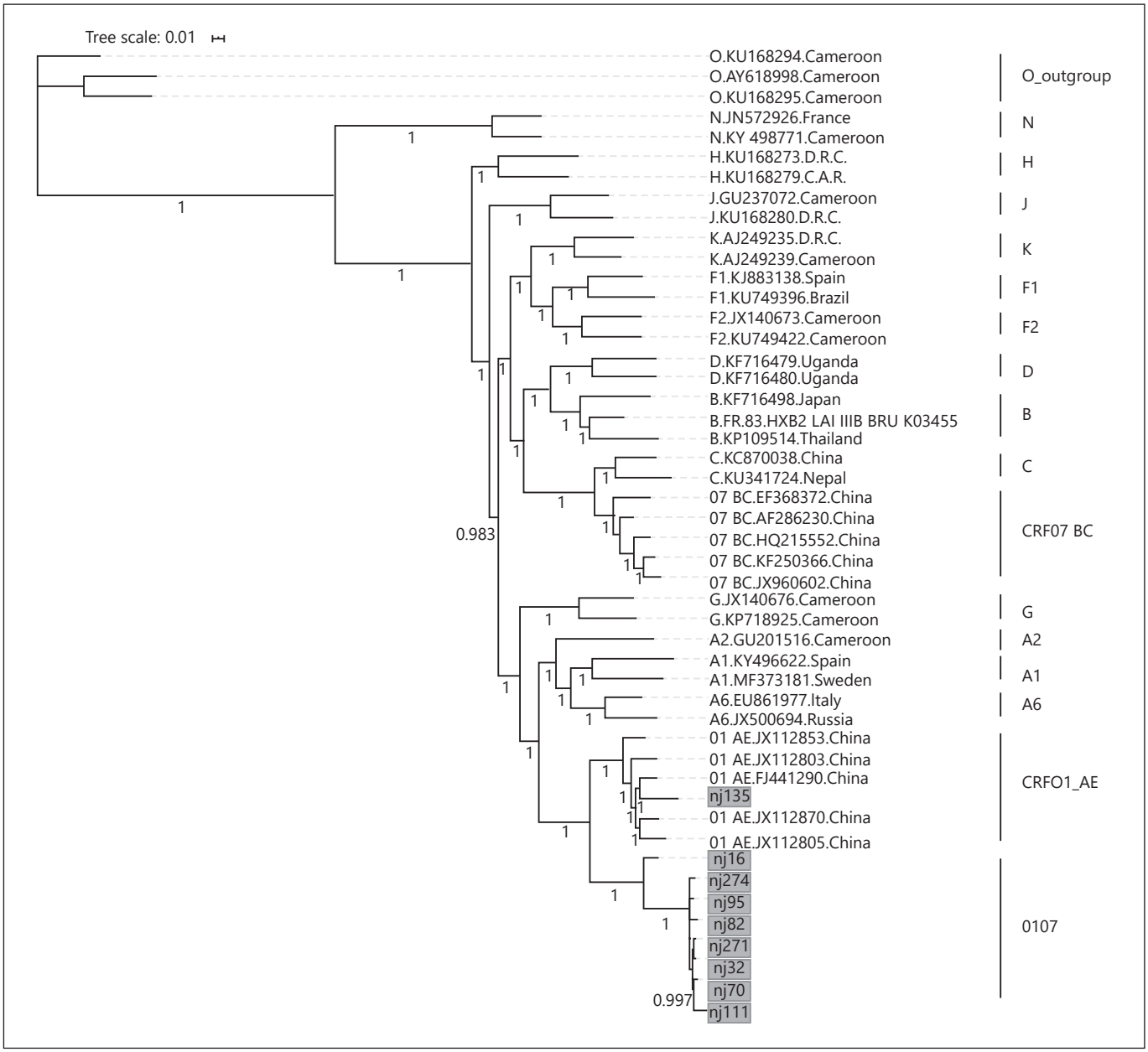
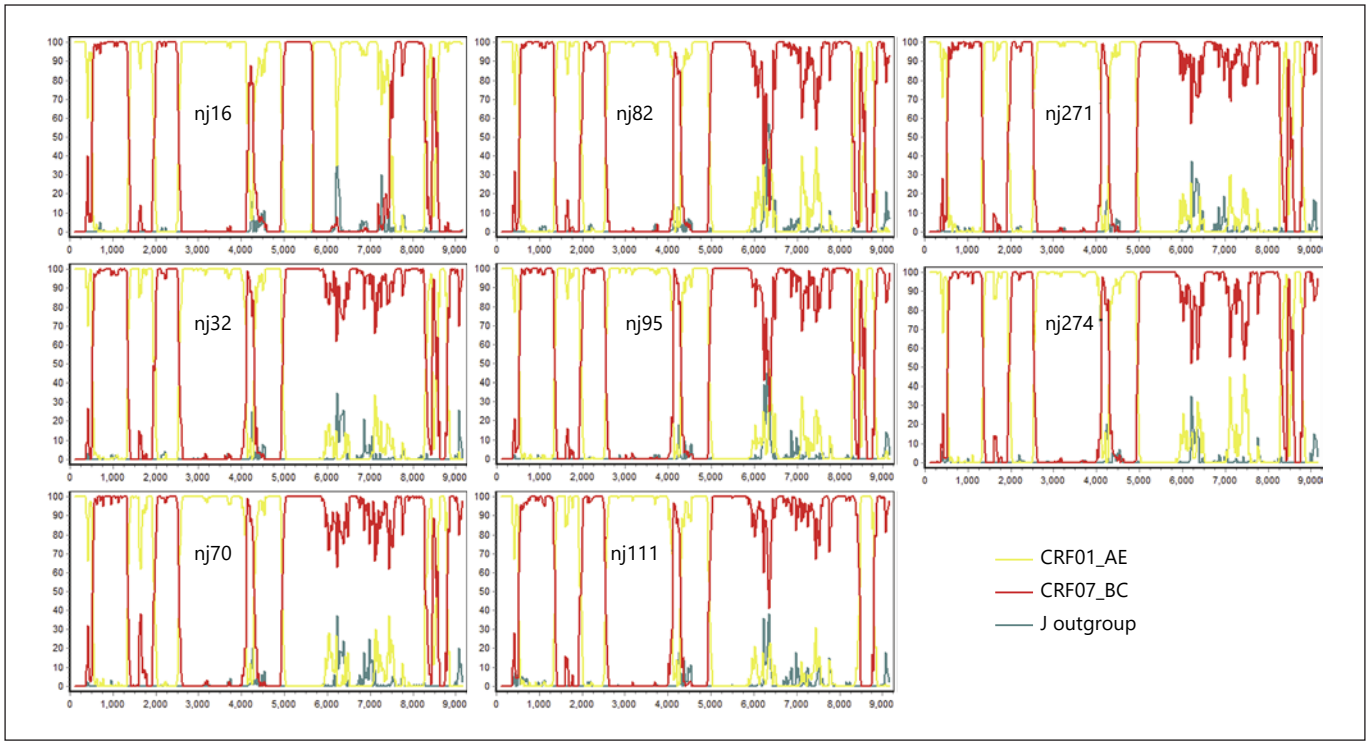


Fig. 1. A ML tree of the NFLG sequences of 9 sample sequences constructed using IQ-TREE. M group of HIV-1 was used as the reference sequences and O group as out-group. The sequences obtained in this study were marked with a shadow under the name. The stability of the phylogenetic nodes was assessed by SH-aLRT branch test with 1,000 replicates, and only significant bootstrap values ≥90% were shown at the corresponding nodes of the tree. The scale bar represents 1% genetic distance. ML, maximum likelihood; NFLG, near-full-length genome.



Color version available online

Fig. 2. The bootscanning plots of 8 NFLG sequences. Sample nj16 was different from the other 7 samples in the second half, and the sample nj32 represented CRF105_0107. The parameters of bootscan analyses were as follows: window size of 200 bp, step size by 20 bp, bootstrap replicate value was 100, distance model was Kimura (2-parameter), and tree algorithm was neighbor-joining. CRF01_AE and CRF07_BC was used as putative parental reference sequences, and subtype J was used as an out-group control. NFLG, near-full-length genome; CRF, circulation recombinant form.

tion analysis was performed using Recombinant Identification Program (RIP, https://www.hiv.lanl.gov/content/sequence/RIP/RIP.html). Recombination break points were determined using SimPlot 3.5.1 and rechecked by jpHMM (jumping profile Hidden Markov Model) tool. The final pattern diagram was drawn by Recombinant HIV-1 Drawing Tool.

Results

Finally, the 9 NFLG sequences were obtained and included into the next analysis, 1 sample was excluded because the quality of sequencing was too bad to be assembled. Table 1 lists the detailed information of the 9 NFLG sequences identified. All clients were found to be infected with HIV via homosexual behavior, and most of them self-reported having multiple sexual partners within 1 year. Except one 58-year-old subject, all 8 other subjects were pretty young (from 19 to 29). Supplementary epidemiological surveys showed that they denied direct transmission to each other.

The 9 NFLG sequences were submitted to BLAST tool and few highly similar reference sequences were found except for 1 sequence highly similar to CRF01_AE. A ML tree was constructed using 48 sequences, consisting of the 9 sample sequences and 39 reference sequences (A1, A2, A6, B, C, D, E, F1, F2, G, H, J, N, CRF01_AE, CRF07_BC, and O as out-group) downloaded from HIV Sequence Database. The ML tree showed all the 9 sequences closely related to CRF01_AE (Fig. 1). Eight samples formed a well-supported single line cluster (bootstrap value = 1), and 1 sample named "nj135" was clustered with reference sequences of CRF01_AE. The results of preliminary recombination analysis showed 8 NFLG sequences to be probably composed of CRF01_AE and CRF07_BC. Further recombination analysis for these sequences in a bootscan analysis showed a clearer recombinant pattern and break points (Fig. 2).

The sequence of "nj16" contained 11 interlaced mosaic sections, including 6 of CRF01_AE (I, III, V, VII, IX_1, XI) and 5 of CRF07_BC (II, IV, VI, VIII_1, X_1). The other 7 NFLG sequences (sample ID: nj32, nj70, nj82,

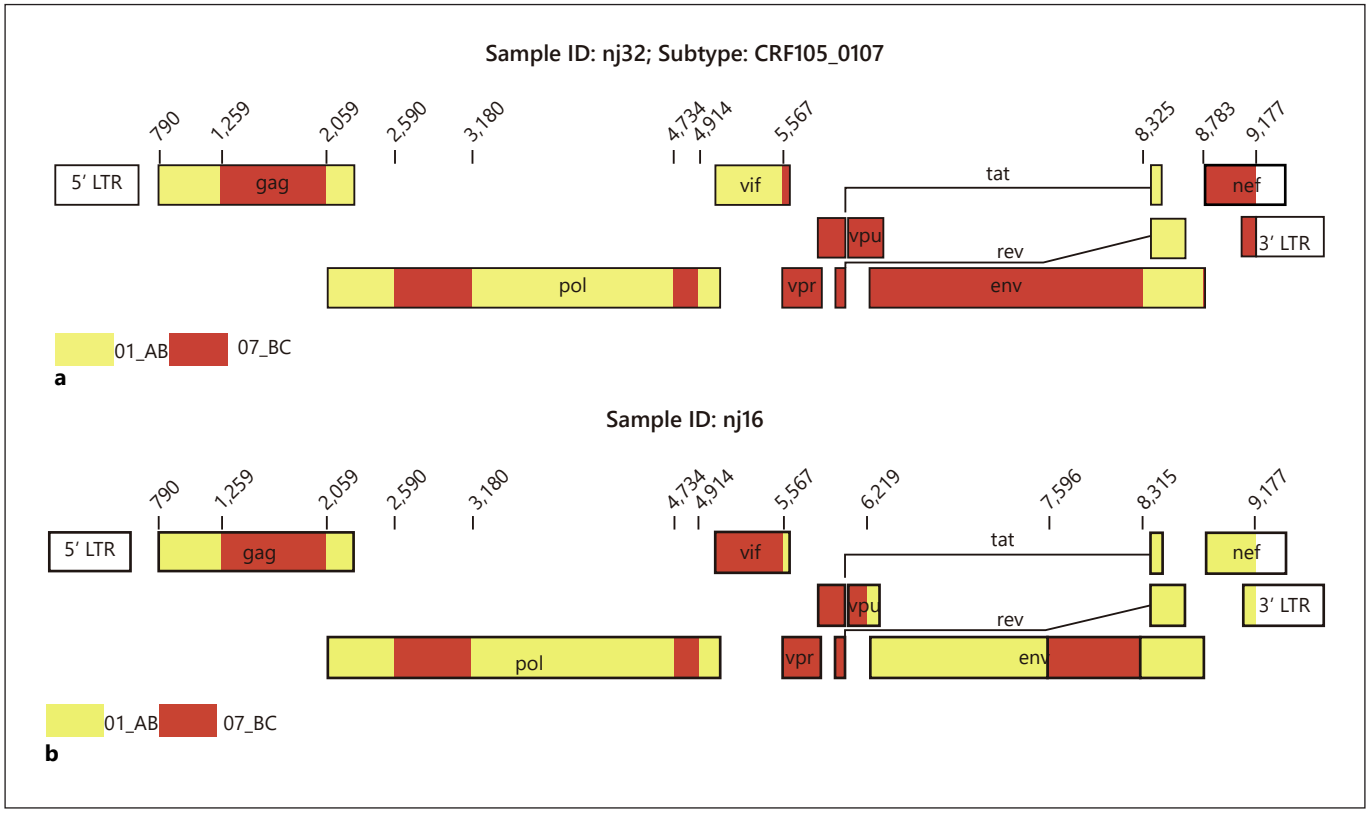


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Fig. 3. The recombinant map results of nj32 (CRF105_0107) and nj16 created using Recombinant HIV-1 Drawing Tool (http://www.hiv.lanl.gov/content/sequence/DRAW_CRF/recom_mapper.html). CRF, circulation recombinant form.

nj95, nj111, nj271, nj274) were highly similar and contained 10 interlaced mosaic sections, consisting of 5 CRF01_AE (I, III, V, VII, IX_2) and 5 CRF07_BC (II, IV, VI, VIII_2, X_2). The "nj16" and other 7 sequences shared the first 7 break points: segment I is CRF01_AE (790–1,258), segment II is CRF07_BC (1,259–2,058), segment III is CRF01_AE (2,059–2,589), segment IV is CRF07_BC (2,590–3,179), segment V is CRF01_AE (3,180–4,733), segment VI is CRF07_BC (4,734–4,913), and segment VII is CRF01_AE (4,914–5,566).

However, the next break points between "nj16" and other 7 sequences were different. For "nj16," segment VIII_1 is CRF07_BC (5,567–6,218), segment IX_1 is CRF01_AE (6,219–7,595), segment X_1 is CRF07_BC (7,596–8,314), and segment XI is CRF01_AE (8,315–9,177). For the 7 other sequences, segment VIII_2 is CRF07_BC (5,567–8,324), segment IX_2 is CRF01_AE (8,325–8,782), and segment X_2 is CRF07_BC (8,783–9,177). The map of novel recombinant virus CRF105_0107 drew reference to sample named "nj32," and the map of "nj16" is also shown (Fig. 3). To confirm the subtypes of different sections, a set of phylogenetic trees of each segment was established (online suppl. Fig. 2, 3). Except for segment VI (4,734–4,913) which was less than 200 bp, all other segments were confirmed well with high bootstrap supports (>0.88, 0.88–1).

Phylogenetic analysis suggested the origins of the recombinants. According to the ML tree of NFLG, the sample of "nj135" was finally identified as CRF01_AE, and it was greatly clustered with the CRF01_AE subcluster 4a lineage, which was reported as mainly circulating among MSM in northern China [14]. However, half of 4a reference sequences (gene accession number: FJ441290 and JX112853) were from Jiangsu, which suggested CRF01_AE 4a subcluster has been spread for more than 15 years in Jiangsu, eastern China. Considering the convenient railway transportation from northern China to Jiangsu province, especially only 3 h from Beijing to Nanjing by Chinese high-speed Rail, the CRF01_AE subcluster 4a lineage could have spread efficiently and formed local cir-

culation in Jiangsu. Besides, subregion tree analyses illustrated that the CRF01_AE segments contained in 8 novel CRF105_0107 sequences all clustered with 4a cluster, which demonstrated that the CRF01_AE 4a cluster in Jiangsu MSM is more likely to recombine with other HIV-1 subtypes. In recent times, finding new sexual partners is much quicker and easier with the help of online dating apps [15], and increased engagement in unprotected sexual behaviors with multiple sexual partners plays an important role in expediting HIV-1 transmission among MSM [16]. These factors coupled together with the ease of transport provide suitable conditions for the formation of novel HIV-1 recombinants.

In addition, 0107 has emerged in different high-risk population in Nanjing (online suppl. Fig. 1). Sequences of Homosexual subjects were more concentrated than sequences of Heterosexual subjects in phylogenetic relationship, and the situation of both transmission ways clustered in 1 cluster exists. Considering the size of clusters in this study, 0107 may spread rapidly among MSM in Jiangsu. Besides, bisexual MSM is a potential bridge for the spread of HIV from MSM to heterosexual population [17]. Therefore, the traceability investigation and targeted prevention should be emphasized and implemented.

Conclusion

To the best of our knowledge, this is the third time a novel 0107 NFLG sequences has been detected in Nanjing city. The first novel 0107 (FJ238521) was identified from an injecting drug user in 2009 [11], and the other 2 0107 novel recombinants (JS150021, JS150029) were identified from 2 males with heterosexual behavior in 2015 [18]. Therefore, this is the first time a cluster of novel CRF105_0107 HIV-1 strains were identified among MSM in Nanjing, Jiangsu province. Considering multiple sex partners was common among MSM and the situation of no direct epidemiology relationships between subjects was confirmed, we deduced that the CRF105_0107 strains identified in this study could have spread and infected few MSM in Jiangsu province. Therefore, it is urgent to identify CRFs and URFs among MSM to monitor the dynamics and complexity of the HIV epidemic.

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Statement of Ethics

All participants involved in this paper signed written informed consent. This work was approved by the ethical review board of the National Center for AIDS/STD Control and Prevention (Project No. X140617334).

Conflict of Interest Statement

No competing financial interests exist.

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Author Contributions

Zhou Y., Wang B., and Fu G. conceived and designed the study. Yin Y., Lu J., and Guo H. obtained and administered the database. Yin Y., Yuan D., and Chen J. performed laboratory work. Hu H., Lu J., and Xu X. performed the analyses and Yin Y. and Zhou Y. interpreted them. Yin Y. and Xuan Y. drafted the manuscript and all authors critically reviewed it. The final version was approved by all authors.

Sequence Data

All of the nucleotide sequences obtained in this study have been submitted to GenBank. MT347589–MT347597.

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