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A Natural Inter-genotypic (2b/1b) Recombinant of Hepatitis C Virus in the Philippines

Shortened title: A 2b/1b recombinant HCV in the Philippines

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ABSTRACT

The prevalence study and the characterization of hepatitis C virus (HCV) have been carried out in the Philippines and the sequence determination of 5'-untranslated region (5'-UTR)-Core and NS5B regions of HCV was performed in this study. An HCV strain (SE-03-07-1689) collected in Metro Manila, Philippines, belonged to discordant subtypes, 2b and 1b in 5'-UTR-Core and NS5B regions, respectively. The 9.3kb-sequence of this strain including the entire open reading frame were compared with those of the reference strains retrieved from the HCV sequences database (GenBank/EMBL/DDBJ) and indicated a recombination event. The computation of the sequence similarity mapped a crossover point within NS3 region. This is the second report on the inter-genotype recombinant of HCV and the third one when an intra-genotype recombinant is included. This recombinant strain, SE-03-07-1689, is tentatively designated as RF3_2b/1b according to the suggestions used for other two HCV recombinants.

INTRODUCTION

Hepatitis C virus (HCV) is a major cause of liver disease worldwide and a potential cause of substantial morbidity and mortality in the future. The prevalence of HCV infection is estimated to be 2%, representing 123 million people in the world. [Shepard et al., 2005]

HCV is characterized by a high degree of genetic heterogeneity like human immunodeficiency virus (HIV). [Zein, 2000] Although it is well known that recombinant forms of HIV-1 have been prevalent in the world, [Peeters, 2001] there have been few reports on recombination of HCV between different genotypes/subtypes, suggesting that these events are rare *in vivo* and that the HCV recombinant form is usually not viable. [Simmonds et al., 1994; Viazov et al., 2000] However, there was a report on the genotype discrepancy between Core and NS5B regions although the recombination crossover point was not clear. [Yun et al., 1996] Besides, the evidences of super-infection have been demonstrated among human population [Herring et al., 2004; Kao et al., 1996] and experimental animals, [Okamoto et al., 1994] suggesting the possible occurrence of recombination. In fact, the natural inter- and intra-genotypic recombinants of HCV (RF1_2k/1b and RF2_1a/1b) were recently found and the crossover points were clearly indicated in NS2 and NS5B regions, respectively. [Colina et al., 2004; Kalinina et al., 2002] The HCV recombination has been reported only in these cases to date. [Simmonds et al., 2005]

Several studies reported that the positive rate for anti-HCV was 2.2% among blood donors (Metro Davao, 1990) [Arguillas et al., 1991], and 4.6% among inmates (Metro Manila) [Katayama et al., 1996] in the Philippines. Recently, it was

shown in our study (Metro Cebu, 2002) that the HCV prevalence was extremely high among injecting drug users (70%), especially among those except the trainees in the rehabilitation centers (100%), as compared to other populations, such as sex workers, antenatal clinic attendees, students and health care workers (0-2%) [Agdamag et al., 2005] Most common HCV genotypes were 1a (55%) followed by 1b (27%), 2a (6%), 2b (3%) in Metro Manila (n=33) [Katayama et al., 1996] and were 1a (65%) followed by 2b (35%) in Metro Cebu (n=23) [Agdamag et al., 2005].

In this study, a natural inter-genotypic recombinant was identified through the determination of the crossover point in the 9.3kb-sequence of a Philippine strain.

MATERIALS AND METHODS

Subjects

HCV genotyping has been conducted at the National Reference Laboratory-STD AIDS Cooperative Central laboratory, San Lazaro Hospital (Metro Manila, Philippines). Samples and/or patients were referred to this laboratory for serologic, viral load testing and for genotyping in some limited cases. The researchers explained the objectives and the procedures of the study by the local language, and confirmed the patient's intent to join the study by his/her signing an informed consent form for the referred patients. The test was done anonymously under unlinked procedure for the referred samples.

Only seven samples from Metro Manila were available for the genotype test on NS5B and 5'-UTR-Core regions, and the possible recombination was analyzed on these samples. Risk factors and the travel history were not documented in the sample profile sheet. Nucleotide sequences of the strains found at Metro Cebu were from the database of STD AIDS Cooperative Central laboratory. [Agdamag et al., 2005]

RNA extraction, cDNA synthesis and amplification

HCV-RNA was extracted from 100μl of plasma using SMITEST EX-R&D (Genome Science Laboratories, Fukushima, Japan), and reverse-transcribed with random primers according to First-Strand cDNA Synthesis protocol (Invitrogen, Carlsbad, CA, USA).

cDNAs were amplified by nested PCR with the primers hep31b/hep32 and hep33b/hep34b in the first and second rounds, respectively, for the phylogenetic

analysis of NS5B as reported before. [White et al., 2000] The nested PCR for the region of 5'-UTR-Core was done with the primers of KY80/C0751R (5'-ATGTACCCCATGAGGTCGGC-3') and hep21b/C0727R (5'-CCACACGTAATGGTATCGATGAC-3'). [White et al., 2000] These amplifications were performed with 20μl reaction mixture containing 2.5mM $MgCl_2$, 200μM each dNTP, 0.5μM primers and one unit of Amplitaq Gold® (Applied Biosystems, Foster City, CA, USA). 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 and 50°C for 5'-UTR-Core for 30 sec, and 72°C for 1min per kb; and a final extension of 72°C for 10 min, respectively. The profile of the second-round PCR was done in the same condition except for the annealing temperature at 60°C for NS5B and 50°C for 5'-UTR-Core, respectively. PCR amplification was confirmed by visualization with ethidium bromide staining of the gel.

Similarly, a putative recombinant HCV-RNA was reverse-transcribed with random primer (Invitrogen) and the cDNAs were amplified with several gene specific primers for the 11 regions (Fig. 1).

Determination of nucleotide sequences

A PCR product was subjected to the determination of nucleotide sequence directly with the primers of hep33b/hep34b for NS5B, and hep21b/C0727R for 5'-UTR-Core, respectively, for the phylogenetic analysis of NS5B and 5'-UTR-Core regions. The sequences were aligned with those retrieved from the HCV sequences database (GenBank/EMBL/DDBJ) through ClustalW after the subsequent inspection and manual modification. [Thompson et al., 1994] The frequency of

nucleotide substitution in each base of the sequences was estimated by the Kimura two-parameter method. A phylogenetic tree was constructed by the neighbor-joining method, and its reliability was estimated by 1000 bootstrap replications. The profile of the tree was visualized with the program of Njplot. [Perriere and Gouy, 1996]

Several PCR-products were cloned into pCR2.1®-TOPO® vector in TOPO TA Cloning® kit (Invitrogen) and the nucleotide sequences were determined with the M13 primers in the kit (Invitrogen), the primers used for PCR, and newly synthesized ones to determine the sequence of a possible recombinant HCV strain (Fig.1). Three plasmids were subjected to the nucleotide sequence determination per each fragment of total 11 parts (Fig.1), and the representative sequence was considered to be one of the 11 tandem parts of the recombinant HCV sequence (9315 bp; 3014 amino acids including the entire open reading frame). The reliability of the determined nucleotide sequences was assessed by the 'Quality Value' in ABI PRIZM® Sequence Analysis Software (Applied Biosystems).

Recombination analysis

The sequence of the recombinant candidate, SE-03-07-1689, was aligned with reference sequences obtained from HCV sequence database, M62321_1a, AB049087_1b, AB191333_1b, AF169004_2a, AY232747_2b, and AF238486_2b (named after the accession numbers and subtypes), through ClustalW after the subsequent inspection and manual modification. [Thompson et al., 1994] The Single-Sequence Viewer of Recombination Analysis Tool (RAT) was used to examine recombination crossover point according to the user's guide available in

the RAT home page (http://cbr.jic.ac.uk/dicks/software/RAT/index.html). [Etherington et al., 2005]

Nucleotide sequence accession number

The sequences reported herein have been deposited in GenBank/EMBL/DDBJ under accession numbers of DQ364460 for SE-03-07-1689, DQ648505 to DQ648517 for 13 NS5B sequences (Fig. 2A), and DQ648495 to DQ648504 for ten 5'-UTR-Core sequences (Fig. 2B).

RESULTS

Phylogenetic analysis of the NS5B and 5'-UTR-Core regions

The NS5B and 5'-UTR-Core regions of seven strains collected in Metro Manila were analyzed (Fig. 2). Those seven strains were classified into subtype-1b (n=4) and 2a (n=3) based on NS5B (Fig. 2A), and subtype-1b (n=3), 2a (n=3) and 2b (n=1) on 5'-UTR-Core (Fig. 2B). One strain (SE-03-07-1689) belonged to the discordant subtypes, 2b (5'-UTR-Core, Fig. 2B) and 1b (NS5B, Fig. 2A).

Identification of the recombination crossover point

The sequence of SE-03-07-1689 from 5'-UTR-Core to the 5' part of NS3 was similar to that of the subtype 2b reference strains (Fig. 3). However, the 3' part sequence of this region was no more belonging to subtype 2b and was rather similar to the sequence of subtype 1b. The computation of similarity was performed to identify the recombination crossover point with RAT application based on the 9.4kb-sequence (1-9394, start-end; 939, window size; 469, increment size), and gave a crossover point within NS3 region. The shorter sequences within the single insert, #6(S4) (Fig.1), were compared with the corresponding ones of reference strains through RAT to analyze more accurate crossover point. The window size and the target sequence length in this analysis were decreased from 939 to 40 and from 9394 to 400, respectively. The crossover point was then located in the position of 3399/3400, corresponding to 3466/3467 of pj6CF strain [Yanagi et al., 1999] (Fig. 3, inset; and Fig. 4).

DISCUSSION

An HCV strain (SE-03-07-1689) belonged to the discordant subtypes, 2b and 1b, based on the sequences of 5'-UTR-Core and NS5B regions, respectively. The subsequent analysis of the genome sequence coding for the entire open reading frame identified the recombination crossover point within NS3 region and demonstrated the existence of natural inter-genotypic HCV recombinant strain (2b/1b, SE-03-07-1689) in the Philippine population. The parental strains of SE-03-07-1689 might exist in the Philippines because the phylogenetic analyses (Fig. 2) suggested that some HCV strains in the Philippines belonged to the same cluster with SE-03-07-1689. However, it is dispensable to perform more intensive investigation on the prevalence of recombinant HCV in Metro Manila and other areas of the Philippines.

The identification of the recombinant crossover point provided the final proof of the existence of a natural recombinant strain. [Kalinina et al., 2002] The recombinant crossover point was located in the NS3 region of the SE-03-07-1689 strain through RAT analysis, and was apparently mapped at 3399/3400 corresponding to 3466/3467 of pj6CF strain [Yanagi et al., 1999] between Glycine and Leucine residues in this study. However, only one strain (SE-03-07-1689) was used for the determination of recombination crossover point. The flanking sequences of the point derived from other recombinants and possible parental strains circulating in Metro Manila are needed to identify a more accurate crossover point of SE-03-07-1689 strain.

The recombination point had been often found in the genome coding the first non-structural protein, e.g., NS2 of HCV recombinants [Kalinina et al., 2002] and

NS1 of Dengue viruses, also members of *Flaviviriae*. [Tolou et al., 2001] However, analysis of the crossover junction of the SE-03-07-1689 was in the NS3 next to NS2 and this is discordant with the previous findings. Recombination crossover point was NS5B in the intra-genotype recombination case found in Peru, not in the genome coding the first non-structural protein. [Colina et al., 2004] Further demonstrations and comparisons must be required to elucidate the regularity principle of an HCV recombination. It is expected that analysis of the recombination crossover points would be accelerated through the recently developed cell culture system. [Heller et al., 2005; Lindenbach et al., 2005; Wakita et al., 2005; Zhong et al., 2005]

The genotype of HCV influences on the outcome of interferon treatment [Zein, 2000] and the interferon sensitivity has been attributed to the sequence variability of NS5A and E2. [Gale et al., 1998; Gale et al., 1997; Pavio et al., 2002; Polyak et al., 2001; Taylor et al., 2005; Taylor et al., 1999] The recombination events at NS2 in St Petersburg [Kalinina et al., 2002] and NS3 in Metro Manila implies that the genotype determination through the analysis of one subgenomic region, such as NS5B, may not be enough to assess the anti-HCV activity of interferon. It might be necessary to analyze the responsible nucleotide sequences in E2 and NS5A regions themselves for the assessment of antiviral activity. This assessment procedure beyond the simple genotyping would become indispensable when the frequency of HCV recombination proves to be a common event.

HCV strains are divided into genotypes, subtypes, and quasispecies, in the current HCV classification system and the recent consensus proposals. [Simmonds et al., 2005; Zein, 2000] However, recombination has not been considered in this

classification. Therefore, this recombinant strain found in the Metro Manila of the Philippines, SE-03-07-1689, is tentatively designated as RF3_2b/1b according to the suggestions used for other two strains (RF1_2k/1b and RF2_1a/1b) already reported as the HCV recombinants. [Colina et al., 2004; Kalinina et al., 2002] The existence of an HCV recombinant in the Philippines in addition to recent findings in St Petersburg and Lima implies that HCV recombination may have an important implication for the pathogenesis, diagnosis and treatment in HCV infection. Further prevalence study of HCV recombinants might have an important role for the discussion on the clinical impact of the HCV recombination event.

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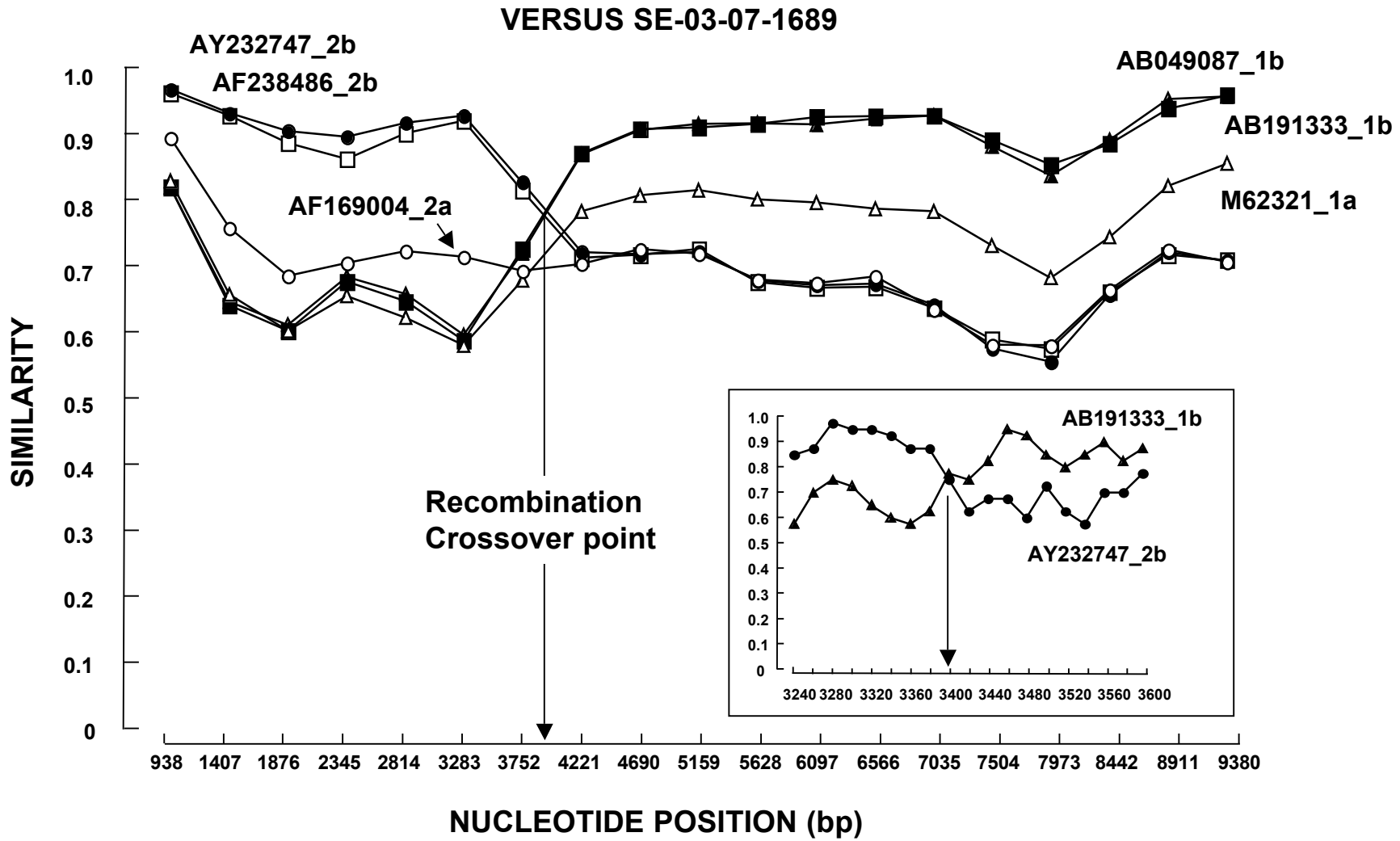
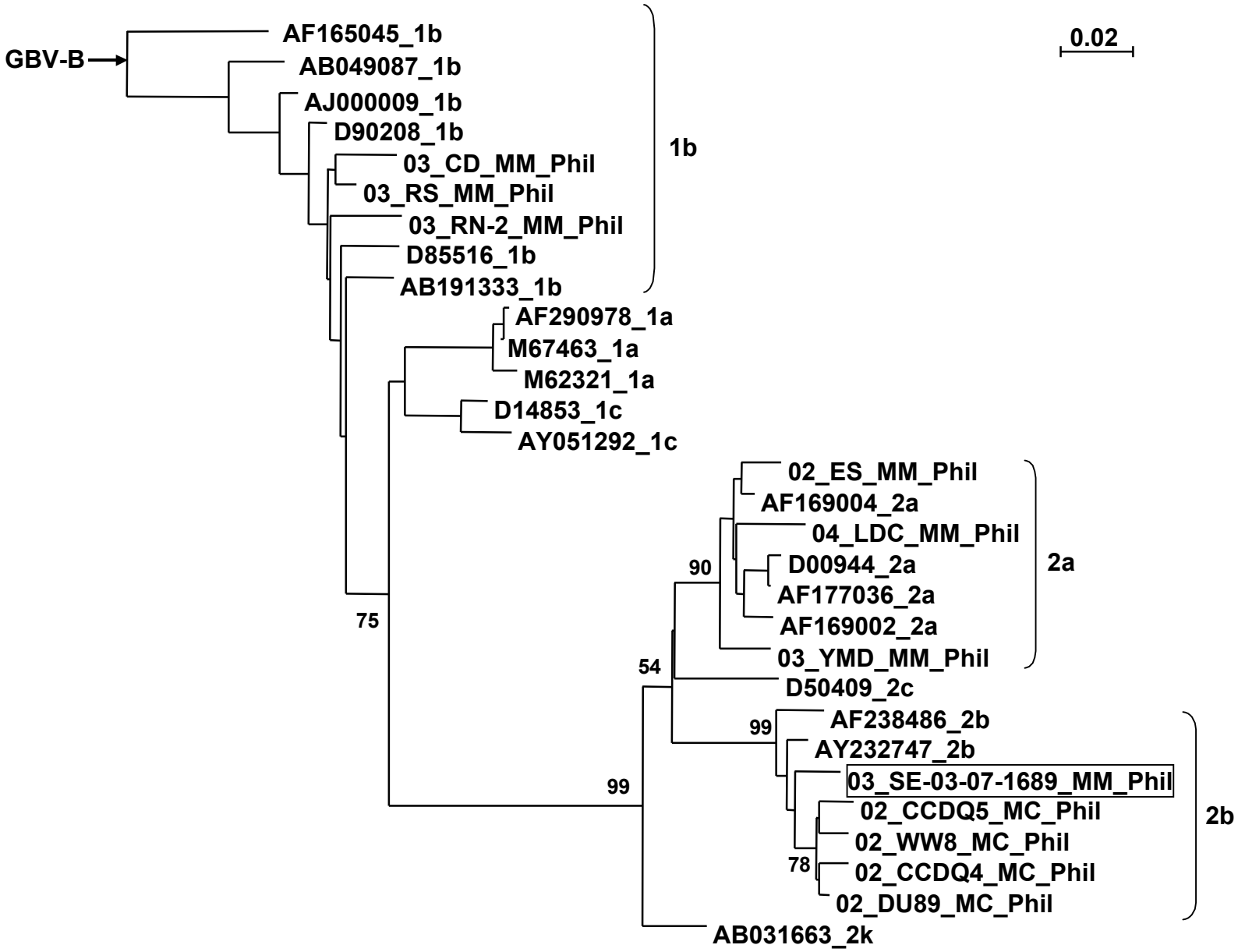

VERSUS SE-03-07-1689
AY232747_2b
AF238486_2b
AB049087_1b
AB191333_1b
M62321_1a
AF169004_2a
SIMILARITY
1.0
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
Recombination
Crossover point
938 1407 1876 2345 2814 3283 3752 4221 4690 5159 5628 6097 6566 7035 7504 7973 8442 8911 9380
NUCLEOTIDE POSITION (bp)
AB191333_1b
AY232747_2b
3240 3280 3320 3360 3400 3440 3480 3520 3560 3600

Fig.3

B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AB049087_1b
AJ000009_1b
D90208_1b
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03_RS_MM_Phil
03_RN-2_MM_Phil
D85516_1b
AB191333_1b
1b
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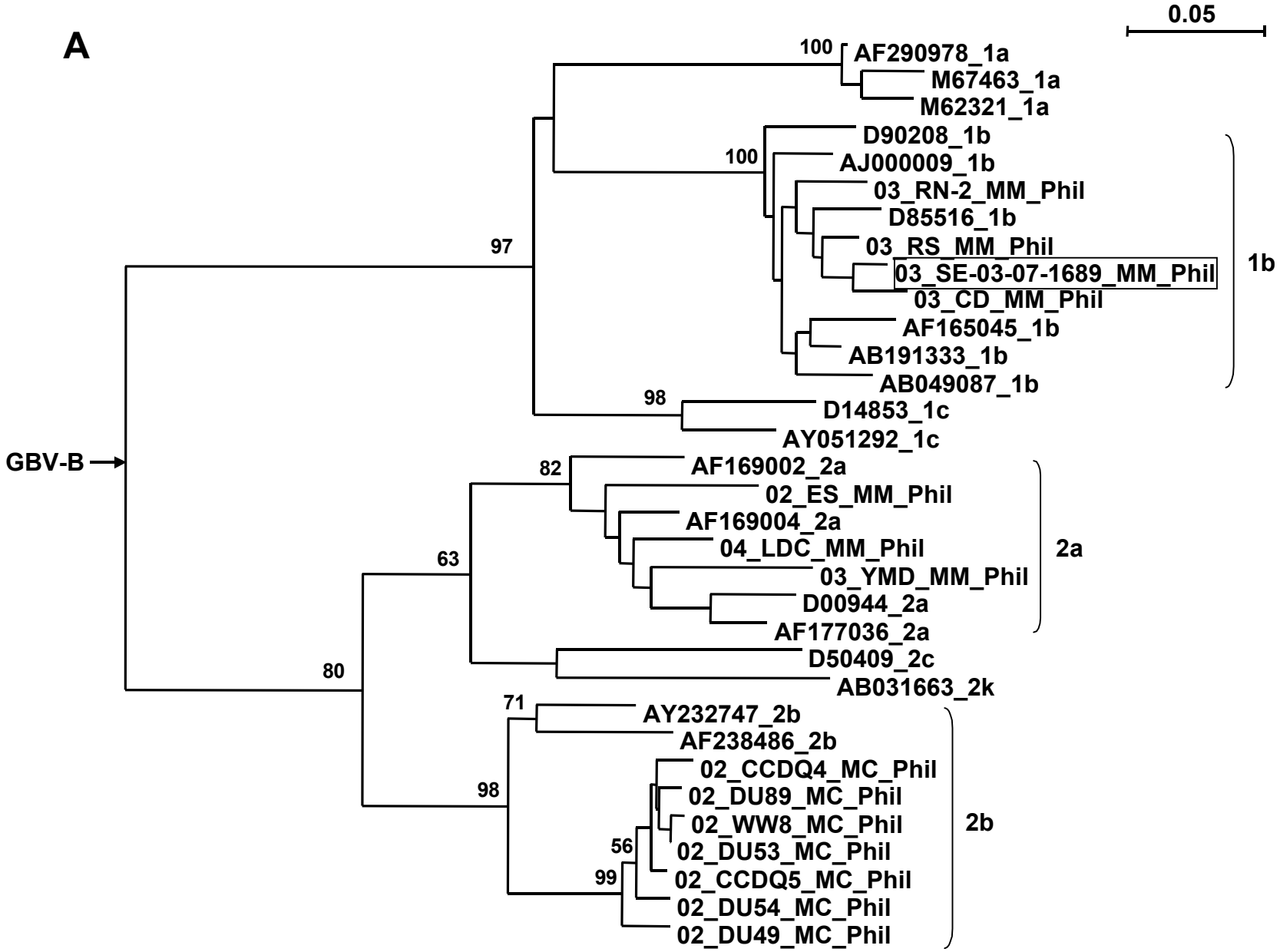
AF290978_1a
M67463_1a
M62321_1a
D14853_1c
AY051292_1c
02_ES_MM_Phil
AF169004_2a
04_LDC_MM_Phil
D00944_2a
AF177036_2a
AF169002_2a
03_YMD_MM_Phil
2a
D50409_2c
AF238486_2b
AY232747_2b
03_SE-03-07-1689_MM_Phil
02_CCDQ5_MC_Phil
02_WW8_MC_Phil
02_CCDQ4_MC_Phil
02_DU89_MC_Phil
2b
AB031663_2k
90
75
54
99
99
78

Fig.2B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AB191333_1b
AB049087_1b
1b
97
98
D14853_1c
AY051292_1c
GBV-B
82
AF169002_2a
02_ES_MM_Phil
AF169004_2a
04_LDC_MM_Phil
03_YMD_MM_Phil
D00944_2a
AF177036_2a
2a
63
D50409_2c
AB031663_2k
80
71
AY232747_2b
AF238486_2b
02_CCDQ4_MC_Phil
98
02_DU89_MC_Phil
02_WW8_MC_Phil
56
02_DU53_MC_Phil
99
02_CCDQ5_MC_Phil
02_DU54_MC_Phil
02_DU49_MC_Phil
2b

Fig.2A

Legend to Figures

Fig. 1 Strategy for the sequence determination of an HCV strain, SE-03-07-1689. A genetic organization of HCV is indicated with blocks (coding regions) and a flanking bar (5'-untranslated region, 5'-UTR). The numbers on the block denote the starting positions of the coding regions except the last one (the last nucleotide position), where the position (+1) corresponds to the 5'-end of Core region. The bar under the block defines the complementary DNA that was subjected to polymerase chain reaction (PCR), cloning into a vector, and a sequence determination. A primer ID was named after the 5'-end position of the nucleotide sequences of the reference strains (accession numbers; D10988 (genotype-2b) and D90208 (genotype-1b) (italic)) used for the primer design. The forward and reverse directions are indicated with F and R, respectively. Primers used for the sequence determination are shown in the parentheses.

Fig. 2 Phylogenetic analysis of the HCV strains on 222 and 538 nucleotides within the NS5B (**A**) and 5'-UTR-Core (**B**) regions. The tree was constructed by the neighbor-joining method using ClustalW program with GBV-B (accession number; NC001655) as the outgroup. Strains including the SE-03-07-1689 (boxed) are indicated with two digits of the collecting year and the location, such as MM Phil and MC Phil, denoting the Metro Manila and Metro Cebu, Philippines (e.g., 03_CD_MM_Phil and 02_CCDQ4_MC_Phil). Accession numbers are used for the reference strains with two digits indicating genotypes at the end of the number. Bootstrap values (>70%) are given on blanches as percentages from 1,000 replicates.

Fig. 3 Similarity plots for a putative recombinant strain, SE-03-07-1689, and reference strains based on the 9.4kb-sequences and a #6(S4) fragment (inset). The plot was performed with the Recombination Analysis Tool (RAT). Four reference sequences of HCV genotype-1b (AB049087 and AB191333) and -2b (AY232747 and AF238486) with the highest sequence scores (through the BLAST program) on the similarity to the sequence of SE-03-07-1689 were selected from GenBank/EMBL/DDBJ sequence database. Two sequences of M62321 (genotype-1a) and AF169004 (2a) were also selected from 'Ready made alignments' of the Los Alamos HCV sequences database. The recombination analysis was performed throughout 9384 bases with a window size of 938 and a step increment of 469, and also 400 bases in the #6(S4) fragment with a window size of 40 and an increment of 20 (inset), respectively. An arrow indicates the crossover point between the nucleotide positions of 3399 and 3400 (inset).

Fig. 4 Alignment of the nucleotide sequences of SE-03-07-1689 and four reference sequences; AF238486, AY232747, AB049087 and AB191333 within #6(S4) fragment. Arrow indicates the possible crossover point (3399/3400). The borderline between NS2 and NS3 was determined on Los Alamos HCV sequences database.

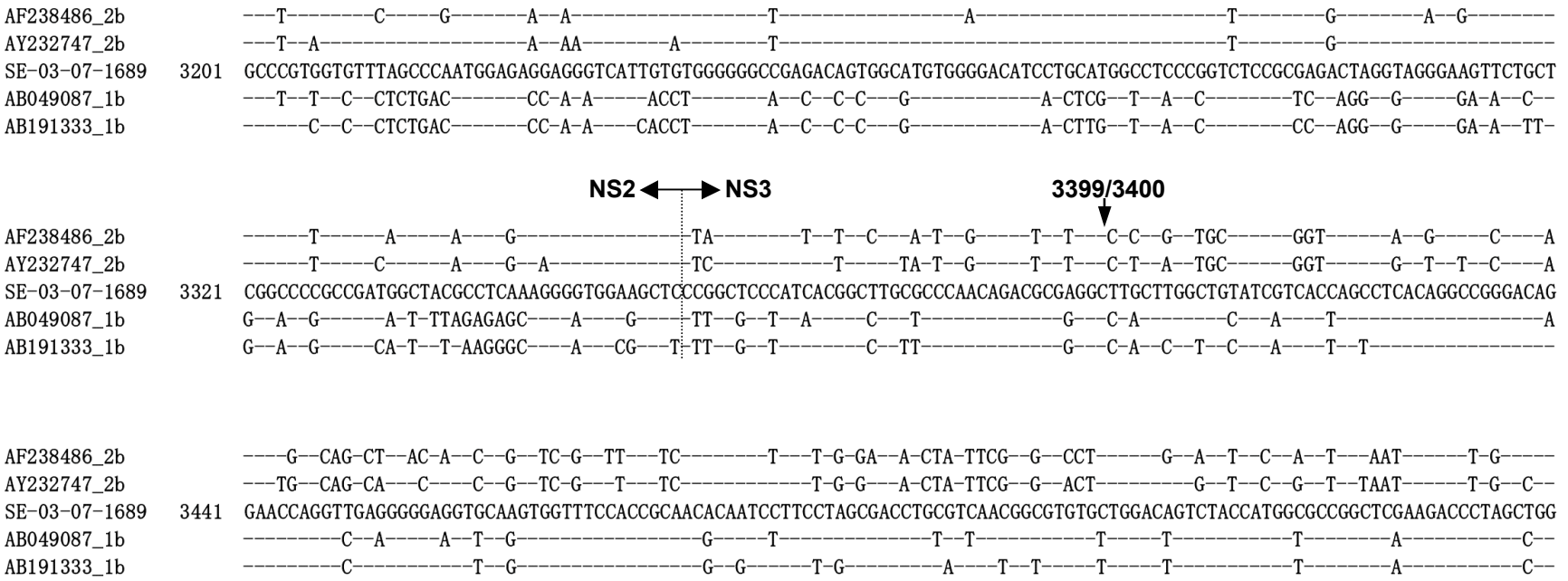
NS2
NS3
3399/3400
AF238486_2b
AY232747_2b
SE-03-07-1689 3201
AB049087_1b
AB191333_1b
SE-03-07-1689 3321
SE-03-07-1689 3441

Fig.4

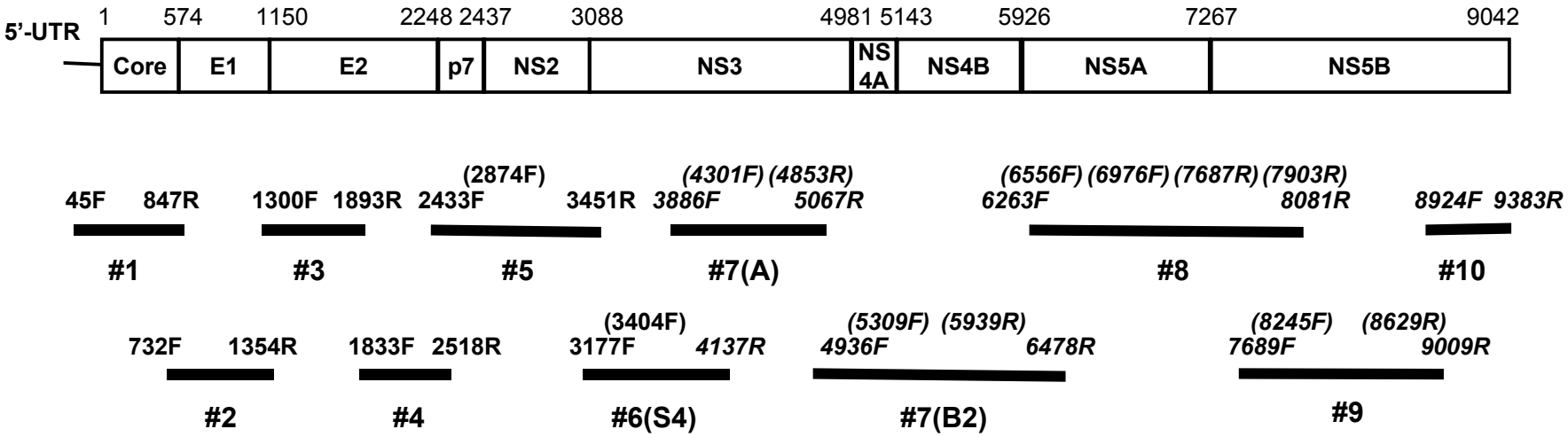
5'-UTR
1
574
1150
2248
2437
3088
4981
5143
5926
7267
9042
Core
E1
E2
p7
NS2
NS3
NS 4A
NS4B
NS5A
NS5B
45F
847R
1300F
1893R
2433F
(2874F)
3451R
3886F
(4301F)
(4853R)
5067R
6263F
(6556F)
(6976F)
(7687R)
(7903R)
8081R
8924F
9383R
#1
#3
#5
#7(A)
#8
#10
732F
1354R
1833F
2518R
3177F
(3404F)
4137R
4936F
(5309F)
(5939R)
6478R
7689F
(8245F)
(8629R)
9009R
#2
#4
#6(S4)
#7(B2)
#9

Fig.1