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Sequence note

Genotypic Characterization of HIV-1 Prevalent in Kepulauan Riau, Indonesia

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Running title: HIV-1 molecular epidemiology in Kepulauan Riau

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Abstract

Kepulauan Riau is a famous tourist destination in Indonesia. The epidemic of human immunodeficiency virus type 1 (HIV-1) infection is gradually increasing in this region. We collected peripheral blood samples from 62 antiretroviral therapy (ART)-experienced individuals. The amplification of viral genomic fragments, HIV-1 subtyping, and the detection of HIV drug resistance (HIVDR) were performed. Viral subtyping revealed that the most prevalent HIV-1 subtype/circulating recombinant form (CRF) was CRF01_AE (55.6%), followed by recombinants between CRF01_AE and subtype B (17.8%) and then subtype B (15.6%). Recombinants containing CRF02_AG gene fragments were also detected (11.1%). Regarding HIVDR, no drug resistance-associated major mutations were found in *pol* genes encoding protease, although minor mutations were frequently detected. Furthermore, major mutations, including M184V (2.2%) and Y188L (2.2%), were identified in the viral *pol* gene encoding reverse transcriptase derived from a study participant, suggesting that the prevalence of HIVDR is low in the region.

Key words: CRF01_AE, HIVDR, ART, Kepulauan Riau, Indonesia

Text

Human immunodeficiency virus type 1 (HIV-1) infection and acquired immunodeficiency syndrome (AIDS) have been a major public health concern in Indonesia. The prevalence of HIV in Indonesia has been increasing yearly. According to a 2017 report by the Directorate General of Disease Prevention and Control, Ministry of Health, Republic of Indonesia, 242,699 individuals were infected with HIV. The annual number of HIV cases in Indonesia has increased from 7,195 in 2006 to 41,250 in 2016¹. Antiretroviral therapy (ART) using combinations of potent antiretroviral drugs in simplified regimens have significantly reduced the morbidity and mortality of HIV-infected individuals, and infected individuals may anticipate an almost normal life expectancy. ART is currently recommended for all HIV-infected individuals, regardless of the CD4 T lymphocyte cell count in Indonesia. The World Health Organization (WHO) recommended that the first-line regimen for adults and adolescents contain two nucleoside/nucleotide reverse transcriptase inhibitors (NRTIs) and one non-nucleoside reverse transcriptase inhibitor (NNRTI). In order to maximize the long-term effectiveness of first-line ART regimens, it is important to ensure that the scaling up of the treatment is accomplished by measures to monitor and improve service quality at the site and program levels². However, HIV drug resistance (HIVDR) has emerged with the expansion of the ART program in Indonesia. In our previous study, drug resistance-associated mutations were detected in *pol* genes encoding reverse transcriptase (RT genes) derived from 37.7% (20/53) of ART-experienced individuals in Surabaya³, while the prevalence of transmitted drug resistance was low (less than 5%) in Surabaya and Sorong^{4,5}.

HIV is divided into two types, HIV-1 and HIV-2. HIV-1 is distributed worldwide, while HIV-2 is confined primarily to West Africa. HIV-1 is subdivided into four groups: M (major), O (outlying), N (new or non-M, non-O), and P, which are prevalent in specific geographic regions. Group M is responsible for the pandemic and has been further divided

into eleven subtypes and subsubtypes, as well as into recombinant forms, which have, in turn, been divided into circulating recombinant forms (CRFs) and unique recombinant forms. Subtype B is the predominant subtype in the Americas, Europe, and Australia, whereas there is a growing epidemic of non-B subtypes and CRFs in Africa and Asia⁶. CRF01_AE is one of the major CRFs of HIV-1 dominating the global epidemic, and is prevalent throughout Southeast Asia. We previously reported that CRF01_AE was a dominant type of HIV-1 in Indonesia, while other recombinants, including the recombinant between CRF01_AE and subtype B, have emerged in Indonesia^{3,5}.

Kepulauan Riau is famous tourist destination in Indonesia. According to its geographic position, the Kepulauan Riau province is located on the borders with Vietnam and Cambodia to the north and Singapore and Malaysia in the west. Kepulauan Riau has 7 islands, Karimun, Bintan, Natuna, Lingga, Anambas, Batam, and Tanjung Pinang⁷. The Kepulauan Riau province is one of the top five provinces in Indonesia for new HIV cases per 100,000 population in 2011 after Papua, West Papua, Bali, and Jakarta⁸. The Directorate General of Disease Prevention and Control, Ministry of Health, Republic of Indonesia reported that the annual number of HIV cases was still increasing in the Kepulauan Riau province in recent years, e.g., 973 cases in 2014, 885 cases in 2015, 1,037 cases in 2016, and 174 cases between January and March 2017. A total of 6,971 HIV cases were reported between 2009 and March 2017. In this province, HIV has spread rapidly among specific population groups that are generally regarded as high risk groups, namely, injecting drug users, commercial sex workers, and men who have sex with men. The proportion of HIV cases by gender was reported to be 57.4% males versus 42.6% females⁹. Effective prevention and treatment programs are needed against HIV infection. In Indonesia, prevention and monitoring programs for HIVDR are limited; however, a HIVDR analysis may provide helpful information for clinicians to decide whether to start ART for new HIV-infected individuals and switch ART regimens for ART-

experienced individuals when treatment failure is suspected. In the present study, we aimed to identify the HIV-1 subtypes and CRFs present in Kepulauan Riau, Indonesia and monitor HIVDR among ART-experienced individuals in this region.

Sixty-two HIV-1-infected, ART-experienced individuals were recruited from the voluntary counseling and testing program in Tanjung Pinang “Kemuning” hospital in Kepulauan Riau. The inclusion criterion for study participants was HIV-1-infected individuals on ART for more than 1 year. Ethical clearance was obtained from the Institutional Ethics Committees of Airlangga University (approval number: 25-995/UN3.14/PPd/2013) and Kobe University Graduate School of Medicine (approval number: 784). An informed consent form and questionnaire were provided to each participant for acceptance prior to their enrollment in the present study. Socio-demographic, behavioral, and clinical data, including opportunistic infections and the recent conditions of these individuals, were retrieved retrospectively from medical records (Table 1). The mean age of participants was 40 years old, whereas the gender distribution was 33 males (53.2%) and 29 females (46.8%). Regarding the ethnicity of participants, most were Melayu (43.5%) and Tionghoa (related geographical location near Singapore and Malaysia) (33.9%). Fifty-two participants (83.9%) had been on ART for more than 3 years. Nineteen participants (30.6%) were prescribed a first-line regimen of lamivudine (3TC), zidovudine (AZT), and nevirapine (NVP). Thirteen participants (21.0%) were prescribed a first-line regimen of 3TC, AZT, and efavirenz (EFV). Twenty-two participants (35.5%) were prescribed a first-line regimen of tenofovir (TDF), 3TC, and the NNRTI, NVP or EFV. Thirty-seven patients (59.7%) had opportunistic infections including tuberculosis and cytomegalovirus.

Ten milliliters of ethylenediaminetetraacetic acid-anticoagulated whole blood samples were collected from each individual. Plasma was separated by centrifugation for 10 min at 2,000 rpm and stored at -80°C for future analyses. Peripheral blood mononuclear cells

(PBMC) were isolated by the BD Vacutainer CPT (Cell Preparation Tube) System (Becton, Dickinson and Company, Franklin Lakes, NJ, USA). DNA was extracted from PBMC using the QIAamp DNA blood mini kit (Qiagen, Hilden, Germany). The HIV-1 *pol* gene encoding protease (PR gene), RT, *gag*, and *env* genes were amplified by the GoTaq green master mix (Promega, Madison, WI, USA) and primer sets as follows. The PR gene was amplified by nested PCR with the primers DRPR05, 5'-AGACAGGYTAATTTTTTAGGGA-3' [corresponding to nucleotides (nt) 2074 to 2095 of the HIV-1 reference strain, HXB2 (GenBank accession no. K03455)] and DRPR02L, 5'-TATGGATTTTCAGGCCCAATTTTTGA-3' (nt 2716 to 2691) in the first round and DRPR01M, 5'-AGAGCCAACAGCCCCACCAG-3' (nt 2148 to 2167) and DRPR06, 5'-ACTTTTGGGCCATCCATTCC-3' (nt 2611 to 2592) in the second round. The RT gene was amplified by nested PCR with the primers RT1L, 5'-ATGATAGGGGGAATTGGAGGTTT-3' (nt 2388 to 2410) and DRRT4L, 5'-TACTTCTGTTAGTGCTTTGGTTCC-3' (nt 4402 to 4380) in the first round, and RT7L, 5'-GACCTACACCTGTCAACATAATTGG-3' (nt 2485 to 2509) and DRRT6L, 5'-TAATCCCTGCATAAATCTGACTTGC-3' (nt 4309 to 4285) in the second round. The viral *gag* gene was amplified by nested PCR with the primers H1G777, 5'-TCACCTAGAACTTTGAATGCATGGG-3' (nt 1231 to 1255) and H1P202, 5'-CTAATACTGTATCATCTGCTGCTCCTGT-3' (nt 2352 to 2325) in the first round, and H1Gag1584, 5'-AAAGATGGATAATCCTGGG-3' (nt 1577 to 1595) and G17, 5'-TCCACATTTCCAACAGCCCTTTTT-3' (nt 2040 to 2017) in the second round. The viral *env* gene was amplified by nested PCR with the primers M5, 5'-CCAATTCCCATAACATTATTGTGCCCCAGCTGG-3' (nt 6858 to 6889) and M10, 5'-CCAATTGTCCCTCATATCTCCTCCTCCAGG-3' (nt 7661 to 7632) in the first round, and M3, 5'-GTCAGCACAGTACAATGIACACATGG-3' (nt 6948 to 6973) and M8, 5'-TCCTTCCATGGGAGGGGCATACATTGC-3' (nt 7547 to 7521) in the second round. PCR

conditions are available upon request. PCR products were electrophoresed on a 1.5% agarose gel and visualized under a UV light after staining with ethidium bromide. The PCR products amplified at the end-point dilution of DNA templates were subjected to a sequencing analysis in order to examine the genomic fragment of the major viral population in a sample.

A sequencing analysis was performed using the BigDye Terminator v3.1 Cycle Sequencing kit and ABI PRISM3500xL genetic analyzer (Applied Biosystems, Foster City, CA, USA). Sequencing data were then assembled and aligned using Genetyx version 10 software (Genetyx, Tokyo, Japan). The sequencing data of 39 PR genes [296 base pairs (bp); nt 2253-2543], the N terminus of 29 RT genes (741 bp; nt 2571-3311), the partial fragment of 24 *gag* genes encoding Gag p24 (390 bp; nt 1633-2002), and the partial fragment of 36 *env* genes encoding the gp120 C2-V3 region (389 bp; nt 7020-7408) were obtained from 62 peripheral blood samples. The nucleotide sequences of the PR, RT, *gag*, and *env* genes have been registered in the GenBank database under accession numbers MG717590 - MG717628 (PR genes), MG717629 - MG717657 (RT genes), MG717566 - MG717589 (*gag* genes), and MG717530 - MG717565 (*env* genes).

HIV-1 subtyping was then performed by a phylogenetic tree analysis, jumping profile Hidden Markov Model (jpHMM)-HIV tools available on the website (http://jphmm.gobics.de/submission_hiv), and recombinant identification program (RIP) available on the HIV sequence database website (<http://www.hiv.lanl.gov/>). Neighbor-joining trees with the Kimura two-parameter model were constructed using MEGA6.2 software^{10, 11}. Bootstrap values (1,000 replicates) for relevant nodes were reported on a representative tree. Phylogenetic trees for the PR, RT, *gag*, and *env* genes are shown in Figure 1. Viral subtyping by RIP, jpHMM-HIV, and phylogenetic trees showed basically consistent results (data not shown); however, if they showed different results, we assigned the viral subtype based on the results of the phylogenetic tree analysis. In addition, if there was an incompatibility in the

subtype or CRF among the PR, RT, *gag*, and *env* genes, the viral gene was considered to be from a recombinant virus. Viral subtyping revealed that the predominant HIV-1 strain in Kepulauan Riau was CRF01_AE (55.6%), followed by recombinants between CRF01_AE and subtype B (17.8%), subtype B (15.6%), and recombinants containing CRF02_AG gene fragments together with the gene fragments of subtypes B, G, and/or CRF01_AE (11.1%). HIV-1 CRF01_AE is widely circulating in Southeast Asia, including Cambodia, Vietnam, Malaysia, and Thailand, as well as in East Asia such as China, Taiwan, Korea, and Japan⁶. In addition, the higher prevalence of CRF01_AE in the Kepulauan Riau province is consistent with our previous findings obtained in Surabaya, Indonesia³. In contrast, subtype B and CRF01_AE were both the major subtype/CRF prevalent in Papua and West Papua^{5, 12}. Recombinant viruses containing the CRF02_AG gene fragments were detected in Kepulauan Riau. Since CRF02_AG was rarely found in Indonesia, the continuous surveillance of HIV-1 subtypes and CRFs appears to be necessary.

Drug resistance mutations were detected in the PR and RT genes based on the International Antiviral Society-United States (IAS-USA) guidelines¹³. No drug resistance-associated major mutations were found in the PR genes (data not shown); however, several minor mutations, such as M36I (an amino acid substitution at position 36 from methionine to isoleucine) (71.8%), L89M (71.8%), I93L (43.6%), L63P (38.5%), K20R (30.8%), and V77I (28.2%), were detected (Table 2). These minor mutations may present as natural polymorphisms, and were detected more frequently in CRF01_AE PR genes than in subtype B PR genes (Table 2), which is consistent with previous findings^{14, 15}. The high frequency of natural polymorphisms in CRF01_AE PR has been reported previously^{14, 15}. In contrast, drug resistance-associated major mutations against NRTI and NNRTI, including M184V (2.2%) and Y188L (2.2%), were detected in a RT gene derived from an individual (sample ID: TP8) (Table 3). The individual (sample ID: TP8) had received a combination of TDF, 3TC, and

EFV since 2012. The mutation, M184V confers resistance to 3TC, while Y188L confers resistance to EFV¹³. In addition, this individual had opportunistic infections (data not shown); therefore, the prescription of other ART regimens to this individual may be needed in order to avoid virological failure.

In conclusion, CRF01_AE viruses were the predominant HIV-1 strains in Kepulauan Riau, Indonesia. The predominance of CRF01_AE was consistent with that in other regions in Indonesia. In addition, recombinants between CRF01_AE and subtype B, subtype B, and other recombinants including CRF02_AG gene fragments were detected; therefore, the continuous surveillance of circulating HIV-1 subtypes and CRFs may be required in this country. Moreover, a low prevalence of drug resistance-associated mutations against NRTIs and NNRTIs was noted. Nevertheless, the prescription of first-line ART regimens with 2 NRTIs and 1 NNRTI has been expanded in Indonesia, and further large-scale and continuous surveillance studies may be required to monitor the appearance of HIVDR.

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Author Disclosure Statement

No competing financial interests exist.

Sequence Data

Nucleoside sequences are available under GenBank accession numbers MG717530 - MG717657.

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Figure legends

FIG. 1. Phylogenetic tree analysis of HIV-1 PR, RT, *gag*, and *env* gene sequences collected in Kepulauan Riau, Indonesia.

Phylogenetic trees were constructed for the HIV-1 PR (A), RT (B), *gag* (C), and *env* genes newly sequenced in the present study (D). The corresponding viral genes of reference HIV-1 strains representing subtypes A1, A2, B, C, D, and G as well as CRF01_AE (01_AE), CRF02_AG (02_AG), CRF15_01B (15_01B), CRF33_01B (33_01B), and CRF34_01B (34_01B) were included in the analyses (shown in bold letters). Sequence IDs are presented as a GenBank accession number, sample ID or the ID of the reference HIV-1 strain, and the subtype or CRF of the reference strain (shown in parentheses) in that order. Bootstrap values were shown if they were >70.

TABLE 1. DEMOGRAPHIC CHARACTERISTICS OF ART-EXPERIENCED INDIVIDUALS IN KEPULAUAN RIAU, INDONESIA

Characteristics	Value (n = 62)
Age, mean years (SD)	40.0 (10.1)
Gender, n (%)	
Male	33 (53.2%)
Female	29 (46.8%)
Marital status, n (%)	
Married	52 (83.9%)
Single	9 (14.5%)
Unidentified	1 (1.6%)
Ethnicity, n (%)	
Jawa	7 (11.3%)
Melayu	27 (43.5%)
Bugis	0 (0.0%)
Tionghoa	21 (33.9%)
Other (Batak, Bali, Sunda, Flores)	6 (9.7%)
Unidentified	1 (1.6%)
Transmission risk category, n (%)	
Heterosexual intercourse	0 (0.0%)
Intravenous drug use	12 (19.4%)
Homosexual intercourse	1 (1.6%)
Unidentified	49 (79.0%)
Types of ART prescribed, n (%)	
3TC+AZT+NVP	19 (30.6%)
3TC+AZT+EFV	13 (21.0%)
TDF+AZT+NVP or EFV	22 (35.5%)
Unidentified	8 (12.9%)
Duration of ART, n (%)	
1 – 3 years	7 (11.3%)
> 3 years	52 (83.9%)
Unidentified	3 (4.8%)
Opportunistic infections, n (%)	
No	24 (38.7%)
Yes	37 (59.7%)
Unidentified	1 (1.6%)

TABLE 2. FREQUENTLY DETECTED DRUG RESISTANCE-ASSOCIATED MINOR MUTATIONS IN PR GENES DERIVED FROM ART-EXPERIENCED INDIVIDUALS IN KEPULAUAN RIAU, INDONESIA

Mutations	Frequency (%)			
	All (n = 39)	CRF01_AE ^a (n = 26)	Subtype B (n = 9)	Others (n = 4)
K20R ^b	12 (30.8)	12 (30.8)	0 (0)	0 (0)
M36I	28 (71.8)	25 (64.1)	0 (0)	3 (7.7)
L63P	15 (38.5)	6 (15.4)	6 (15.4)	3 (7.7)
V77I	11 (28.2)	7 (17.9)	4 (10.3)	0 (0)
L89M	28 (71.8)	24 (61.5)	0 (0)	4 (10.3)
I93L	17 (43.6)	8 (20.5)	9 (23.1)	0 (0)

^a The subtype of PR genes was assigned based on jpHMM, RIP, and phylogenetic analyses.

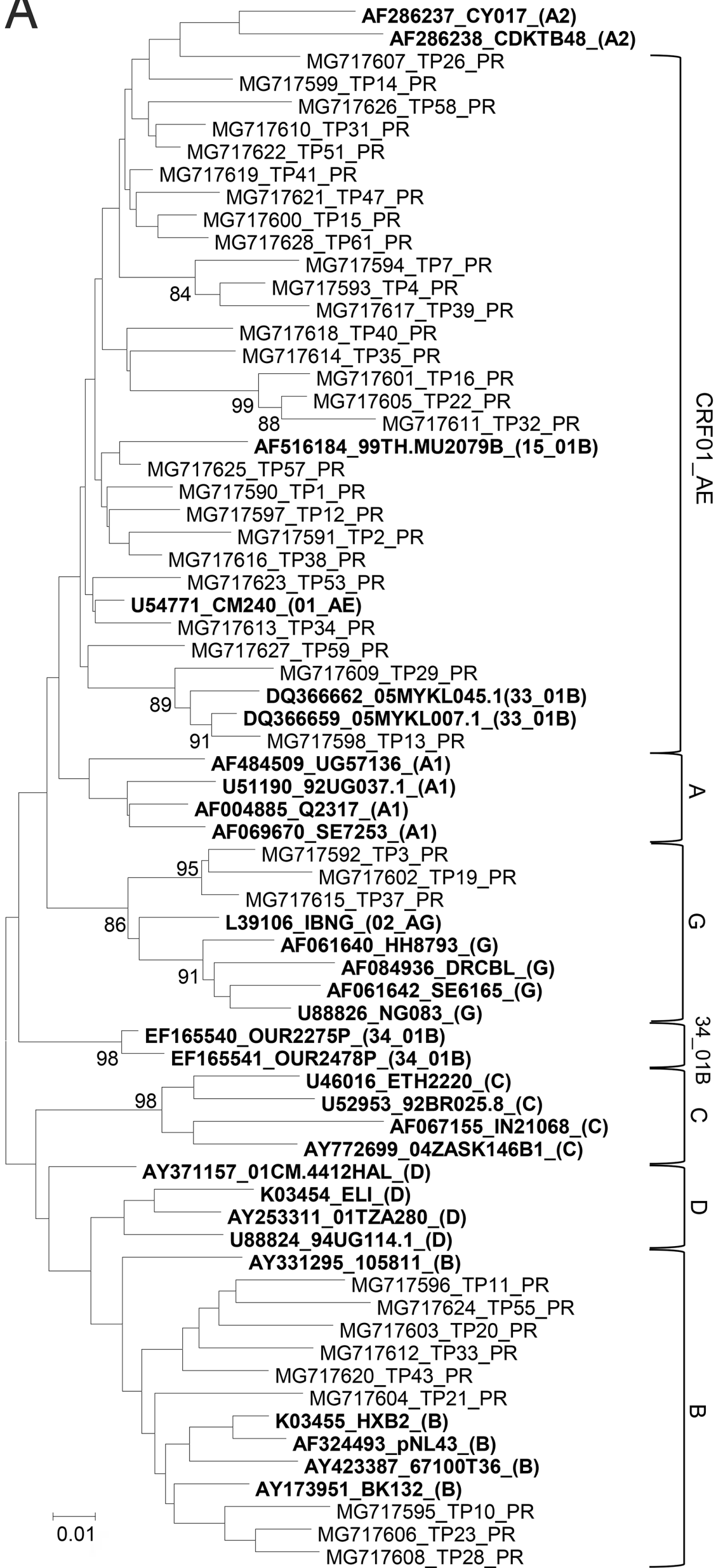
^b The identification of drug resistance-associated mutations was based on the guidelines published by the International Antiviral Society-United States (IAS-USA).

TABLE 3. DRUG RESISTANCE-ASSOCIATED MUTATIONS IN RT GENES DERIVED FROM ART-EXPERIENCED INDIVIDUALS IN KEPULAUAN RIAU, INDONESIA

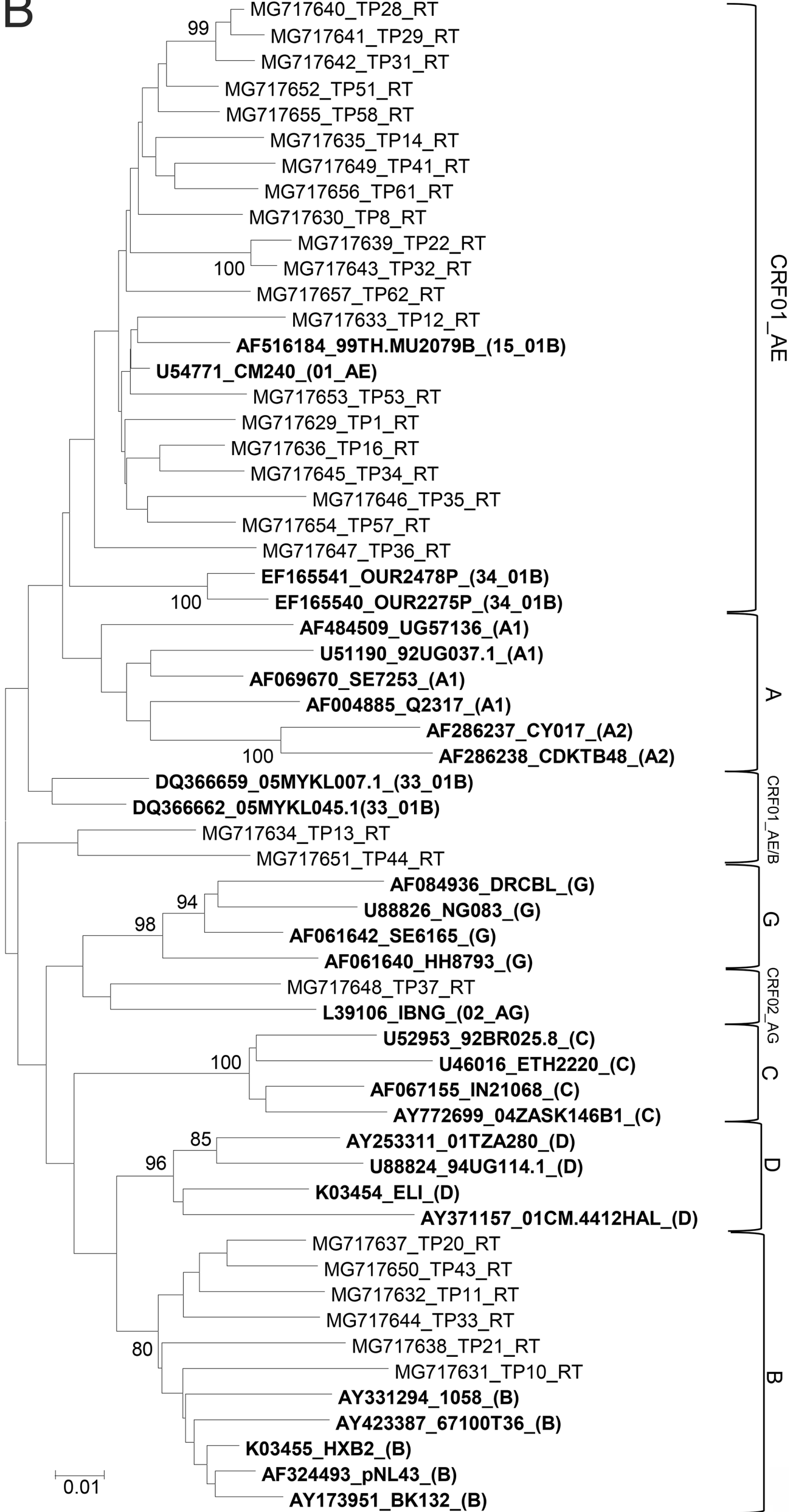
Sample ID	Types of ART drugs	Drug Resistance Mutations ^a	
		NRTI	NNRTI
TP 8	TDF+3TC+EFV, 2012	M184V	V106I Y188L
TP 11	3TC+AZT+NVP, 2012 TDF+3TC+EFV		V179D
TP 22	TDF+3TC+EFV, 2010		V106I
TP 32	3TC+AZT+EFV, 2011		V106I

^aMutations associated with high levels of drug resistance are shown according to the guidelines published by the International Antiviral Society-United States (IAS-USA). Drug resistance-associated major mutations are shown in bold.

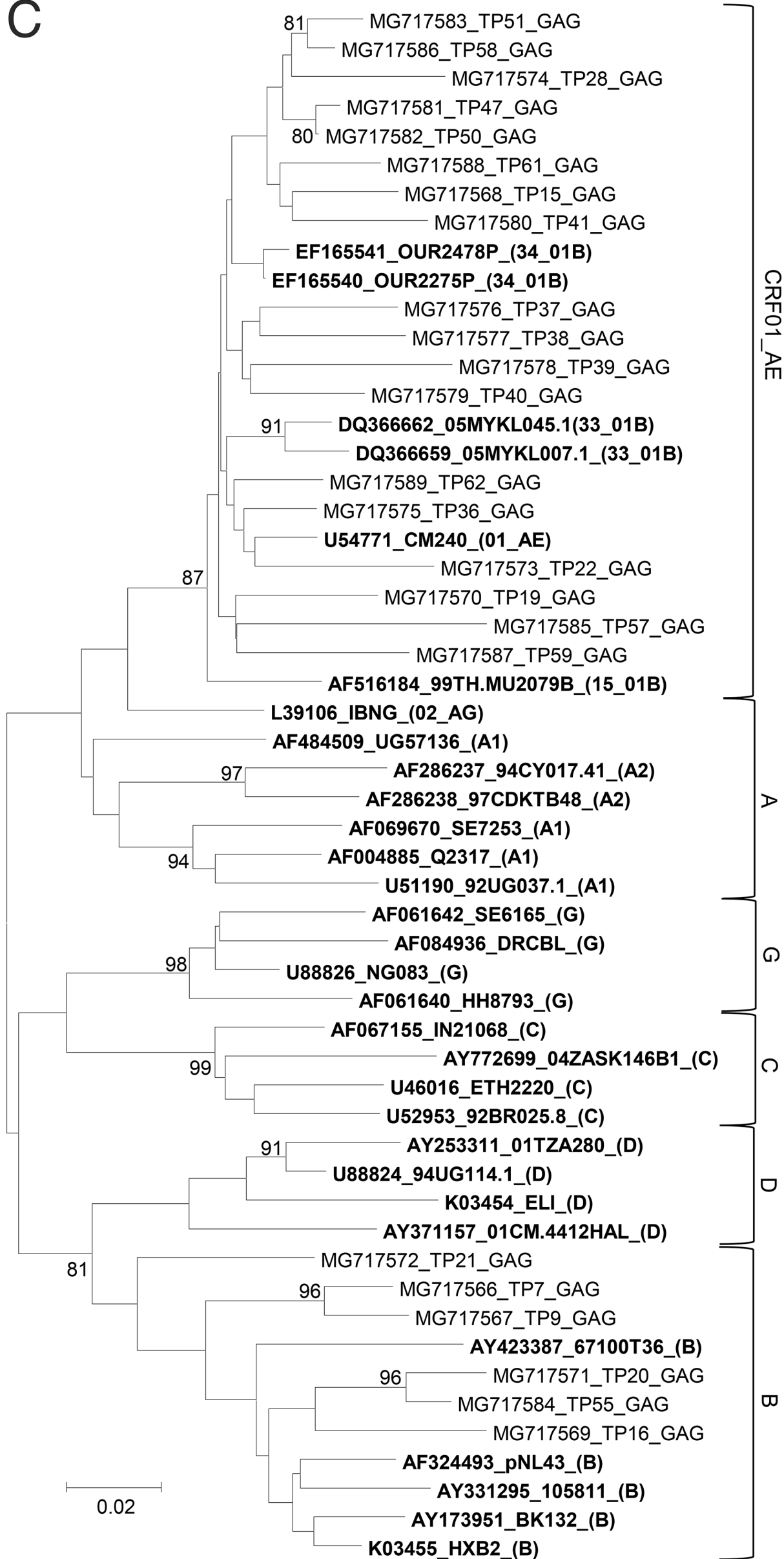
A



B



C



D

