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Multiple routes of hepatitis C virus transmission among injection drug users in Hai Phong, Northern Vietnam

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ABSTRACT

To identify hepatitis C virus (HCV) transmission routes among injection drug users in Northern Vietnam, plasma samples were collected from 486 drug users in Hai Phong. Plasma viral RNA was extracted from 323 (66.5%) samples that were positive for anti-HCV antibodies. Portions of the HCV 5'-untranslated (5'*UTR*)-*Core* and *NS5B* genes were amplified by reverse-transcriptase polymerase chain reaction, sequenced directly, and genotyped in 194 and 195 specimens, respectively. Both regions were genotyped in 137 specimens. In the 5'*UTR*-*Core* region, genotype 6a was predominant (32.5%), followed by genotype 1a (23.7%), genotype 1b (20.6%), and genotype 6e (14.4%). In the *NS5B* region, genotype 1a was predominant (42.6%), followed by genotype 1b (24.1%), genotype 6a (14.4%), genotype 3b (7.2%), and genotype 6e (5.1%). Of the 137 specimens with both regions genotyped, 23 (16.8%) showed discordant genotyping results between the two regions, suggesting possible recombination and/or dual infection. Phylogenetic analysis revealed close associations between Hai Phong strains and strains from Southern China: the Yunnan province for genotype 3b; the Guangxi province for genotype 6e; the USA for genotype 1a; and Southern Vietnam for genotypes 1a and 6e. The human immunodeficiency virus (HIV) infection rate among HCV-infected injection drug users was 52.6-55.4% and did not differ significantly by HCV genotype. Most drug users infected with HIV-1 [98.8% (171/173)] were co-infected with HCV. These results suggest multiple routes of HCV transmission among injection drug users in Northern Vietnam that may also be HIV transmission routes.

Key words: HIV, genotype, molecular epidemiology, Southeast Asia

INTRODUCTION

Hepatitis C virus (HCV; a member of the *hepacivirus* genus in the family of *Flaviviridae*) is a major causative agent of chronic liver disease. The prevalence of HCV infection is estimated to be around 2–2.2% worldwide [Shepard et al., 2005; The Global Burden of Hepatitis C Working Group, 2004]. HCV is classified into 6 genotypes, most of which comprise multiple sub-genotypes. Genotype 1a and genotype 1b are distributed widely, while other genotypes are specific to geographic regions. Genotype 3 and genotype 6 are associated with South/Southeast Asia [Simmons et al., 2005], and genotype 6 is thought to have developed in Asia [Pybus et al., 2009].

The prevalence of HCV in Vietnam is estimated to be 6.1% [WHO, 1999]. However, according to previous studies, the prevalence is not uniform throughout the country. The anti-HCV-antibody rate in blood donors is 20.6% in Ho Chi Minh City in Southern Vietnam and 0.8% in Hanoi in Northern Vietnam [Nakano et al., 2004]. This rate is 9% in individuals without liver disease in Ho Chi Minh City and 4% in Hanoi [Nakata et al., 1994]. These differences in prevalence may reflect the proportion of injection drug users in the target populations. In Ho Chi Minh City, injection drug use has been common since the Vietnam War (1965-1975), while it has only been common recently in Hanoi [Pham et al., 1994]. The HCV prevalence in Northern Vietnam has been reported as 1.0% in the general population and 31-97% in injection drug users [Hoang et al., 2003; La et al., 1995; Quan et al., 2009; Nguyen et al., 2007].

Tran et al. (2003) studied the genetic distribution of HCV in Ho Chi Minh City and reported that genotype 2a was predominant (33.3%), followed by genotype 1a (23.8%), genotype 1b (19.0%), and genotype 6a (14.3%). However, there are few published

molecular epidemiological analyses of HCV in Northern Vietnam. Pham et al. (2009) reported recently that in blood donors in Hanoi, HCV genotype 6a was predominant (37.1%), followed by genotype 1a (30.0%) and genotype 1b (17.1%).

Northern Vietnam and Southern China are heroin-trafficking routes that lead from the "Golden Triangle" of poppy cultivation. As a result, these areas are thought to be transmission routes for the human immunodeficiency virus (HIV; a member of the *lentivirus* genus in the family of *Retroviridae*) among the injection drug user population. Several reports suggest that HIV-1 CRF01_AE was either introduced to the northern provinces of Vietnam from the Guangxi province in Southern China, reaching Hanoi by this route [Beyrer et al., 2000; Kato et al., 1999; Kato et al., 2001; Yu et al., 1999], or the converse [Liao et al., 2009]. It has also been reported that the majority of HIV-1 strains circulating among injection drug users in Hai Phong, a city in Northern Vietnam, are associated closely with those from the Guangxi province [Ishizaki et al., 2009].

In the current study, HCV transmission among the injection drug user population in Hai Phong was investigated genetically and compared with HIV infection routes identified previously.

SUBJECTS AND METHODS

Subjects

Plasma specimens were collected from 486 injection drug users (all male; mean age, 34.4 years; age range, 20-65 years) at drug rehabilitation centers in Hai Phong in April 2007 after ethical clearance was obtained. All subjects provided written informed consent. The samples had been used previously for the analysis of HIV-1 prevalence and drug resistance among injection drug users in the city [Ishizaki et al., 2009]. The study protocol was reviewed and approved by the ethical committees of Hanoi Medical University in Vietnam and Kanazawa University in Japan.

RNA extraction, cDNA synthesis, and amplification

The plasma specimens were tested for anti-HCV antibodies using a test kit (HCV PHA; Abbott Japan). HCV RNA was extracted from 100 µl of anti-HCV-positive plasma using SMITEST EX-R&D (Genome Science Laboratories) and reverse-transcribed with random primers using the First-Strand cDNA synthesis kit (Invitrogen) according to the manufacturer's instructions. A portion of the *NS5B* gene was amplified by nested polymerase chain reaction (PCR) with the primers hep31b/32 and hep33b/34b in the first and second rounds, respectively [White et al., 2000]. The 5'*UTR-Core* was amplified by nested PCR with the primers KY80/C0751R and hep21b/C0727R in the first and second rounds, respectively [Kageyama et al., 2006]. The first round amplification was performed in 50-µl reaction mixtures containing 2.5 mM $MgCl_2$, 200 µM each dNTP, 0.5 µM primers, and 2.5 U AmpliTaq Gold® (Applied Biosystems). The thermal profile of the first-round PCR included one cycle of 94°C for 10 min; 40

cycles of 94°C for 30 sec, 55°C for *NS5B* or 50°C for 5'*UTR*-Core for 30 sec, and 72°C for 1 min per kb; and a final extension of 72°C for 10 min. The first-round products were then subjected to second-round amplification in 20 µl reaction mixtures containing 2.5 mM $MgCl_2$, 200 µM each dNTP, 0.5 µM primers, and 1 unit AmpliTaq Gold®. The thermal conditions of the second-round PCR were the same as the first-round, except the annealing temperature was 60°C for *NS5B* and 50°C for the 5'*UTR-Core*. PCR amplification was confirmed by visualization on an ethidium bromide-stained gel.

Determination of nucleotide sequences and phylogenetic analysis

The nucleotide sequences of the PCR products were determined directly for phylogenetic analysis. The primers hep33/hep34 were used for the *NS5B* region and hep21b/C0727R for the 5'*UTR-Core*. Because direct sequencing of the 4 PCR products amplified from the *NS5B* region was unsuccessful, the products were cloned into a vector using the TOPO TA cloning kit (Invitrogen) for clonal sequence determination. The sequences were aligned with sequences retrieved from the HCV sequences database (GenBank/ EMBL/ DDBJ) using ClustalW followed by subsequent visual inspection and manual modification [Thompson et al., 1994]. The frequency of nucleotide substitution at each base of the sequence was estimated by the Kimura two-parameter method. A phylogenetic tree was constructed by the neighbor-joining method, and its reliability was estimated by 1,000 bootstrap replications. The profile of the tree was visualized with the program Njplot [Perriere and Gouy, 1996]. A Fisher's exact test was used to compare the proportions of the genotypes found in the two regions i.e. *5'UTR-Core* and *NS5B*.

RESULTS

Prevalence of anti-HCV antibodies and HCV RNA

Of the 486 injection drug users tested in Hai Phong, 323 (66.5%) were positive for anti-HCV antibody. Of these 323 drug users, 256 (79.3%) were positive for HCV RNA in the 5'*UTR-Core* and/or *NS5B* regions.

Genotype distribution

The sequences and genotypes of the 256 HCV RNA-positive specimens were determined. Sequences in the 5'*UTR-Core* and *NS5B* regions were analyzed in 194 and 195 specimens, respectively, and sequences in both of regions were analyzed in 137 specimens.

Table I shows the genotype distribution for these two regions. In the 5'*UTR-Core* region, genotype 6a was predominant (32.5%; 63/194), followed by genotype 1a (23.7%), genotype 1b (20.6%), and genotype 6e (14.4%). In the *NS5B* region, genotype 1a was predominant (42.6%; 83/195), followed by genotype 1b (24.1%), genotype 6a (14.4%), genotype 3b (7.2%), and genotype 6e (5.1%). One case (HPA017) was found to be infected with two HCV genotypes, genotypes 6a and 6e, by clonal sequencing.

Of the 137 specimens in which sequences in both regions were analyzed, 23 (16.8%) showed discordant genotyping results between the two regions, suggesting possible recombination and/or dual infections. Age group 46-50 had the highest discordant rate (50%; 2/4), followed by age groups 41-45 (27.3%; 6/22))), 36-40 (18.8%; 6/32))), and 31-35 (18.4%; 7/38))). Table II shows the genotyping profiles of concordant (a) and discordant (b) specimens. Combination between genotypes 6a and 1a at the 5'*UTR-Core*

and *NS5B* regions, respectively, was highest (26.1%; 6/23), followed by genotypes 6e and 1a (17.4%). No combination was found between the most prevalent genotypes, genotype 1a and genotype 1b.

Phylogenetic analysis

The phylogenetic trees based on the 5'*UTR-Core* and *NS5B* sequences are shown in Fig. 1 and 2, respectively. Clonal sequence determination was done for four samples (HPA003, HPA016, HPA017, and HPA035)), which population sequences were not successful. In HPA017 two clonal groups corresponding to genotype 6a and genotype 6e were identified, while no such diversity was found in the remaining three samples. Therefore, a representative clone for each sample/group was used for the analysis. Analysis of genotype 1a, based on the 5'*UTR-Core* region, showed that most of the Hai Phong strains both formed independent clusters and clusters with isolates from the USA (Fig. 1a). In the *NS5B* tree, Hai Phong strains formed independent clusters and clusters with isolates from Hanoi in Northern Vietnam, Ho Chi Minh City in Southern Vietnam, and/or the USA (Fig. 2a). In the genotype 1b phylogenetic tree, based on the 5'*UTR-Core* and *NS5B* regions, Hai Phong strains formed an independent cluster and clusters with isolates from Hanoi, Ho Chi Minh City, and the USA (Fig. 1b and 2b). In the genotype 3b phylogenetic tree, Hai Phong strains formed an independent cluster and a cluster with isolates from Hanoi and Yunnan in Southern China (Fig 1c and 2c). In the genotype 6a phylogenetic tree, the Hai Phong isolates were similar to those from Hanoi, Ho Chi Minh City, Hong Kong, and Yunnan province in Southern China. In the phylogenetic trees of genotype 6e, genotype 6h, and genotype 6l, most of the Hai Phong

isolates formed clusters with Vietnamese strains, except for genotype 6e, which was indistinguishable from an isolate from the Guangxi province in China (DQ314805). For genotype 6h and genotype 6l, phylogenetic trees based on the 5'*UTR-Core* sequences distinct clusters but trees based on the *NS5B* region did not (Fig 1d and 2d).

HIV prevalence by HCV genotypes

The HIV prevalence according to HCV genotype is shown in Table III. Generally the prevalence did not differ significantly by HCV genotype (52.6–55.4% in 5'*UTR-Core–NS5B)*, though it was higher for genotype 3b (78.6–100%), and lower for genotype 6h (0%).

Sequence data

The sequences described in this report have been deposited in GenBank/EMBL/DDBJ under accession numbers AB522972-AB523361.

DISCUSSION

In this study, nine HCV genotypes were detected among injection drug users in Hai Phong, Northern Vietnam. There were close phylogenetic associations between Hai Phong strains and strains from Southern China: the Yunnan province for genotype 3b; the Guangxi province for genotype 6e; the USA for genotype 1a; and Southern Vietnam for genotype 1a and genotype 6e. These findings suggest that there are multiple transmission networks for HCV among injection drug users in Hai Phong.

Table I shows a comparison of prevalent HCV genotypes among injection drug users in Hai Phong and Ho Chi Minh City [Tran et al, 2003], the Yunnan province, Southern China [Xueshan Xia et al, 2008], and the Guangxi province, Southern China [Garten et al., 2005]. The HCV genotype distribution in Hai Phong was similar to that reported recently in Hanoi [Pham et al, 2009]. In Hai Phong, genotype 1a was more prevalent than genotype 1b, and most of the strains were related phylogenetically to strains in Hanoi, Ho Chi Minh City and/or the USA. This is consistent with previous findings that after the Vietnam War, the prevalence of genotype 1a in Northern Vietnam increased exponentially through injection drug use and blood transfusions [Nakano et al., 2004]; prior to this, genotype 1b was more prevalent in the general population. Some genotype 1b strains showed a close relationship with strains from the USA, suggesting an ongoing spread of virus between Vietnam and the USA. Genotype 1a and genotype 1b were detected in Ho Chi Minh City and the Yunnan and Guangxi provinces, as well as in Hai Phong (Table 1). This is consistent with a previous report that these HCV genotypes are widely distributed [Simmonds et al., 2005].

As shown in Table I, genotype 3 was not found in Ho Chi Minh City but was found frequently in Southern China. The prevalence of genotype 3b (4.1– 7.2%) in Hai Phong in this study suggests that genotype 3 may have been transmitted from Southern China. This is supported by the phylogenetic analysis (Fig. 1c and 2c): genotype 3b strains formed two distinct clusters, one of which showed similarity to Yunnan strains.

In the phylogenetic analysis of genotype 6a, most Hai Phong strains showed similarity to the isolates from Hanoi, Ho Chi Minh City, the Yunnan province in China, and/or Hong Kong, indicating widespread genotype 6a infection around these regions. A previous study found that genotype 6 was spread widely through Southeast Asia and was not limited to injection drug users [Pybus et al., 2009]. Genotype 6e showed lower prevalence in Hai Phong and Guangxi and was not found in Yunnan province. Considering that genotype 6e is thought to have emerged in Vietnam [Pybus et al., 2009], this genotype may have spread from Northern Vietnam to the Guangxi province.

In this study, HIV prevalence was 52.6–55.4% (5'*UTR-Core–NS5B*) among injection drug users infected with HCV in Hai Phong. Prevalence did not differ significantly among HCV genotypes, though it was higher for genotype 3b (78.6–100%), and nonexistent for genotype 6h (0%). HCV genotype 3b strains in injection drug users in Hai Phong were related closely to those in injection drug users in the Yunnan province, while the majority of HIV-1 strains prevalent in this group (CRF01_AE) are related closely to strains in the Guangxi province [Ishizaki et al., 2009]. These results suggest that HIV and HCV have not always shared routes of infection. However, most of the HIV-1-infected injection drug users [98.8% (171/173)] were co-infected with HCV. Therefore, HIV intervention programs for injection drug users should take HCV

transmission routes into consideration and use HCV infection as a prediction marker for HIV infection [Garten et al., 2005].

In the current study, 16.8% (23/137) of the subjects showed possible dual infections and/or recombination of HCV. In addition, clonal sequence analysis identified dual infection of HCV genotypes in one case (HPA017). The frequency of dual infection and/or recombination in this study was higher than expected; however, it was within the range reported in other countries (3.8% in Australia [Bowden et al., 2005]; 4% in USA [Rosen et al., 1999]; 14.2% in USA [Nolte et al., 2003]; 14.8% in Argentina [Quarleri et al., 2000]; 19% in United Kingdom [Buckton et al., 2006]; 20% in USA [Herring et al., 2004]). The efficiency of detecting multiple infections may depend on the number of HCV genotypes circulating in the study population. Nine genotypes were found in this study population, which may have made the detection of dual infections and/or recombination easier.

The probability of multiple infections and/or recombination is expected to increase as the risk of HCV superinfection increases, with the cumulative risk depending on the duration of injection drug use. In this study, the older group had a higher rate of multiple infections and/or recombination than younger groups, though data on how long each subject had used injection drugs was not available. The profile of dual infections and/or recombination seemed to reflect HCV genotype frequency in this population. However, no dual infections were identified for genotype 1a and genotype 1b, the most prevalent genotypes. This suggests that the infection routes of these HCV genotypes were different in this study population.

Several HCV recombinants have been reported worldwide, and a mosaic HCV recombinant was reported recently [Ross et al., 2008; Sentandreu et al., 2008]. However, the mechanism of HCV recombination is not understood well. Possible recombination and/or dual infection events in the 23 specimens in this study are under investigation currently, and the results may provide insights into the mechanism of HCV recombination.

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FIGURE LEGENDS

Fig. 1. Phylogenetic analysis of the HCV strains based on 525 nucleotides within the 5'*UTR-Core* region. The tree was constructed with the neighbor-joining method using the Clustal W program with GBV-B (accession number NC001655) as the outgroup. ● denotes isolates from Hai Phong (this study); ○, □, and • denote isolates from Vietnam, China, and Hong Kong, respectively, using sequences obtained from the GenBank/EMBL/DDBJ database. Accession numbers were used for the reference strains, and the two digits preceding the number indicate the genotypes. Bootstrap scores ≥70% are shown on branches as percentages from 1,000 replicates. (a) Genotype 1a; (b) genotype 1b, (c) genotype 3; (d) genotype 6. (a) - (d): For the Hai Phong specimens (this study), HIV status (+ or -) is denoted after the specimen ID.

Fig. 2. Phylogenetic analysis of HCV strains based on 324 nucleotides within the *NS5B* region. The tree was constructed with the neighbor-joining method using the Clustal W program with GBV-B (accession number NC001655) as the outgroup. ● denotes isolates from Hai Phong (this study); ○, □, and • denote isolates from Vietnam, China, and Hong Kong, respectively, using sequences obtained from the GenBank/EMBL/DDBJ database. Accession numbers were used for the reference strains, and the two digits preceding the number indicate the genotypes. Bootstrap scores ≥70% are given on branches as percentages from 1,000 replicates. (a) Genotype 1a; (b) genotype 1b, (c) genotype 3; (d) genotype 6. (a) - (d): For the Hai Phong

specimens (this study), HIV status (+ or -) is denoted after the specimen ID. HPA017-1 and HPA017-4 are different clones isolated from the same sample, HPA017.

Table I: HCV genotype distribution based on the 5'*UTR-Core* and *NS5B* regions compared to neighboring regions

	Hai Phong (Vietnam)				Ho Chi Minh (Vietnam)	Yunnan (China)	Guangxi (China)
Genotype	***5'UTR-Core***	***NS5B***	**%difference**	***p* value**	Tran *et al.* (2003)	Xia *et al.* (2008)	Garten *et al*. (2005)
1a	46 (23.7%)	83 (42.6%)	-18.9%	0.0001	5 (23.8%)	1 (1.2%)	24 (19.0%)
1b	40 (20.6%)	47 (24.1%)	-3.5%	0.4823	4 (19.0%)	16 (19.5%)	1 (0.8%)
2	0	1 (0.5%)	-0.5%	1.0000			
2a	1 (0.5%)	1 (0.5%)	0	1.0000	7 (33.3%)	0	0
3a	3 (1.5%)	3 (1.5%)	0.2%	1.0000	0	19 (23.2%)	2 (1.6%)
3b	8 (4.1%)	14 (7.2%)	-3.1%	0.2777	0	24 (29.3%)	46 (36.5%)
4	0	0	0	ND	1 (4.8%)	0	0
5	0	0	0	ND	0	0	0
6a	63 (32.5%)	28 (14.4%)	18.1%	<0.0001	3 (14.3%)	4 (4.9%)	48 (38.1%)
6e	28 (14.4%)	10 (5.1%)	9.3%	0.0031	0	0	5 (4.0%)
6e, 6a*	0	1 (0.5%)	-0.5%	1.0000	0	0	0
6h	3 (1.5%)	7 (3.6%)	-2.1%	0.3416	0	0	0
6l	2 (1.0%)	0	1%	0.4961	0	0	0
6n	0	0	0	ND	0	9 (11.0%)	0
6u	0	0	0	ND	0	7 (8.5%)	0
Unclassified	0	0	0	ND	1 (4.8%)	2 (2.4%)	0
Total	194	195			21	82	126

* dual infection (6a and 6e)
ND: Not Determined

Table II: HCV genotype distribution of concordant (a) and discordant (b) samples between the 5'*UTR-Core* and *NS5B* regions

(a) Concordant samples

Genotype	n
1a	42 (30.7%)
1b	33 (24.1%)
2a	1 (0.7%)
3a	2 (1.5%)
3b	8 (7.0 %)
4	0
5	0
6a	17 (12.4%)
6e	8 (5.8%)
6h	3 (2.2%)
Total	114 (83.2%)

(b) Discordant samples

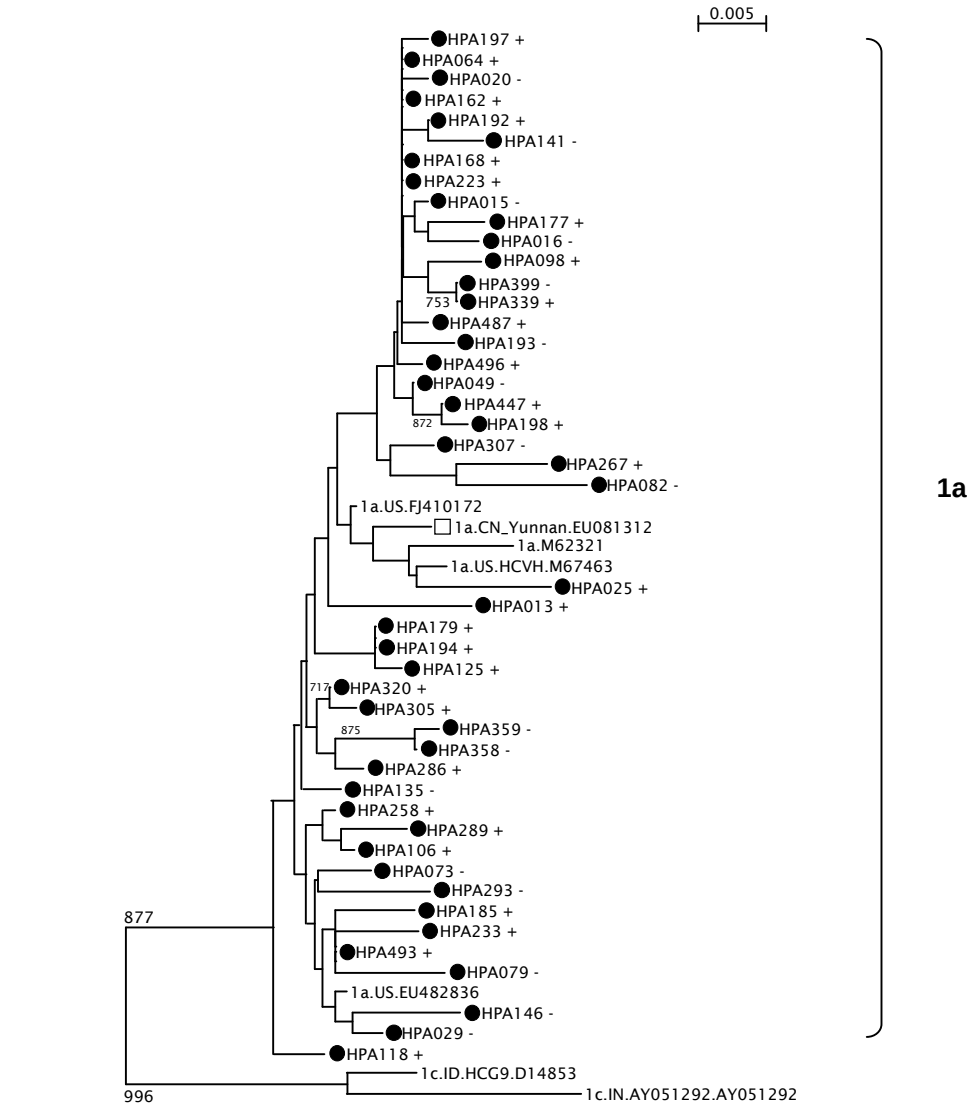
Genotype		n
NS5B	5'UTR-Core	
1a	3a	1
	6a	6
	6e	4
	6l	1
1b	6a	2
	6e	2
2	6e	1
3a	6e	1
6a	1a	2
6e	6a	1
6h	6a	1
	6e	1
		23

Table III: HIV prevalence according to HCV genotype

Genotype	5'UTR-Core	NS5B
1a	29/46 (63.0%)	50/83 (60.2%)
1b	21/40 (52.5%)	24/47 (51.1%)
2	0	1/1 (100%)
2a	0/1 (0%)	0/1 (0%)
3a	1/3 (33.3%)	2/3 (66.7%)
3b	8/8 (100%)	11/14 (78.6%)
4	0	0
5	0	0
6a	29/63 (46.0%)	15/28 (53.6%)
6e	13/28 (46.4%)	5/10 (50.0%)
6e, 6a*	0	0/1 (0%)
6h	0/3 (0%)	0/7 (0%)
6l	1/2 (50.0%)	0
6n	0	0
6u	0	0
Total	102/194 (52.6%)	108/195 (55.4%)

* dual infection (6a and 6e)

Fig. 1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HPA162 +
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HPA141 -
HPA168 +
HPA223 +
HPA015 -
HPA177 +
HPA016 -
HPA098 +
HPA399 -
753
HPA339 +
HPA487 +
HPA193 -
HPA496 +
HPA049 -
HPA447 +
872
HPA198 +
HPA307 -
HPA267 +
HPA082 -
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HPA179 +
HPA194 +
HPA125 +
717
HPA320 +
HPA305 +
875
HPA359 -
HPA358 -
HPA286 +
HPA135 -
HPA258 +
HPA289 +
HPA106 +
HPA073 -
HPA293 -
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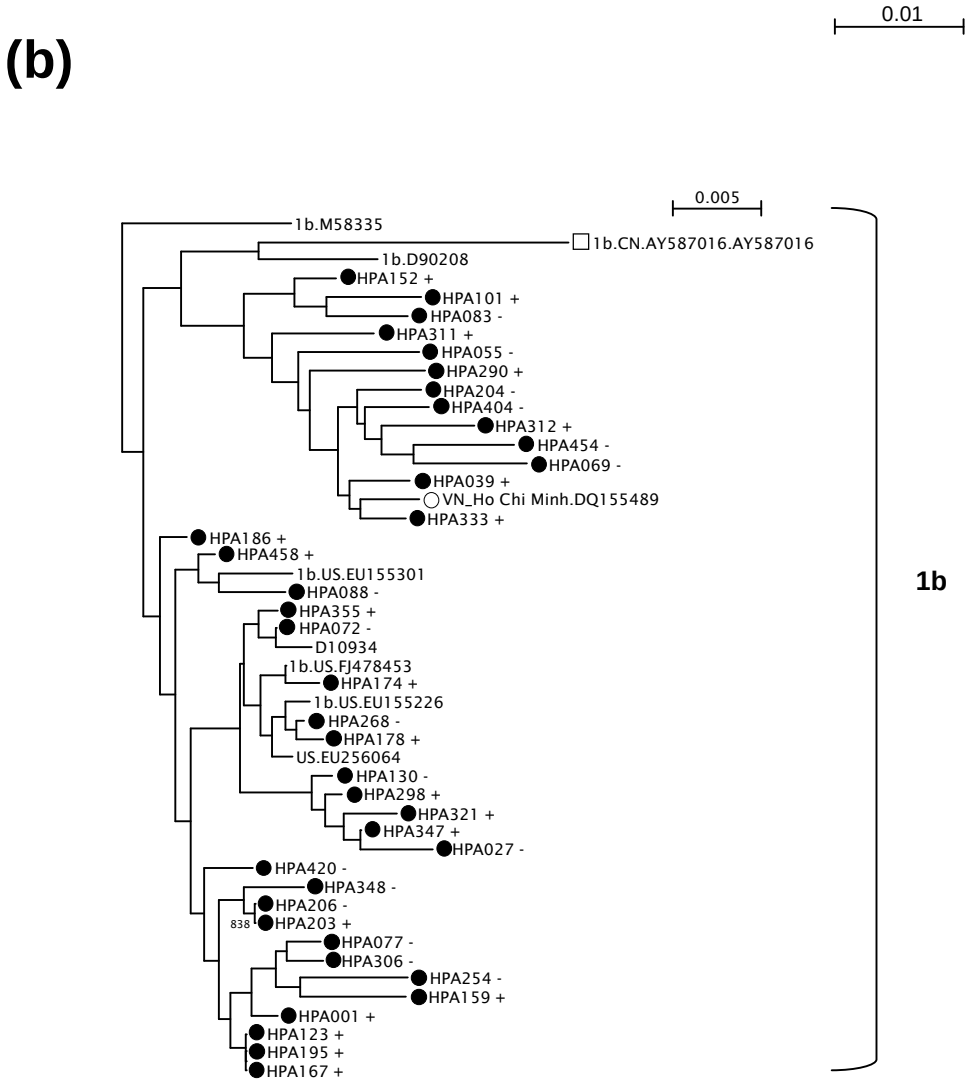
1a

Fig. 1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HPA454 -
HPA069 -
HPA039 +
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HPA458 +
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HPA178 +
US.EU256064
HPA130 -
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HPA347 +
HPA027 -
HPA420 -
HPA348 -
HPA206 -
838
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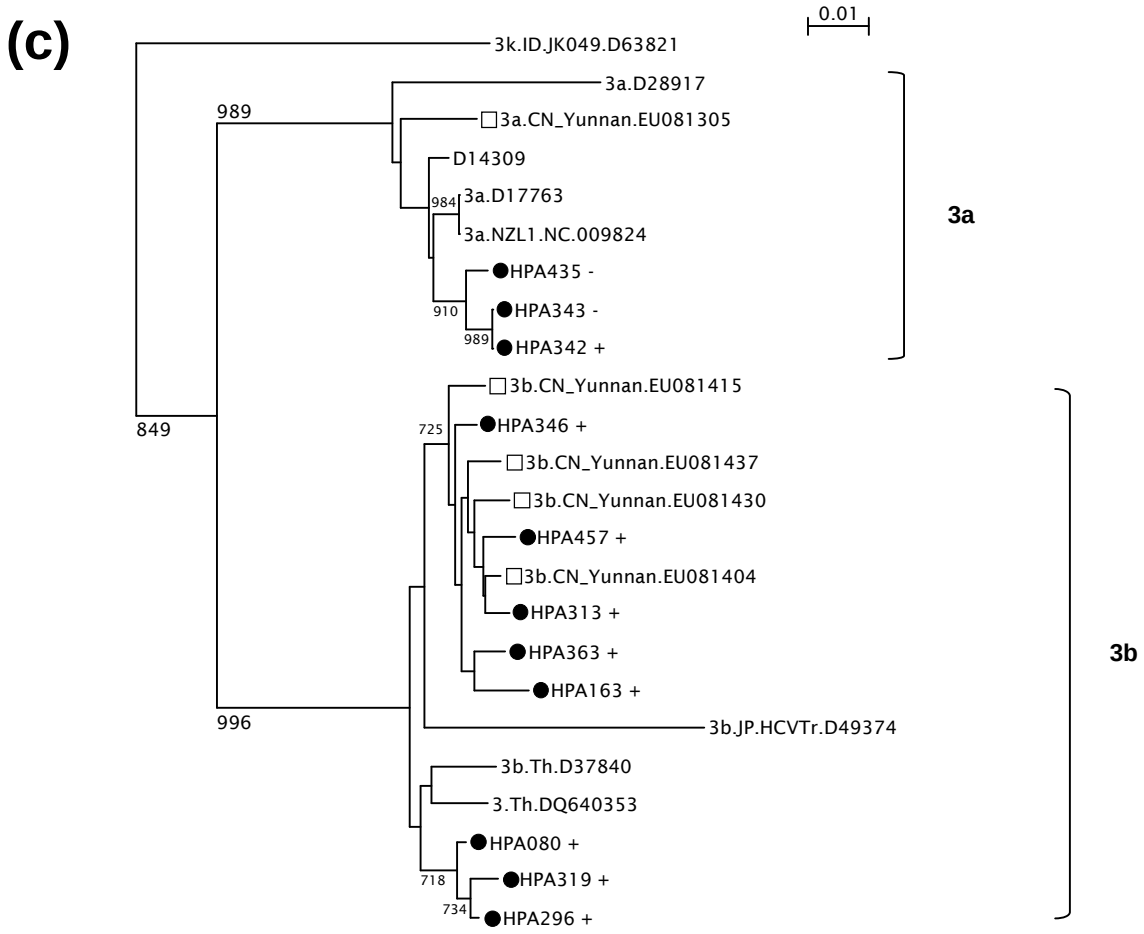
1b

Fig. 1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3a
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996
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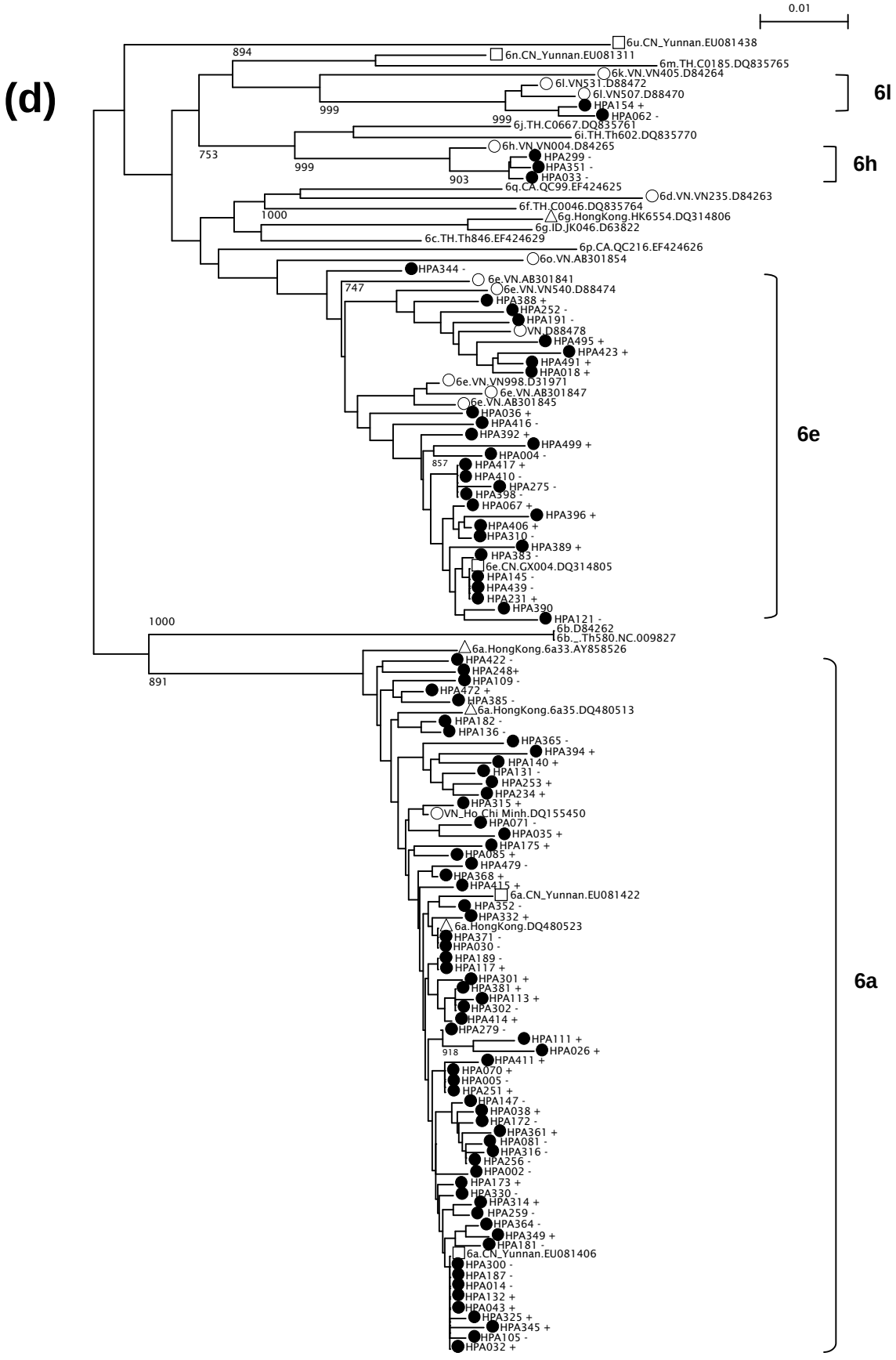
Fig. 1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6e
6a

Fig. 2

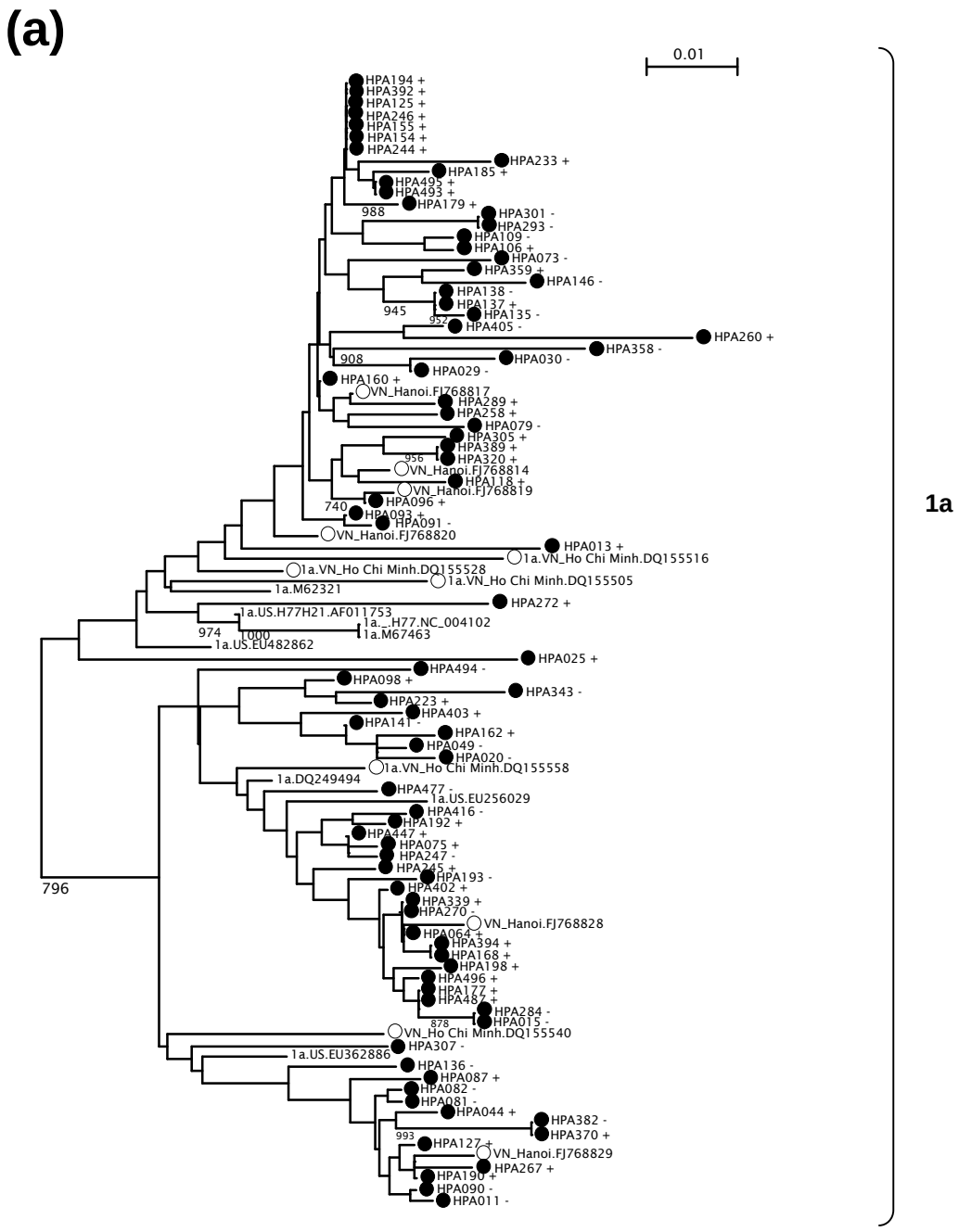
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Fig. 2

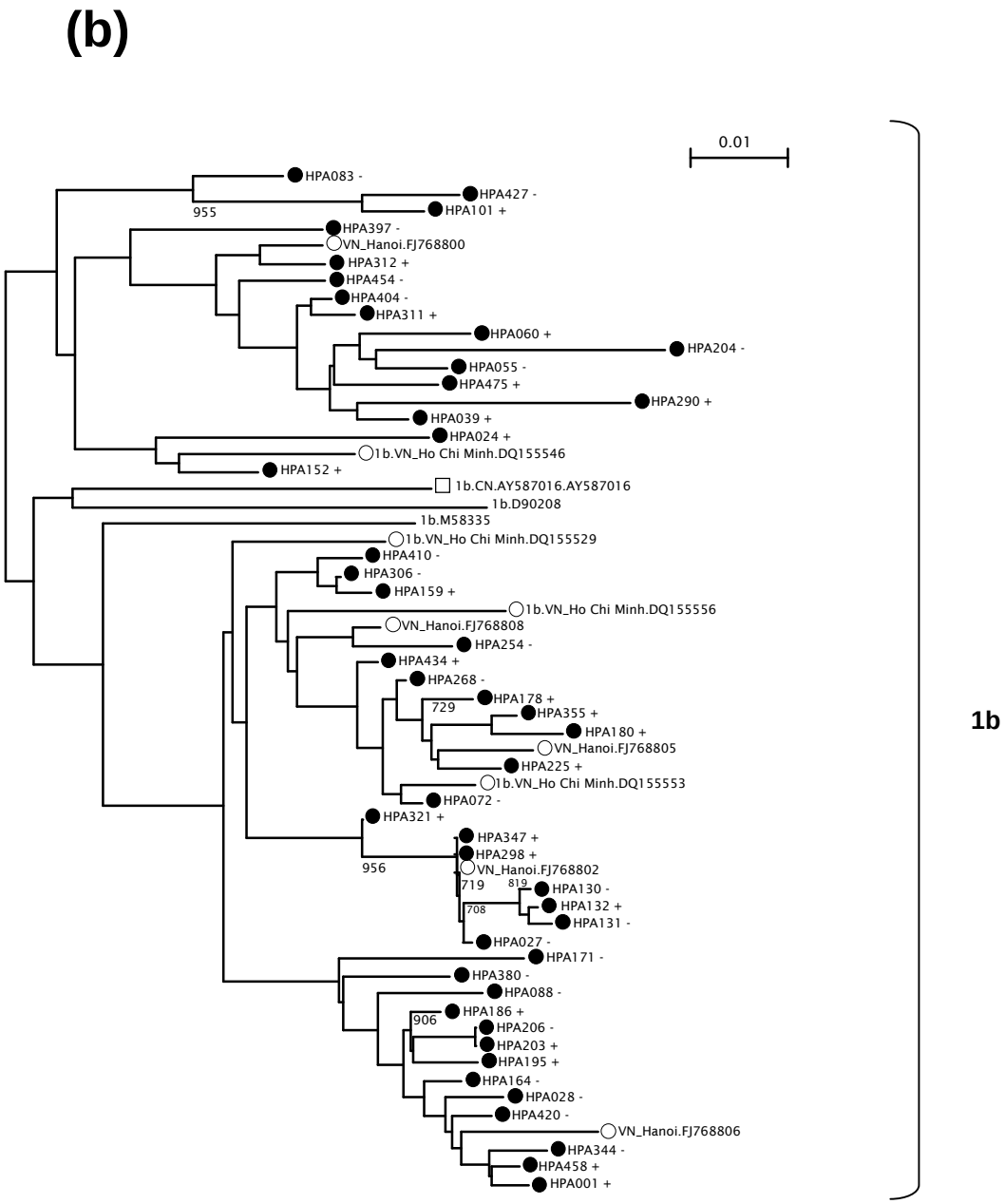
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Fig. 2

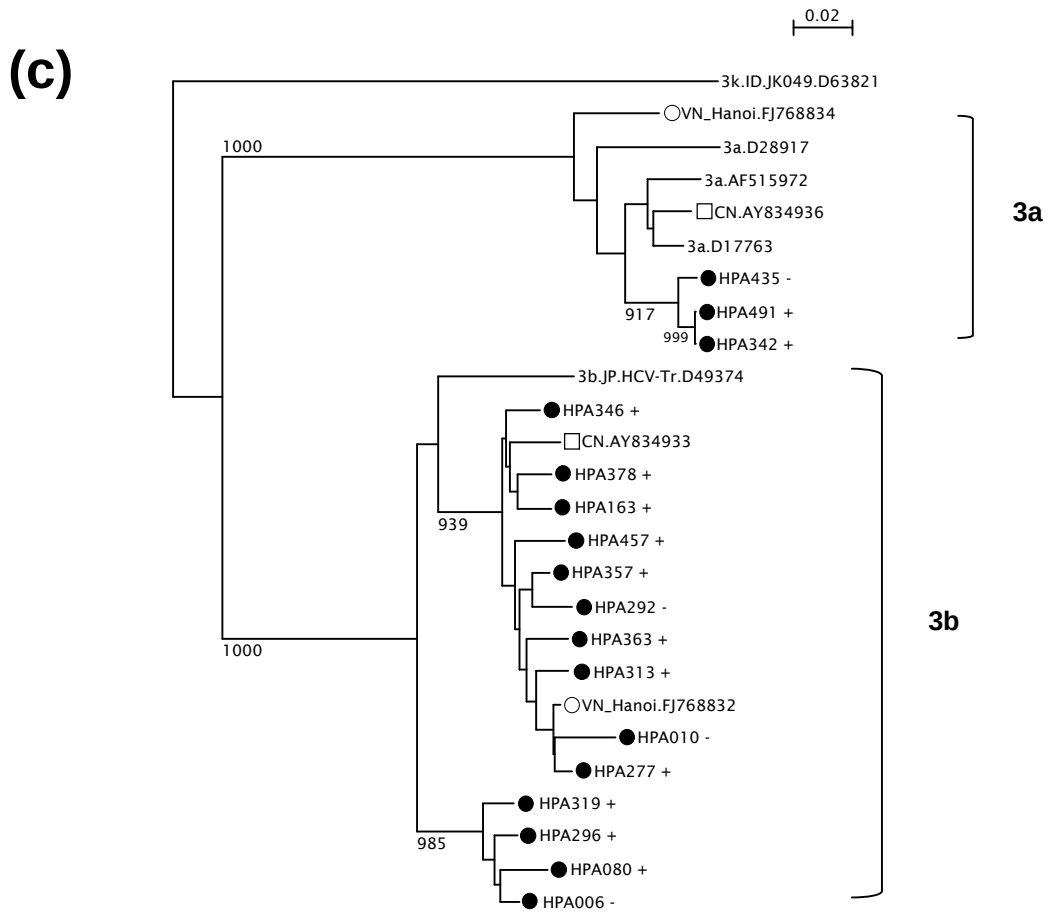
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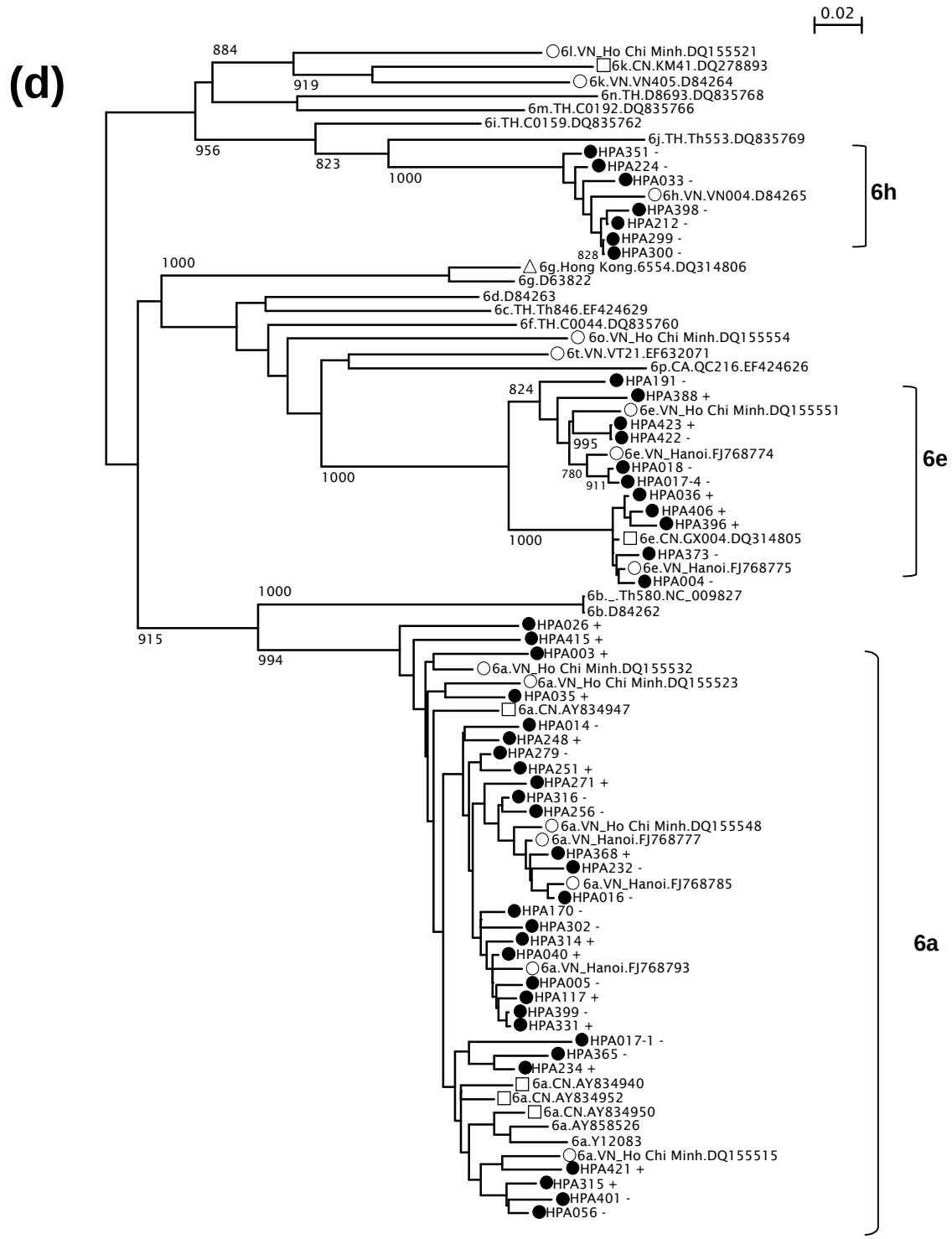
Fig. 2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