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Genetic Diversity and Drug Resistance of HIV-1 Circulating in North Sulawesi, Indonesia

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Running title: HIV-1 molecular epidemiology in North Sulawesi

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Abstract

Manado, the capital city of North Sulawesi, is a unique region in Indonesia because of its religion. We collected peripheral blood samples from 63 individuals on antiretroviral therapy (ART). The amplification of viral genomic fragments, viral subtyping, detection of human immunodeficiency virus (HIV) drug resistance-associated mutations (DRAMs), and phylogenetic analyses were performed. Viral subtyping revealed that the most prevalent HIV type 1 (HIV-1) subtype/circulating recombinant form (CRF) was CRF01_AE (84.1%), followed by subtype B (6.8%) and recombinants between CRF01_AE and CRF02_AG (4.5%). Although no major DRAMs were present in PR genes, they were detected in RT genes. Nine out of 38 samples (23.7%) had major DRAMs against nucleoside RT inhibitors (NRTIs) and/or non NRTIs. The results of phylogenetic analyses indicated that CRF01_AE in North Sulawesi is related to that in Bali. Therefore, Bali may play an important role in circulating CRF01_AE in North Sulawesi.

Key words: CRF01_AE, ART, North Sulawesi, Indonesia

Text

Human immunodeficiency virus type 1 (HIV-1) and acquired immunodeficiency syndrome (AIDS) represent a major public health concern in Indonesia. According to UNAIDS data, approximately 630,000 (540,000 - 740,000) people are living with HIV in Indonesia. Although the number of new HIV infections each year has been decreasing, that of individuals newly infected with HIV remains high, with 49,000 (43,000 - 57,000) being reported in 2017[1].

Circulating recombinant form (CRF) 01_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 subtypes and recombinants, including subtype B and the recombinant between CRF01_AE and subtype B, have emerged in Indonesia[2-7].

Indonesia has many islands, each of which has different cultures, religions, and ethnicities. Manado is the capital city of the North Sulawesi province. Although the major religion in Indonesia is Islam, it is Christianity in Manado. Thus, Manado is a unique region in Indonesia. Limited information is currently available on the genetic diversity of HIV-1 strains in HIV/AIDS patients in Manado.

In the present study, we examined current HIV-1 genetic diversity and the appearance of mutations associated with viral resistance to protease (PR) inhibitors (PIs)

and reverse transcriptase (RT) inhibitors in HIV-1-infected individuals living in Manado. We also investigated the molecular evolutionary dynamics of HIV-1 in Manado using phylogenetic analyses.

Sixty-three HIV-1-infected, anti-retroviral therapy (ART)-experienced individuals were recruited in Manado. 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). Informed consent and a questionnaire were provided to each participant for acceptance prior to their enrollment in the present study. Sociodemographic, behavioral, and clinical data, including information on opportunistic infections, were retrospectively retrieved from medical records. The mean age of participants was 34.4 ± 8.28 years, whereas the gender distribution was 35 males (55.6%) and 28 females (44.4%) (Table 1). Regarding the ethnicity of participants, most individuals were Minahasa (68.3%). Minahasa is a local ethnic group in North Sulawesi. Fifty-seven participants (90.5%) were prescribed two types of nucleoside RT inhibitors (NRTIs) and one non-nucleoside RT inhibitor (NNRTI). Four participants (6.3%) were prescribed two types of NRTIs and a combination of protease inhibitors, lopinavir plus a booster dose of ritonavir (LPV/r). Twenty-eight participants (44.4%) were heterosexual. Eleven (17.5%) and 6 (9.5%) participants were homosexual and injecting drug users, respectively.

Ten milliliters of whole blood samples were collected from each individual. Peripheral blood mononuclear cells (PBMC) were isolated using 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 PR (PR gene) and RT (RT gene) as well as the *gag* and *env* genes were amplified by the EX Taq (TAKARA, Shiga, Japan) or 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 2691 to 2716) in the first round and DRPR01M, 5'-AGAGCCAACAGCCCCACCAG-3' (nt 2148 to 2167) and DRPR06, 5'-ACTTTTGGGCCATCCATTCC-3' (nt 2592 to 2611) in the second round. The RT gene was amplified by nested PCR with the primers RT1L, 5'-ATGATAGGGGGAATTGGAGGTTT-3' (nt 2388 to 2410) and RT4L, 5′-TACTTCTGTTAGTGCTTTGGTTCC-3′ (nt 3402 to 3425) in the first round, and RT7L, 5'-GACCTACACCTGTCAACATAATTGG-3' (nt 2485 to 2509) and RT6L, 5′-TAATCCCTGCATAAATCTGACTTGC-3′ (nt 3348 to 3372) 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’-CTAATACTGTATCATCTGCTCCTGT-3’ (nt 2328 to 2352) in the first round, and H1Gag1584, 5'-AAAGATGGATAATCCTGGG-3’ (nt 1577 to 1595) and G17, 5’-TCCACATTTCCAACAGCCCTTTTT-3’ (nt 2017 to 2040) in the second round. The viral *env* gene was amplified by nested PCR with the primers M5, 5’-CCAATTCCCATACATTATTGTGCCCCAGCTGG-3’ (nt 6858 to 6889) and M10, 5’-CCAATTGTCCCTCATATCTCCTCCTCCAGG-3’ (nt 7632 to 7661) in the first round, and M3, 5’-GTCAGCACAGTACAATGCACACATGG-3’ (nt 6948 to 6973) and M8, 5’-TCCTTGGATGGGAGGGGCATACATTGC-3’ (nt 7521 to 7547) in the second round. PCR conditions are available upon request. PCR products were electrophoresed on a 1% 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 33 PR genes (297 base pairs (bp); nt 2253-2549), the N terminus of 37 RT genes (762 bp; nt 2550-3311), the partial fragment of 38 *gag* genes encoding Gag p24 (381 bp; nt 1627-2007), and the partial

fragment of 36 *env* genes encoding the gp120 C2-V3 region (390 bp; nt 7020-7409) were obtained from 63 peripheral blood samples. The nucleotide sequences of the PR, RT, *gag*, and *env* genes have been registered in the GenBank database under accession numbers MH727243-MH727275 (PR genes), MH727276-MH727312 (RT genes), MH727313-MH727350 (*gag* genes), and MH727351-MH727386 (*env* genes).

HIV-1 subtyping was performed using a phylogenetic tree analysis 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 MEGA6.06 software[8,9]. 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 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 North Sulawesi was CRF01_AE (84.1%), followed by subtype B (6.8%), recombinants between CRF01_AE and CRF02_AG (4.5%), subtype C (2.3%), and CRF07_BC (2.3%). HIV-1 CRF01_AE is widely circulating in South-East Asia, including Thailand,

Malaysia, Vietnam, and Cambodia[10]. The higher prevalence of CRF01_AE in the North Sulawesi province is consistent with our previous findings obtained in Surabaya, Kepulauan Riau, and Maumere, Indonesia[2,6,7]. Recombinant viruses containing CRF02_AG gene fragments were detected in North Sulawesi. Since some CRF02_AG recombinant viruses were also detected in other regions in Indonesia, such as Kepulauan Riau, the continuous surveillance of the HIV-1 subtype and CRFs appears to be necessary.

Major drug resistance-associated mutations (DRAMs) were searched for in PR and RT genes based on the Stanford University HIV Drug Resistance Database (https://hivdb.stanford.edu/hivdb/by-mutations/). Although no major DRAMs were present in PR genes, they were detected in RT genes. Nine out of 38 samples (23.7%) had major DRAMs against NRTIs and/or NNRTIs (Table 2). Highly frequent DRAMs against NRTI and NNRTI were M184V and K103N, respectively. Multiple drug resistance against RT inhibitors was detected in two individuals (sample ID: MN43 and MN58), who have been taking anti-retroviral drugs for 5 and 3 years, respectively. Since the high DRAMs against RT were found in North Sulawesi, the continuous surveillance of HIV-1 DRAMs appears to be necessary.

Phylogenetic analyses were conducted to investigate the relationship of CRF01_AE between North Sulawesi and the rest of the world. We included HIV-1 CRF01_AE *pol* sequences (PR 1-99 and RT 1-250 amino acids) from our new samples collected in Manado. Twenty-five CRF01_AE sequences covering 1054 base pairs (HXB2: 2253-3306) were included. All closely related publicly available CRF01_AE sequences were downloaded from the HIV sequence database of the Los Alamos National Laboratory (LANL, https://www.hiv.lanl.gov/) after a BLAST search against the 25 Manado CRF01_AE sequences. A total of 143 reference sequences were selected based on the highest similarities to the Manado sequences after manually removing closely related sequences from the same areas or sequences that have no information on collecting regions. A maximum likelihood tree was constructed using the general time reversible (GTR) + Γ + I model in PhyML in order to examine phylogenetic interrelationships among viral sequences. The reliability of the phylogenetic tree was evaluated using the approximate Likelihood Ratio Test (aLRT) of SH-like supports. The final tree was generated using FigTree v1.4.3. Monophyletic groups with aLRT support > 0.85 were regarded as a clade. A phylogenetic analysis demonstrated that North Sulawesi CRF01_AE was similar to Asian strains, including those from Thailand, China, and Malaysia. Among the Asian strains, some North Sulawesi CRF01_AE formed a sub-clade (sub-clade 1), and they were similar to Malaysian CRF01_AE (Figure 2a).

In order to reveal the potential source and transmission route of North Sulawesi CRF01_AE, Bayesian phylogeographic inferences were conducted using BEAST v.1.8.4. Closely related sequences from the same areas or sequences that have no information on collecting dates and regions were manually removed and African CRF01_AE sequences were included. The selected models were the GTR + Relaxed clock (uncorrelated) with a constant size model. The Markov chain Monte Carlo (MCMC) analysis was performed for 100 million generations and sampled every 1000 steps. The output was assessed for convergence by means of an effective sample size (ESS) after a 10 % burn-in using Tracer. To minimize the effects of standard errors, only ESS > 200 was included. Maximum clade credibility (MCC) trees summarized the posterior distribution, and were generated with TreeAnnotator and visualized in FigTree v1.4.3. A posterior probability >0.9 was regarded as a cluster. North Sulawesi CRF01_AE strains were grouped into two distinct clusters (clusters 1 and 2) (Figure 2b).The dates of the common ancestor of the African CRF01_AE, Thailand and Chinese CRF01_AE, CRF01_AE clusters 1, and CRF01_AE clusters 2 were estimated to be 1972.4 (95% credible region, 1960.6 to 1981.8), 1981.9 (1976.6 to 1986.3), 1997.4 (1993.9 to 2000.4), and 1999.6 (1994.2 to 2004.6), respectively (Figure 2b). Estimations of the common ancestor of African CRF01_AE and Thailand and Chinese CRF01_AE were essentially similar to those reported previously[11]. The results of temporal and spatial dynamics analyses showed that

the two clusters were both rooted within the Bali sequences. Although the introduced date from Bali to North Sulawesi was in the late 1990s, the two CRF01_AE clusters appeared to have been independently introduced into the North Sulawesi population. Cluster 1 contained additional CRF01_AE sequences from Malaysia, Thailand, China, and others. On the other hand, cluster 2 spread to Indonesia only. No significant differences in transmission risks (heterosexual/homosexual and injecting drug users) were identified between clusters 1 and 2. Cluster 1 strains appear to have high epidemic potential.

In conclusion, CRF01_AE viruses were the predominant HIV-1 strains in North Sulawesi, Indonesia. The predominance of CRF01_AE was consistent with that in other regions in Indonesia. In addition, subtype B, CRF07_BC, and recombinants between CRF01_AE and CRF02_AG were detected. A high prevalence of DRAMs against NRTIs and NNRTIs was also noted. The results of phylogenetic analyses indicated that CRF01_AE in North Sulawesi was related to that in Bali. Therefore, Bali may play an important role in circulating CRF01_AE in North Sulawesi. Further continuous surveillance studies are needed to identify the potential source and transmission route of Indonesian CRF01_AE and to develop effective treatments for HIV.

Acknowledgments

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Author Disclosure Statement

No competing financial interests exist.

Sequence Data

Nucleoside sequences are available under GenBank accession numbers MH727243 to MH727386.

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Figure legends

FIG.1. Phylogenetic tree analyses of HIV-1 PR, RT, *gag*, and *env* gene sequences collected in North Sulawesi, Indonesia.

Phylogenetic trees were constructed for the HIV-1 PR (A), RT (B), *gag* (C), and *env* (D) genes newly sequenced in the present study. 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), CRF07_BC (07_BC), CRF15_01B (15_01B), CRF33_01B (33_01B), and CRF34_01B (34_01B) were included in the analyses. Sequence IDs are presented as a sample ID or the ID of the reference HIV-1 strain, a GenBank accession number, and the subtype or CRF of the reference strain (shown in parentheses) in that order. Bootstrap values were shown if they were >70.

FIG.2. Phylogenetic analyses of HIV-1 CRF01_AE in North Sulawesi.

The phylogenetic tree was constructed using the approximately maximum likelihood method based on the pol region (HXB2: 2253 to 3306 nt) in PhyML (A). HIV-1 subtype C sequences were used as the outgroup in the rooted tree. The nucleotide substitution model was GTR + Γ + I. The maximum clade credibility (MCC) tree of CRF01_AE in North Sulawesi was estimated by a Bayesian MCMC approach (B). The times of the most

recent common ancestors (tMRCAs) for the phylogenetic clusters of interest are indicated as mean dates and 95% highest posterior density (HPD) regions. The country of origin of each sequence is indicated by the ISO 3166 two-letter code (https://www.iso.org/obp/ui/#iso:pub:PUB500001:en) as follows: CN, China; ID, Indonesia; IR, Iran; MY, Malaysia; TH, Thailand; US, the United States of America; AU, Australia; JP, Japan; BE, Belgium; KR, Korea; SG, Singapore; VN, Viet Nam; FR, France; AT, Austria; KW, Kuwait; SE, Sweden; CZ, Czech Republic; PK, Pakistan; CF, Central African Republic.

TABLE 1. DEMOGRAPHIC CHARACTERISTICS OF ART-EXPERIENCED INDIVIDUALS IN NORTH SULAWESI, INDONESIA

Characteristics	Value (n=63)
Age, mean years (SD)	34.4 (8.28)
Gender	
Male	35 (55.6%)
Female	28 (44.4%)
Marital status	
Married	32 (50.8%)
Single	26 (41.3%)
Unidentified	5 (7.9%)
Ethnicity	
Minahasa	43 (68.3%)
Sanger	3 (4.8%)
Jawa	2 (3.2%)
Gorontalo	5 (7.9%)
Arab	1 (1.6%)
Chinese	1 (1.6%)
Bolaang mongondo	1 (1.6%)
Maluku	1 (1.6%)
Unidentified	6 (9.5%)
Transmission risk	
Heterosexual	28 (44.4%)
Homosexual	11 (17.5%)
Injecting drug users	6 (9.5%)
Others	1 (1.6%)
Unidentified	6 (27.0%)
Types of ART	
3TC+AZT+NVP	18 (28.6%)
3TC+AZT+EFV	17 (27.0%)
3TC+TDF+EFV	22 (34.9%)

3TC+TDF+LPV/r	4 (6.3%)
Unidentified	2 (3.2%)

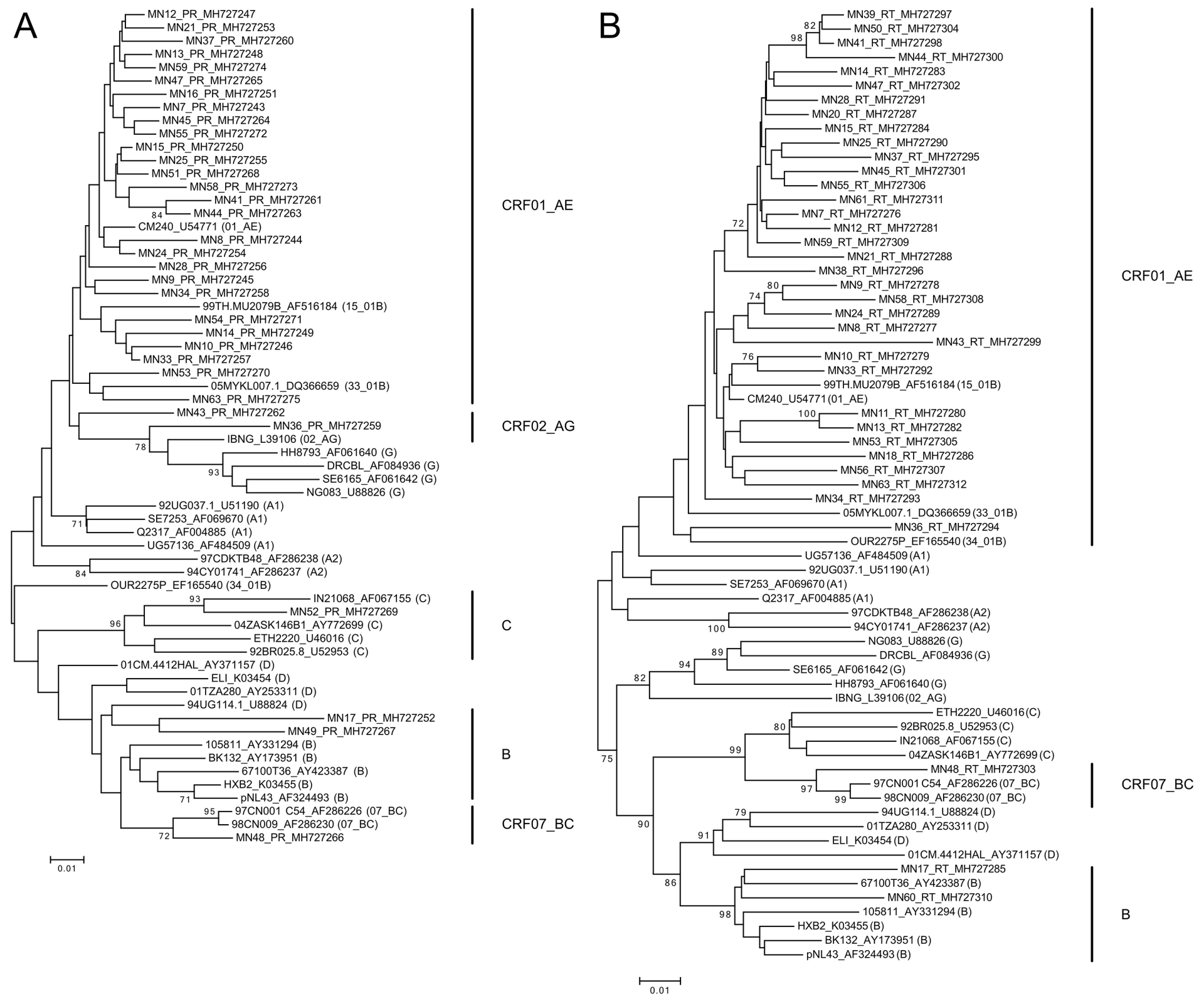
SD, standard deviation; ART, antiretroviral therapy; 3TC, lamivudine; AZT, zidovudine; NVP, nevirapine; EFV, efavirenz; TDF, tenofovir; LPV/r, lopinavir plus a booster dose of ritonavir.

TABLE 2. FREQUENTLY DETECTED DRUG RESISTANCE-ASSOCIATED MAJOR MUTATIONS IN RT GENES DERIVED FROM ART-EXPERIENCED INDIVIDUALS IN NORTH SULAWESI, INDONESIA

Sample ID	Types of ART	Drug Resistance Mutations[a]	
		NRTI	NNRTI
MN9	3TC, AZT, NVP	M184V	K103N
MN18	3TC, AZT, NVP	-	V179T
MN21	3TC, AZT, NVP	M184V	K103N
MN24	3TC, TDF, LPV/r	M184V	K103N
MN43	3TC, AZT, EFV	A62V, K65R, D67H, V75I, F77L, F116Y, Q151M, M184V, K219E	K103N
MN44	3TC, AZT, NVP	M184V, T215F	Y181C, G190A
MN48	3TC, AZT, NVP	L74V	-
MN58	3TC, AZT, NVP	M41L, A62V, M184V, L210W, T215Y	K103N, P225H
MN60	3TC, AZT, NVP	-	K103N

[a]Mutations associated with drug resistance are shown according to the Stanford University HIV Drug Resistance Database.

ART, antiretroviral therapy; NRTI, nucleoside/nucleotide reverse transcriptase inhibitor; NNRTI, non-nucleoside reverse transcriptase inhibitor; 3TC, lamivudine; AZT, zidovudine; NVP, nevirapine; TDF, tenofovir; LPV/r, lopinavir plus a booster dose of ritonavir; EFV, efavirenz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MN13_PR_MH727248
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MN47_PR_MH727265
MN16_PR_MH727251
MN7_PR_MH727243
MN45_PR_MH727264
MN55_PR_MH727272
MN15_PR_MH727250
MN25_PR_MH727255
MN51_PR_MH727268
MN58_PR_MH727273
MN41_PR_MH727261
MN44_PR_MH727263
CM240_U54771 (01_AE)
MN8_PR_MH727244
MN24_PR_MH727254
MN28_PR_MH727256
MN9_PR_MH727245
MN34_PR_MH727258
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MN54_PR_MH727271
MN14_PR_MH727249
MN10_PR_MH727246
MN33_PR_MH727257
MN53_PR_MH727270
05MYKL007.1_DQ366659 (33_01B)
MN63_PR_MH727275
MN43_PR_MH727262
MN36_PR_MH727259
IBNG_L39106 (02_AG)
HH8793_AF061640 (G)
DRCBL_AF084936 (G)
SE6165_AF061642 (G)
NG083_U88826 (G)
92UG037.1_U51190 (A1)
SE7253_AF069670 (A1)
Q2317_AF004885 (A1)
UG57136_AF484509 (A1)
97CDKTB48_AF286238 (A2)
94CY01741_AF286237 (A2)
OUR2275P_EF165540 (34_01B)
IN21068_AF067155 (C)
MN52_PR_MH727269
04ZASK146B1_AY772699 (C)
ETH2220_U46016 (C)
92BR025.8_U52953 (C)
01CM.4412HAL_AY371157 (D)
ELI_K03454 (D)
01TZA280_AY253311 (D)
94UG114.1_U88824 (D)
MN17_PR_MH727252
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BK132_AY173951 (B)
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HXB2_K03455 (B)
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92UG037.1_U51190 (A1)
SE7253_AF069670 (A1)
Q2317_AF004885 (A1)
97CDKTB48_AF286238 (A2)
94CY01741_AF286237 (A2)
NG083_U88826 (G)
DRCBL_AF084936 (G)
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HH8793_AF061640 (G)
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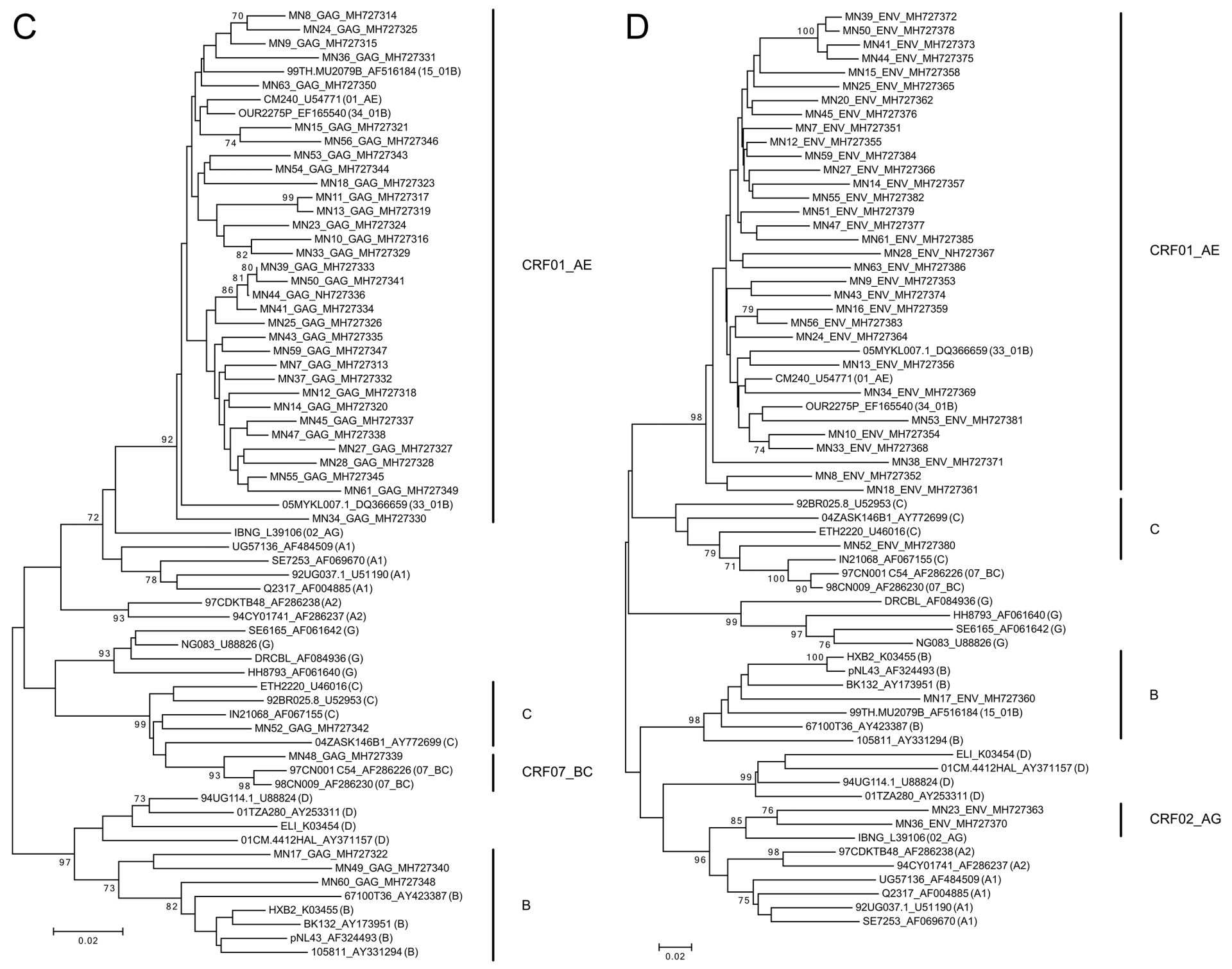
B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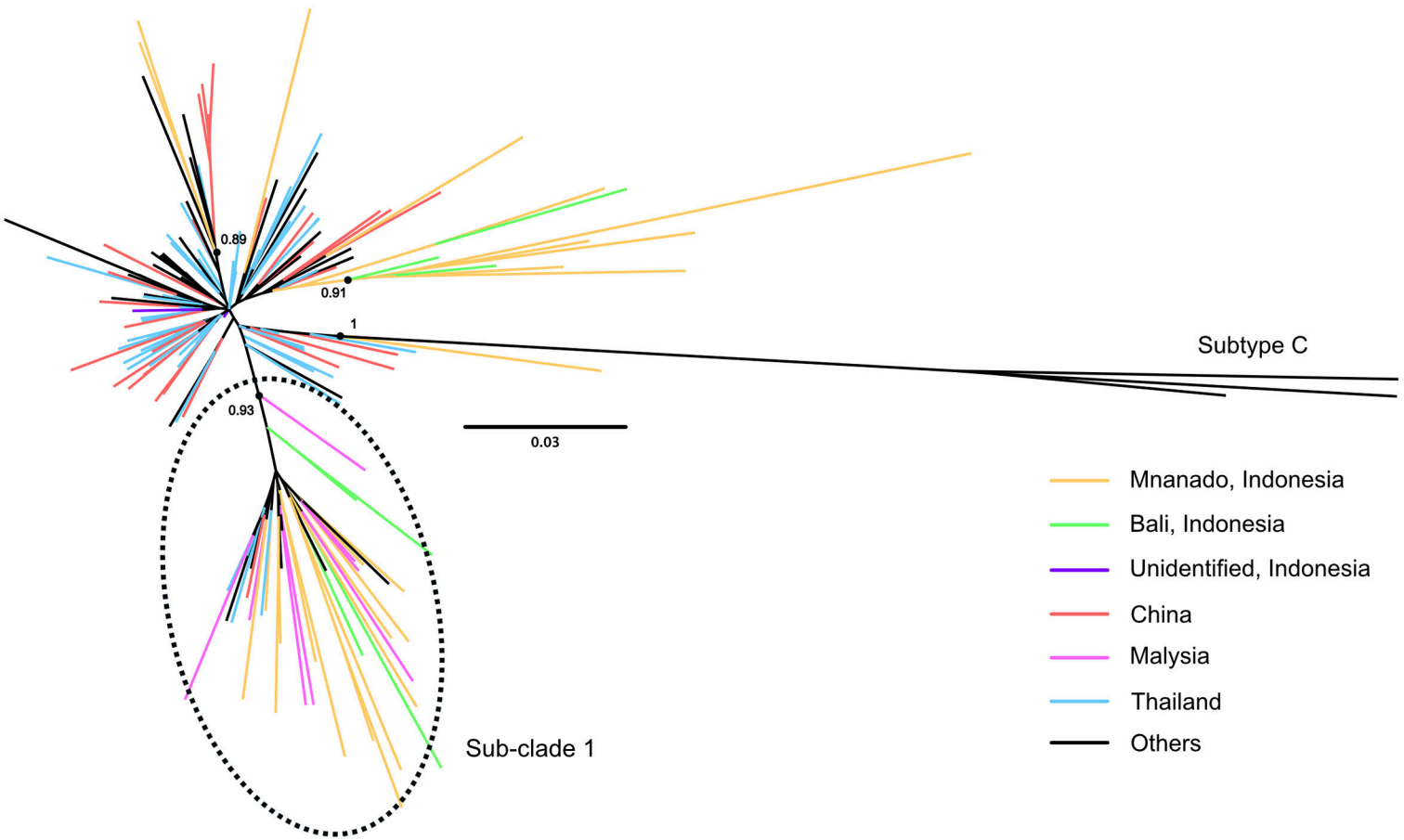
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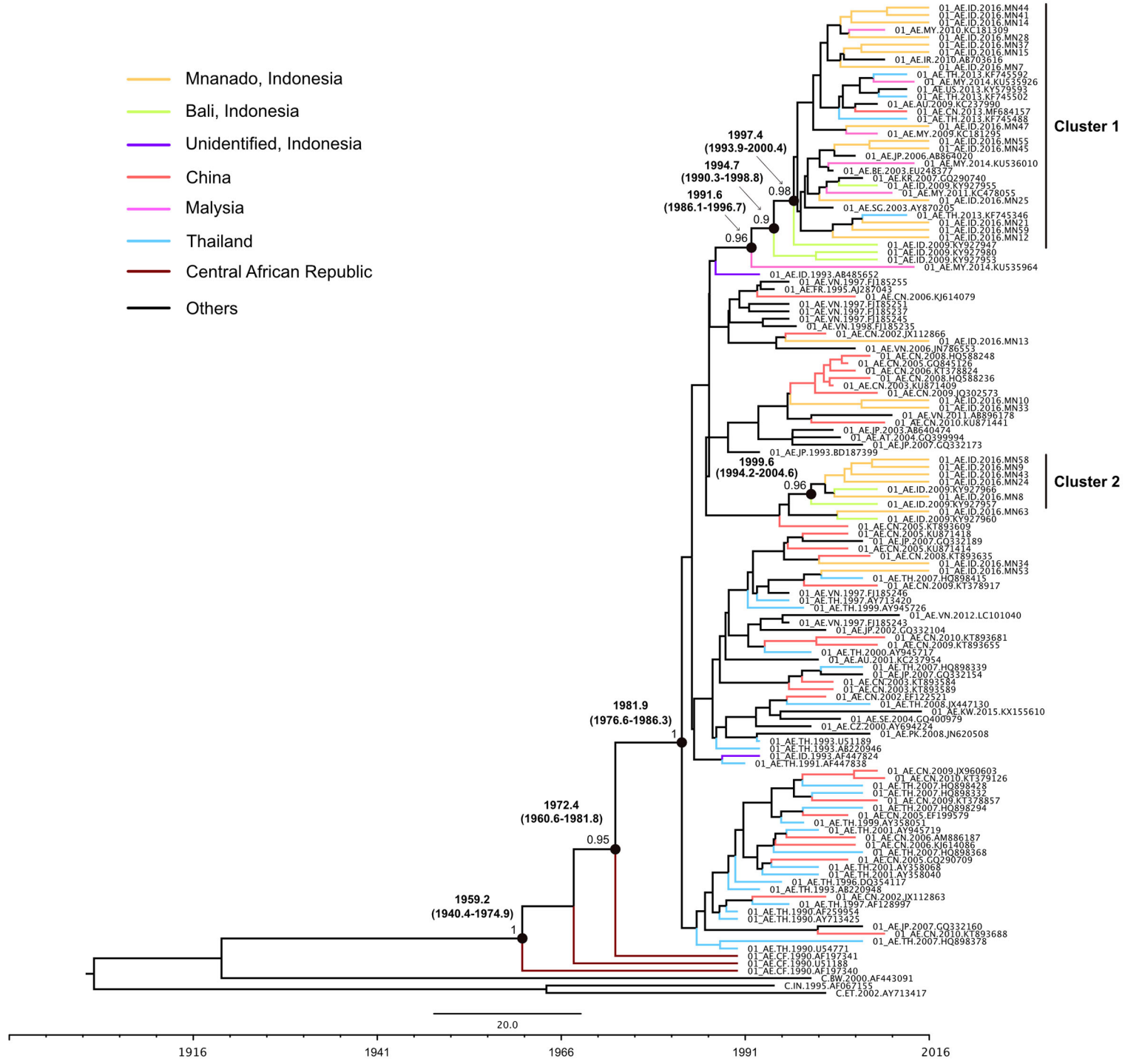
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