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NOTE

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Phylogenetic analysis of *env* gene of bovine leukemia virus strains spread in Miyazaki prefecture, Japan

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ABSTRACT. To understand how the latest dominant bovine leukemia virus (BLV) strains were introduced and spread in the Miyazaki prefecture, we collected blood samples from 3 geographic areas (north, central and south) and carried out sequence analysis of the BLV *env* gene. Two genotypes, genotype I, and III, were identified and the majority of the strains belonged to genotype I (71/74). To clarify a route of BLV introduction, we divided the strains into 20 subgenotypes based on their nucleotide sequences and performed phylogenetic analysis. Our study indicated that common BLV strains were comparatively evenly distributed even in the area, where the farmers have not introduced cattle from other areas and the cattle have limited exposure to BLV infection in grazing fields.

KEY WORDS: BLV, genotype, phylogenetic analysis

Bovine leukemia virus (BLV) belongs to the family *Retroviridae* genus *Deltaretrovirus* and is the causative agent of enzootic bovine leukosis (EBL). BLV infection is distributed worldwide and leads to significant economic loss for the cattle industry [3, 13, 15, 20]. A nationwide survey of BLV infection indicated that 35.2% of cattle in Japan, including dairy and beef breeding cattle, were seropositive for BLV [13]. The survey further indicated that the Kyushu is one of the areas with highest seroprevalence in Japan, urging the need to control BLV infection in the area. Most importantly, cattle industry in Miyazaki prefecture in Kyushu is the third-largest in Japan, and thus, BLV infection has a great economic impact.

To control BLV infection, understanding distribution of current BLV strains and identifying the introduction route of BLV infection are critical. Sequence analysis of BLV *env* gene can provide precious epidemiological information for developing a BLV control strategy. Analysis of BLV gp51 *env* gene sequences from different locations throughout the world revealed the presence of different genetic groups that correlated with geographic origin [12, 21], and thus, the nucleotide and amino acid sequences composition of gp51 are useful genomic markers of BLV in studies of geographical distribution [9]. A phylogenetic study of BLV *env* gene has so far identified at least 10 genotypes in the world [5, 7, 8, 14, 16, 19].

As part of our BLV control program in the Miyazaki prefecture, we previously conducted phylogenetic analysis of BLV strains in the north area of central Miyazaki, where the outbreak of foot-and-mouth disease (FMD) occurred and all the cattle were culled in 2010 [11]. The dominant genotype was genotype I (94%), and the rest was genotype III. The other study reported that genotype I has been recognized as the dominant type and accounted about 48.8% in Japan [1]. To further dissect how the latest dominant BLV strains were introduced and spread, we investigated the distribution of BLV genotypes among 3 geographic areas (north, central and south) in the Miyazaki prefecture and carried out phylogenetic analysis of BLV *env* gene.

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Table 1. BLV genotypes and *env* gene sequences used in phylogenetic analysis

Country	Subgenotype	Genotype	Accession no.	References
Japan	Miya 1[a, b)]	I	KU600017	This study
Japan	Miya 2[a)]	I	KU600018	This study
Japan	Miya 3[a, b)]	I	KU600019	This study
Japan	Miya 4	I	KU600020	This study
Japan	Miya 5	I	KU600021	This study
Japan	Miya 6	I	KU600022	This study
Japan	Miya 7	I	KU600023	This study
Japan	Miya 8	I	KU600024	This study
Japan	Miya 9	I	KU600025	This study
Japan	Miya 10	I	KU600026	This study
Japan	Miya 11	I	KU600027	This study
Japan	Miya 12	I	KU600028	This study
Japan	Miya 13	I	KU600029	This study
Japan	Miya 14	I	KU600030	This study
Japan	Miya 15	I	KU600031	This study
Japan	Miya 16	I	KU600032	This study
Japan	EBL 1[a, b)]	III	KX538928	This study
Japan	EBL 2[a)]	III	KX538929	This study
Japan	EBL 3[a)]	I	KX538930	This study
Japan	EBL 4[a)]	I	KX538931	This study
Japan	Jref	I	K02120	Sagata *et al.,* 1985
U.S.A.	FLK-BLV	I	M35242	Mamoun *et al.,* 1990
U.S.A.	VDM	I	M35239	Mamoun *et al.,* 1990
Australia	BLV-Aust.	I	D00647	Coulston *et al.,* 1990
Croatia	ELG Cro/BEM/08	VIII	JN990069	Balic *et al.,* 2012
Argentina	B19	II	AF257515	Rodriguez *et al.,* 2009
U.S.A.	USCA-1	III	EF065647	Zhao and Buehring, 2007
Brazil	151	VI	AY185360	Camargos *et al.,* 2002
Chile	1	VII	AY515280	Felmer *et al.,* 2005
Belgium	344	IV	AF503581	Willems *et al.,* 1993
Costa Rica	CRGC	V	EF065639	Zhao and Buehring, 2007
Bolivia	Por20	IX	LC080665	Polat *et al.,* 2016
Thailand	Sa8-H1	X	KU233561	Lee *et al.* 2016

a) Subgenotypes identified from samples obtained at a slaughterhouse (Miya 1, 2, 3 were also identified from the field samples). b) Subgenotypes identified from animals raised in a neighboring prefecture, Kagoshima (Miya 1 and 3 were also identified from animals raised in the Miyazaki prefecture).

Blood samples were collected from animals using collection tubes containing EDTA and centrifuged at 3,000 rpm for 5 min at 4°C, and the plasma was obtained for a serological diagnosis for BLV infection. BLV enzyme-linked immunosorbent assay (ELISA) kit (JNC, Tokyo, Japan) was used for the serological test for BLV gp51 protein according to the manufacturer's instruction. Genomic DNA was also extracted from the whole blood with the seropositive samples using a Wizard Genomic DNA purification kit (Promega, Fitchburg, MA, U.S.A.) according to the manufacturer's instructions. The DNA concentration was determined using the NanoDrop 1000 spectrophotometer (Thermo Fisher Scientific, Waltham, MA, U.S.A.) and diluted to 25 *ng*/*μl* and amplification of BLV *env* gene. A total of 74 genomic DNA samples (north: 26; central: 25; and south: 23) from BLV-seropositive animals were used for sequencing and phylogenetic analyses. For PCR amplification of the BLV *env* gene fragment, the following forward and reverse primers were used [1]. The forward primer was env 5032f (5́-TCTGTGCCAAGTCTCCCAGATA-3́), and the reverse primer was env 5608r (5́-AACAACAACCTCTGGGAAGGGT-3́). The target product was amplified in 20 *μl* reaction mix consisting of 2 *μl* of 10X Ex Taq Buffer, 1.6 *μl* of dNTP mixture (2.5 mM each dNTP), 0.1 *μ*M of forward primer, 0.1 *μ*M of reverse primer, 0.1 unit of Ex Taq HS (TaKaRa Bio Inc., Kusatsu, Japan) and 1 *μl* of the DNA in final concentrations. Amplification reaction was carried out using thermo cycler in the following cycles; initial denaturation 94°C for 9 min and 40 cycles of denaturation 95°C for 30 sec, annealing 60°C for 30 sec, extension 72°C for 1 min, then final extension at 72°C for 4 min and holding at 4C°. The PCR products were electrophoresed on 1% gel plates and visualized using ultraviolet light.

The PCR amplicons were extracted from the gels using QIAquick Gel Extraction Kit (QIAGEN, Hilden, Germany) according to the manufacturer's protocol. The sequencing was performed using forward primer env 5032f with a Big Dye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Forster City, CA, U.S.A.), purified using a Big Dye X Terminator Purification Kit (Applied Biosystems) and analyzed with an Applied Biosystems 3730 DNA Analyzer. The sequences of the BLV *env* gene were aligned using BioEdit software program V.7.2.5. [4], and neighbor-joining (NJ) tree was constructed using the MEGA 7 software program

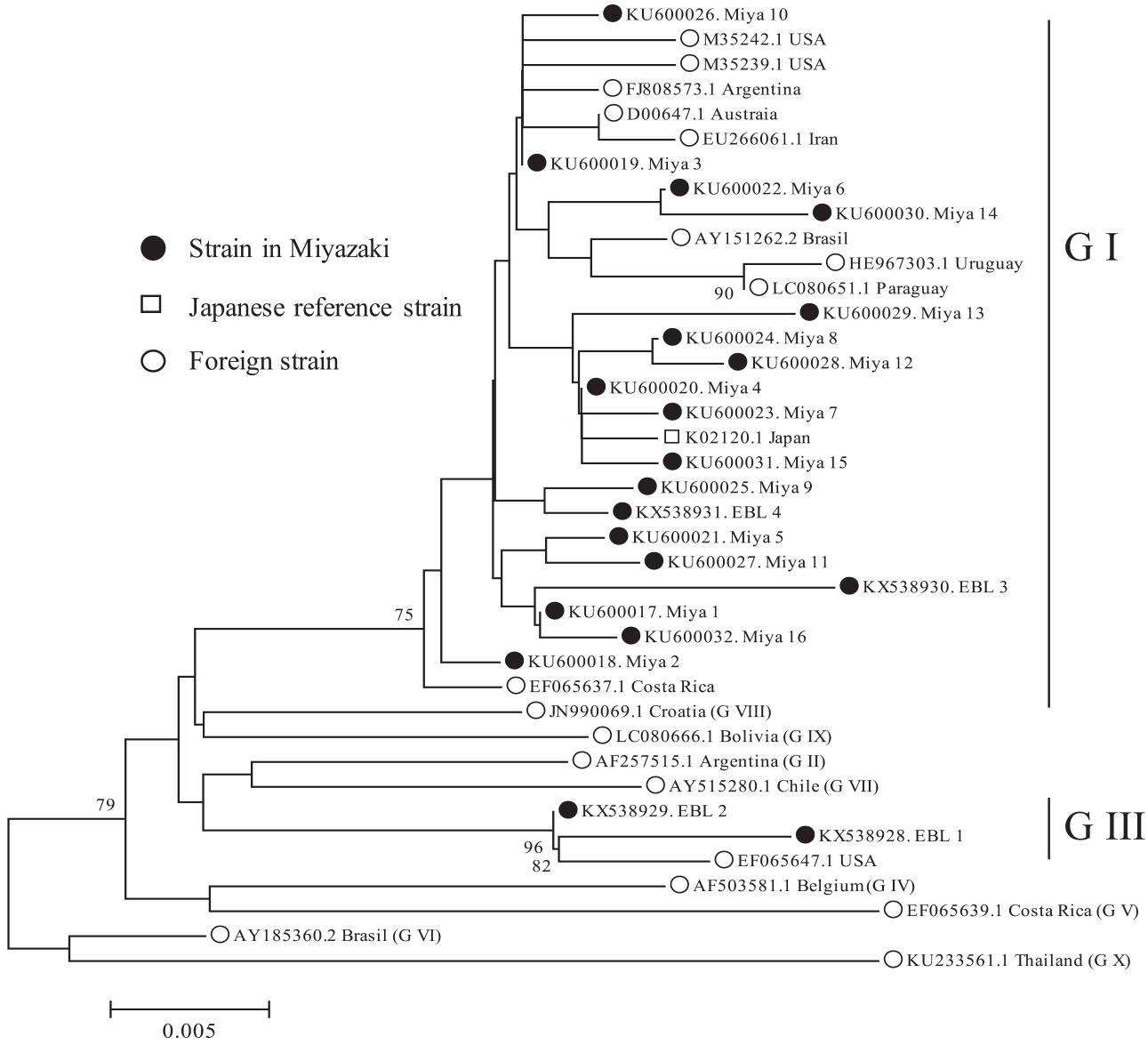
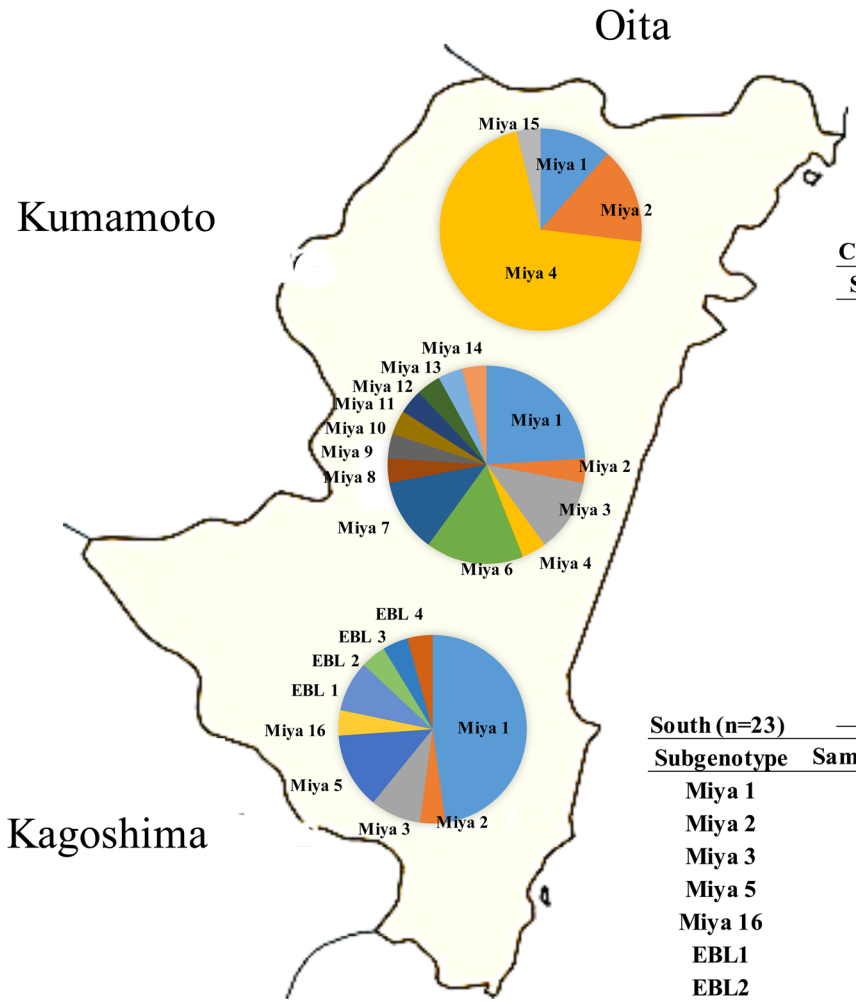


Fig. 1. Phylogenetic tree of the BLV genotypes. The phylogenetic tree was based on partial sequences (423 nt) of the *env* gene. The sequences from this study labeled with black circle and from Genbank labeled with white square (Japanese strain) or circle (foreign strain). The BLV genotypes are indicated on the right (G I and G III) or in parentheses (G II, G III and G IV-G X). The evolutionary history was inferred using the Neighbor-Joining method with bootstrap probability more than 70%. The bootstrap values, more than 70%, are shown next to branches. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree.

with the evolutionary model set to Kimura 2-parameter [6]. The reliability of the phylogenetic relationships was evaluated using nonparametric bootstrap analysis with 1,000 replicates for NJ analysis [17, 18]. Bootstrap values exceeding 70% were considered well supported [2]. The sequences found in this study were submitted to GenBank, and the accession numbers were KU600017-32 and KX538928-31. Statistical analysis was carried out by ANOVA followed by Tukey test as a post hoc test using GraphPad Prism V.4.0 (GraphPad, San Diego, CA, U.S.A.) [10].

Among 74 samples analyzed in this study, a total of 20 different sequences were identified and designated as 20 subgenotypes (Miya 1 through Miya 16 and EBL 1 through EBL 4). These included 10 samples (EBL 1: 2 samples, EBL 2, EBL 3, EBL 4, Miya 1: 3 samples, Miya 2 and Miya 3) obtained at a slaughterhouse located in the southern area. As indicated in Table 1, these 10 samples include 5 samples from animals largely raised in a neighboring prefecture, Kagoshima, very close to the Miyakonojo-city in the southern area of the Miyazaki prefecture. These samples were included, because of their proximity to Miyakonojo, making it relevant to this study. The phylogenetic analysis indicated that the genotype I (71 strains: 96%) was dominant and the rest belonged to genotype III (3 strains: 4%) as shown in Fig 1. Although 2 genotype III strains (both belong to the subgenotypes EBL 1) were derived from animals that were raised in the Kagoshima prefecture and slaughtered in the Miyazaki prefecture, we included the samples as strains in the Miyazaki prefecture as described above. All 3 genotype III strains were found in the southern area. These



North (n=26)

Subgenotype	Sample no.	Prevalence (%)
Miya 1	3	11.5
Miya 2	4	15.4
Miya 4	18	69.2
Miya 15	1	3.8

Central (n=25)

Subgenotype	Sample no.	Prevalence (%)
Miya 1	6	24.0
Miya 2	1	4.0
Miya 3	3	12.0
Miya 4	1	4.0
Miya 6	4	16.0
Miya 7	3	12.0
Miya 8	1	4.0
Miya 9	1	4.0
Miya 10	1	4.0
Miya 11	1	4.0
Miya 12	1	4.0
Miya 13	1	4.0
Miya 14	1	4.0

South (n=23)

Subgenotype	Sample no.	Prevalence (%)
Miya 1	11	47.8
Miya 2	1	4.3
Miya 3	2	8.7
Miya 5	3	13
Miya 16	1	4.3
EBL1	2	8.7
EBL2	1	4.3
EBL3	1	4.3
EBL4	1	4.3

Fig. 2. Prevalence and distribution of different subgenotypes in 3 different localities of the Miyazaki prefecture.

3 genotype III strains were from animals with EBL. However, the number of samples in this study was limited for any conclusion on EBL association.

Distribution of BLV subgenotypes in the Miyazaki prefecture is shown in Fig. 2. The statistical analysis revealed that the subgenotype Miya 1 is spread in all areas of the Miyazaki prefecture and the number is significantly higher than the other subgenotypes ($P<0.05$). This is consistent with our previous study in which one of the dominant strains contained the identical sequence with the subgenotype Miya 1 [11].

It had been an interesting question what strains were prevalent in the Nishiusuki-gun of the north area where the prevalence of BLV infection was about 1%, far below average in Japan, without any BLV control measures in the past. Because the farmers had been introducing cattle raised within the area, it was speculated that the area was probably free from BLV infection for a long time and BLV infection occurred recently leading to the low prevalence. It was known that several farmers in the area were raising cattle in grazing fields located in the Kumamoto prefecture where BLV infection was common and those animals were considered to be at risk of BLV infection. Although the subgenotype Miya 4 was found to be dominant in the Nishiusuki-gun (18/19) and these strains possibly entered from the Kumamoto prefecture, additional information regarding BLV strains in the Kumamoto prefecture are required for the conclusion. The subgenotypes Miya 1 and Miya 2 were found outside of the Nishiusuki-gun and in all other areas, indicating that these strains are circulating and probably dominant in Kyushu.

BLV transmission from BLV-infected animals to non-infected animals occurs either horizontally or vertically. However, main BLV transmission between geographically separated areas is an exclusively horizontal transmission from newly introduced BLV-infected animals to the herd. Therefore, it is important to understand dominant BLV strains that were potentially the source of BLV infection and spread in endemic areas. In the present study, we have investigated the latest dominant BLV strains introduced and spread in the Miyazaki prefecture. We speculate that most of the BLV strains identified in the Miyazaki prefecture are the strains

circulating not only within the prefecture but also among other prefectures, though BLV genotype I strains are more dominant in the Miyazaki prefecture (96% in this study) compared with the average in Japan (48.8%) [1]. However, it was difficult to draw a conclusion, because we could not find strains unique to each area. Modernized transportation allows moving cattle from many different prefectures far from the Miyazaki prefecture, and it may complicate our study to obtain the accurate information on where the animals came from. To more accurately understand what BLV strains were introduced from where or how, it is important to carry out more detailed sequence analysis covering other prefectures including neighboring prefectures and/or analysis using a next generation sequencer. It is also conceivable to use the Japanese traceability system of cattle for tracing back to cattle providing the source of BLV strains. Nonetheless, elimination of BLV in one prefecture is a long-term project and we need to continue monitoring the strains to understand how the situation could be changing.

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