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Detection of bovine leukemia virus in beef cattle kept in the Central Coast Regions of Vietnam

Dung Thi LE[1)#], Son Vu NGUYEN[2)#], Thu Anh Nu LE[3,4)], Van Huu NGUYEN[3)], Phung Dinh LE[3)], Dung Van DINH[3)], Hai Thanh DUONG[3)], Hai Van VU[3)], Yuri FUJIMOTO[5)], Tetsuo KUNIEDA[4)], Takeshi HAGA[1,5)*]

[1)]Division of Infection Control and Disease Prevention, Graduate School of Agricultural and Life Sciences, The University of Tokyo, Tokyo, Japan
[2)]Department of Veterinary Pathology, Faculty of Veterinary Medicine, Vietnam National University of Agriculture, Hanoi, Vietnam
[3)]Faculty of Animal Science and Veterinary, University of Agriculture and Forestry, Hue University, Hue, Vietnam
[4)]Faculty of Veterinary Medicine, Okayama University of Science, Ehime, Japan
[5)]Laboratory of OSG Veterinary Science for Global Disease Management, Graduate School of Agricultural and Life Sciences, The University of Tokyo, Tokyo, Japan

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ABSTRACT. Bovine leukemia virus (BLV) is the etiologic agent of enzootic bovine leucosis. Our previous study showed the BLV existence in cattle kept in the Red River Delta Region of Vietnam. However, no positive samples were identified in beef cattle. Besides, information related to the BLV circulation in the remained parts of Vietnam is limited. Therefore, we tested the existence of BLV in 48 beef cattle kept in the Central Coast Regions. Nested PCR targeting the *BLV-env-gp51* confirmed the prevalence of 14.6% in investigated regions. Phylogenetic analysis suggested the co-existence of genotypes 1 and 10. The close relationship between strains found in Vietnam, Thailand, Myanmar, and China was revealed suggesting the possibility of BLV transmission through the movement of live cattle.

KEYWORDS: beef cattle, bovine leukemia virus, genotyping, Vietnam

Bovine leukemia virus (BLV) belongs to the genus *Deltaretrovirus* of the *Retroviridae* family and is the etiologic agent of enzootic bovine leucosis (EBL). EBL is a common neoplastic disease in cattle characterized by BLV-induced B-cell lymphosarcoma and results in both direct and indirect economic losses [4, 29].

The clinical course of BLV infection can be categorized into four stages: Primary infection, persistent infection (PI), persistent lymphocytosis (PL), and lymphosarcoma stage (LS) [11]. Most BLV-infected cattle remain in the primary infection and PI stages as asymptomatic virus carriers for a few months or several years and infected cattle can only be detected by the presence of antibodies against BLV and/or proviral DNA. After the latent period, approximately 30% of BLV infected cattle develop an abnormally large number of lymphocytes in the PL stage characterized by the disruption of the immune system [33]. The PL stage in affected animals persists for several years and can progress to the malignant LS. Development of tumor occurs in a small proportion of infected animals (0.1–10%), mostly in older cattle being infected with BLV for a long period [4, 33].

The genome of BLV contains the structural genes *gag*, *pro*, *pol*, and *env* and the two regulatory genes named *tax* and *rex*, accessory genes (*R3*, *G4*), and two identical long terminal repeats (LTR). *env* is translated as the precursor ($Pr72^{env}$) and processed to generate two mature proteins gp51 (the surface glycoprotein) and gp30 (the transmembrane protein). The env proteins have essential and indispensable roles in the viral lifecycle, viral infectivity [4, 28]. In addition, the surface gp51 glycoprotein is thought to be the major target of host immune responses [31]. Because of the important biological functions, the *env-gp51* region has been widely used for BLV genotyping, phylogenetic and epidemiological studies. The phylogenetic analysis based on *env* sequences demonstrated that BLV strains could be classified into eleven distinct genotypes [1, 16, 27, 37].

Currently, BLV is present in a high percentage of livestock cattle worldwide. Despite the global BLV presence, the majority of western European Union member countries and New Zealand have been declared free of EBL [33]. Australia has successfully

*Correspondence to: Haga T: ahaga@g.ecc.u-tokyo.ac.jp, Division of Infection Control and Disease Prevention, Graduate School of Agricultural and Life Sciences, The University of Tokyo, 1-1-1 Yayoi, Bunkyo-ku, Tokyo 113-8657, Japan
#These authors contributed equally to this work.
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eradicated BLV from their dairy cattle herds by 2012 [7]. By contrast, many Asian countries are still confronting with the burden of BLV infection [6, 10, 13, 16, 18, 22, 23, 25, 26, 37].

Vietnam, located in Southeast Asia, is a country that thrives on agriculture. Beef cattle have been raised for draught power, fertilizer, reproduction and meat for centuries. A rapid increase in the population of cattle has been seen in Vietnam over the recent years. According to General Statistics Office of Vietnam, in 2021, Vietnam's cattle herd was estimated at 6.3 million heads. However, Vietnam remains dependent on imported live cattle and beef due to the high demand of the domestic market. Especially, the importation of live cattle from the Southeast Asian countries such as Thailand and Myanmar have increased notably in recent years [32]. Australia, United States, and India are also the beef meat suppliers to Vietnam [24, 32]. Local cattle, as well as livestock from Thailand and Australia, are predominantly used to satisfy local beef demands [32].

Recently, we have reported the widespread of BLV in Vietnamese cattle population kept in the Red River Delta Region (Fig. 1) and the prevalence was 21.1% at the individual level confirmed by nested PCR [15]. However, we could not identify any positive beef cattle. To date, no information related to circulation of BLV in the remained parts of the country is available. Realizing the limitation of available data, we investigated the detailed prevalence and molecular characteristics of BLV in beef cattle kept in the Central Coast Regions by using PCR, sequencing and phylogenetic analysis. Blood samples were randomly collected from 48 beef cattle kept in 16 herds in four provinces located in the Central Coast Regions of Vietnam, including Quangtri, Binhdinh, Hue, and Danang (Fig. 1B). Beef cattle breeds included Yellow (n=10), Brahman crossbred (n=20), and Laisind (n=18) cattle. Permission was obtained from farm owners before sampling.

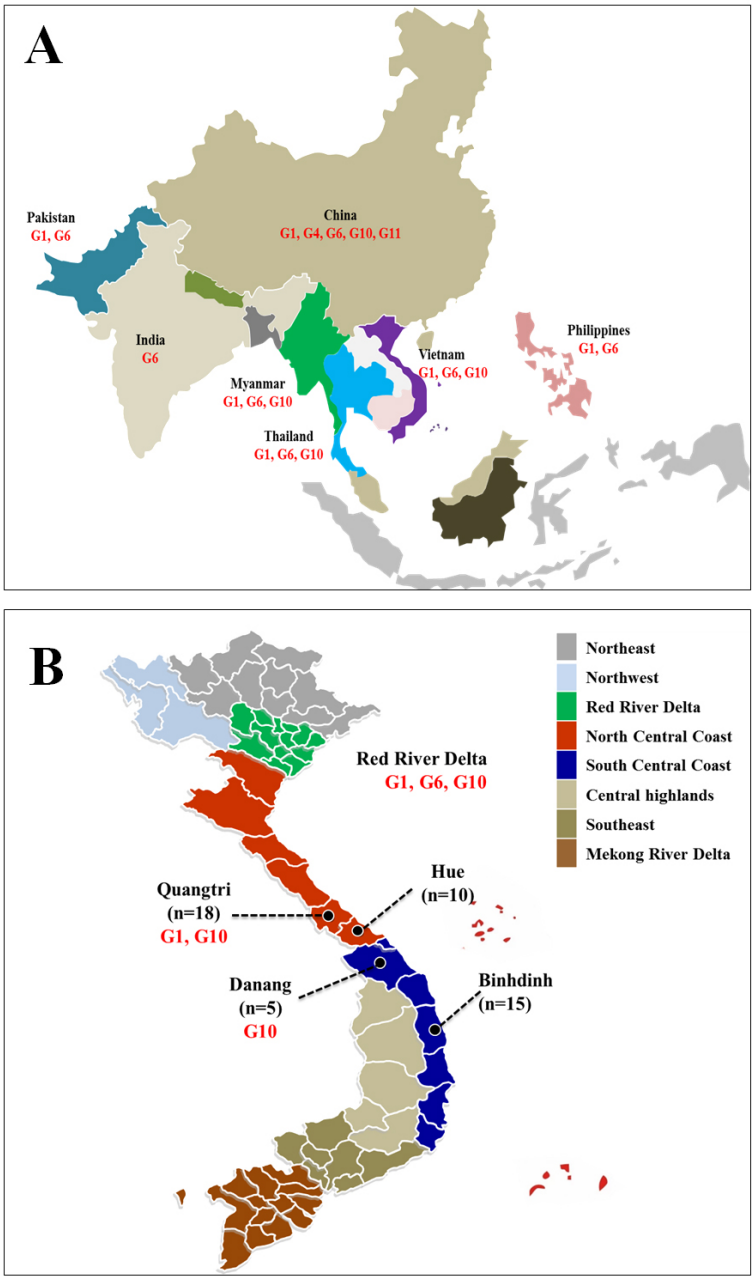


Fig. 1. The map showing the distribution of bovine leukemia virus (BLV) genotypes (G) in Southeast Asian and neighboring countries compiled from the previous studies [15, 16, 18, 19, 23, 25, 30, 34, 36, 37] (**A**); the map showing the list of regions in Vietnam, number of cattle (n) collected in this study and BLV genotypes detected in Vietnam (**B**). Blood sampling was performed in four provinces (indicated by dot).

DNA extraction from blood was performed by standard phenol-chloroform method. Nested PCR test was used to amplify 444 bp fragment of *BLV-env-gp51* gene as described previously [9]. PCR products of nested PCR were purified using NucleSpin Gel and PCR Clean up kit (Macherey Nagel GmbH & Co., KG, Duren, Germany) and were sequenced on an ABI 3130xl Genetic Analyzer (Thermo Fisher Scientific, Waltham, MA, USA) using BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, CA, USA). Nucleotide sequences were obtained by using MEGA 7 software [14].

The partial sequence of the *BLV-env-gp51* gene (423 bp) obtained in the present study were aligned in parallel with BLV *env* sequences of the all known BLV genotypes available in GenBank of National Center for Biotechnology Information (GenBank). Subsequently, a maximum likelihood phylogenetic tree was constructed using Kimura-2 parameter model of nucleotide of substitution with gamma distribution (K2+G) in MEGA 7. The reliability of phylogenetic relationships was evaluated using bootstrap analysis with 1,000 replicates.

Genotype 1 and 10 nucleotide sequences isolated from Vietnamese cattle were aligned to reference genotype 1 and 10 sequences. Then, deduction of amino acid sequences through translation of nucleotide to amino acid sequences was performed by MEGA 7.

A pairwise identity matrix of sequences was inferred using Sequence Dermarcation Tool Version 1.2 (SDTv1.2) software [20].

Among 48 samples analyzed in this study, the amplification of *BLV-env-gp51* gene was found in 7 cases (14.6%, 95% confidence interval=6.1–27.8%). BLV infection was detected only in the animals kept in two provinces, Danang (1/5 animals) and Quangtri (6/18 animals), while those from the remained provinces (Hue and Binhdinh) tested BLV-negative. Interestingly, BLV infection was identified in Vietnamese native cattle (Yellow cattle) for the first time. The prevalence of BLV in Yellow cattle was 33.3%, while only one positive animal belonged to Brahman crossed breed and no positive sample was found in Laisind breed. No significant difference was found between Yellow and Brahman (P=0.07), Yellow and Laisind (P=0.11), and Brahman and Laisind (P=1.00).

In order to analyze the genetic variability among the BLV strains in Vietnamese beef cattle, we performed sequencing of the *BLV-env-gp51* gene corresponding to the nucleotide positions 5104 to 5547 of the full length BLV genome of the reference strain pBLV-FLK (accession number LC164083). The obtained partial sequences from positive samples (423 bp) were submitted to the DDBJ/EMBL/GenBank databases (under accession numbers from LC672033 to LC672039). A phylogenetic tree based on the partial *env-gp51* gene sequence (423 bp), including the seven Vietnamese strains and 81 corresponding sequences from all of the eleven BLV genotypes available in GenBank was constructed (Fig. 2). The results of phylogenetic analysis suggested that the BLV genotype circulating in the Central Coast Regions of Vietnam belonged to genotype 1 and genotype 10. Genotype 10 was dominant (86%) and the rest belonged to genotype 1 (14%).

Among seven strains isolated in the present study, three strains, named VT20, VT21, VT30, showed 100% nucleotide identity. Therefore, the nucleotide sequences from the five typical Vietnamese BLV isolates were aligned with those from BLV strains representing two genotypes 1 and 10 deposited in GenBank. The alignment of *gp51* gene sequences identified a total of 23 single nucleotide polymorphism, including 5 unique mutations (the substitutions are different from all of the reference strains) in strains isolated in the present study (Supplementary Fig. 1A). Six of these 23 substitutions were nonsynonymous (T317C, A397G, C410T, A428G, T431C, and C529T), leading to amino acid substitutions and the remaining substitutions were synonymous mutations (Supplementary Fig. 1A, 1B). Alignment of gp51 deduced amino acid sequences demonstrated that all substitutions found in Vietnamese strains were localized at signal peptides, V106A was found in overlapping region of $CD4^+$ Epitope and ND1, four substitutions (T133A, S137F, Q143R, and I144T) were localized in the ND2, three substitutions (S137F, Q143R, and I144T) were seen in Zinc binding zone, finally, substitution P177S was observed in overlapping region of $CD8^+$ and E Epitopes [38].

The phylogenetic analysis, nucleotide and amino acid alignment also indicated the close relationship between Chinese, Thailand, Myanmar and Vietnamese genotypes 1, 6, and 10 strains. To further investigate the relationship between BLV strains isolated in Vietnam and those found in the other countries, we performed the pairwise comparison of 423 bp long sequences obtained from Vietnamese cattle in the present and the previous studies with reference strains representative for genotypes 1, 6 and 10 from different countries. Numerical values and the SDT color-coded matrix of pairwise identify scores are shown as Supplementary Table 1 and Fig. 3, respectively.

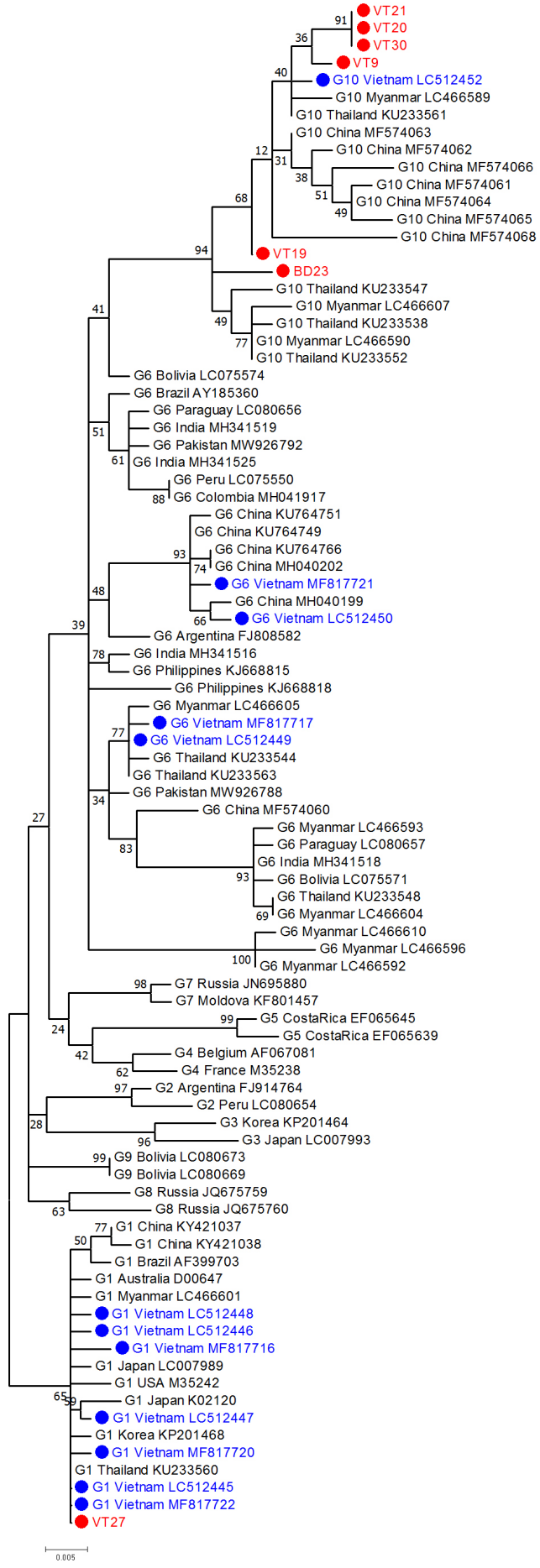
The sequence identity among Vietnamese genotype 10 strains ranged from 98.1 to 99.5%. Among these genotype 10 strains, VT21, VT9 and LC512452 exhibited closely related to the sequences from Thailand (KU233561). The strain VT19 showed the highest identity with sequences found in China (MF574063) and Thailand (KU233561). The strain BD23 were the most divergent from other Vietnamese genotype 10 sequences and was similar with sequences found in Thailand (KU233552) and Myanmar (LC466590).

Pairwise comparison of 423 bp long sequences belonged to genotype 1 was calculated. The sequence identity of eight sequences representing Vietnamese BLV strains was 99.3% to 100.0%. The strain, VT27, found in the present study and the strains, LC512445 and MF817722, KU233560 found in Vietnamese and Thai cattle reported in the previous studies were identical. While the remained Vietnamese genotype 1 sequences were 99.5–99.8% identity to sequence from Thailand (KU233560).

Sequence identity of genotype 6 sequences found in Vietnamese cattle was 97.6–99.8%. The strains MF817721, LC512450 showed high identity (99.5–99.8%) to sequences from China (KU764749). The sequence MF817717 showed the close relationship with sequence found in Thailand (KU233563). The highest identities (99.8% and 100%) were observed when comparison between Vietnamese genotype 6 sequence (LC512449) and sequences from Thailand (KU233563, KU233544), Myanmar (LC466605), and Vietnam (MF817717). Overall, the phylogenetic tree and the pairwise comparison clearly indicated the close relationship between BLV strains found in Vietnam, Thailand, China, and Myanmar.

Our recent study, investigating the circulation of the BLV in cattle kept in the Red River Delta Region of Vietnam, indicated that BLV infection of dairy cattle was prevalent in 22.1%. We could not identify any BLV-positive beef cattle [15]. However, a study performed simultaneously confirmed the existence of BLV in beef cattle (Laisind and Brahman crossed breed) reared in the same region [8]. The limited number of samples collected from a small area and tested in the previous study (n=67) might have prevented us from identifying positive animals. In the current study, we reported for the first time the prevalence of BLV in the beef cattle kept in the Central Coast Regions of Vietnam, demonstrating the widespread of BLV in beef cattle in Vietnam. The BLV infection proportion found in the present study (14.6%) was lower than that reported in Vietnamese dairy cattle in the previous study [15]. The higher prevalence of BLV in dairy cattle compared to beef cattle was also reported in cattle population worldwide [2, 3, 12, 17, 21, 22, 35]. Among three breeds of beef cattle involved in this study, positive samples were only observed in Brahman crossed cattle and Yellow cattle. This is the first time the positive samples have been confirmed in Vietnamese indigenous cattle (Yellow cattle). The present study includes a limited number of cattle. A larger number of animals from different breeds involved would offer a more accurate insight into BLV infection in Vietnamese beef cattle.

Previous study demonstrated the co-existence of genotypes 1, 6, and 10 in cattle kept in in the Red River Delta Region of Vietnam [8, 15]. Among the three genotypes, genotype 1 was found as the dominant genotype and only one BLV strain isolated in Vietnam was confirmed belonging to genotype 10. The results obtained in the current study confirmed the distribution of two BLV genotypes



1 and 10. Interestingly, in the present study, genotype 10 has been recognized as the dominant type and accounted about 86% of strains isolated in the Central Coast Regions of Vietnam. However, we could not find any strains belonged to genotype 6. The limited number of samples investigated may be the reason for isolating the only two genotypes.

Most of the nucleotide and amino acid substitutions of Vietnamese strains were located within the epitopes. These results were consistent with previous reports on the BLV genotypes 1, 6, and 10 strains isolated elsewhere [15, 16, 19].

Over the last century, a rapid increase in the population of cattle has been seen in Vietnam. However, the productivity is not satisfied to the high demand of the domestic market. Therefore, live cattle and their products were imported from several countries to Vietnam. Especially, the importation of live cattle from the neighboring countries such as Thailand and Myanmar through official and unofficial border gates into Vietnam have increased notably in recent years [32]. Vietnam is a transit country for animals moving from Thailand, destined for China [32]. The co-existence of the three genotypes 1, 6, and 10 was confirmed in Vietnam, Thailand, Myanmar, and China (Fig. 1A). Furthermore, our phylogenetic analysis and the pairwise comparison revealed that Vietnamese BLV isolates were closely related with the BLV strains circulating in Thailand, Myanmar and China. The introduction of BLV-infected cattle has been considered as the major driver for BLV infection [5]. Therefore, we speculate that the BLV strains may have been transmitted to Vietnam through cattle trade from Thailand and Myanmar and there was a transmission of BLV along with the movement of live cattle between Southeast Asian countries and China.

In conclusion, this study was the first to report the prevalence and molecular characteristic of BLV among beef cattle kept in the Central Coast Regions of Vietnam, although Dao *et al.* previously reported the prevalence in Red River Delta Region (Fig. 1) [8]. The data indicated the prevalence of BLV infection (14.6%) among beef cattle, which is lower than the prevalence reported in dairy cattle group in our earlier study. A high infection rate (33.3%) was observed for the first time in Vietnamese Yellow cattle. The circulation of two genotypes 1 and 10 was confirmed in the Central Coast Regions with the dominance of genotype 10. The phylogenetic tree and the pairwise comparison demonstrated the Vietnam BLV strains isolated in the previous studies and in this study exhibited homology to Thailand, Myanmar, and China strains. BLV may have been transmitted to Vietnam through the import of live cattle from Myanmar and Thailand. The movement of cattle from Southeast Asian countries destined for China might be closely related with the transmission of BLV among these countries. Investigating the prevalence of BLV among imported cattle will give us further information about the transmission of BLV through the movement of live cattle.

Fig. 2. Maximum likelihood phylogenetic tree based on the 423 bp nucleotide sequence of the bovine leukemia virus (BLV) *env-gp51* of the seven BLV strains isolated in the Central Coast Regions of Vietnam and 81 reference sequence representing eleven known BLV genotypes isolated worldwide. Sequences are named with their genotypes, countries of origin, and accession numbers. The BLV strains isolated from Vietnamese cattle are indicated by filled circles. The strains found in the present study are in red and the remained Vietnamese strains reported in the previous studies are in blue. Genotype (G).

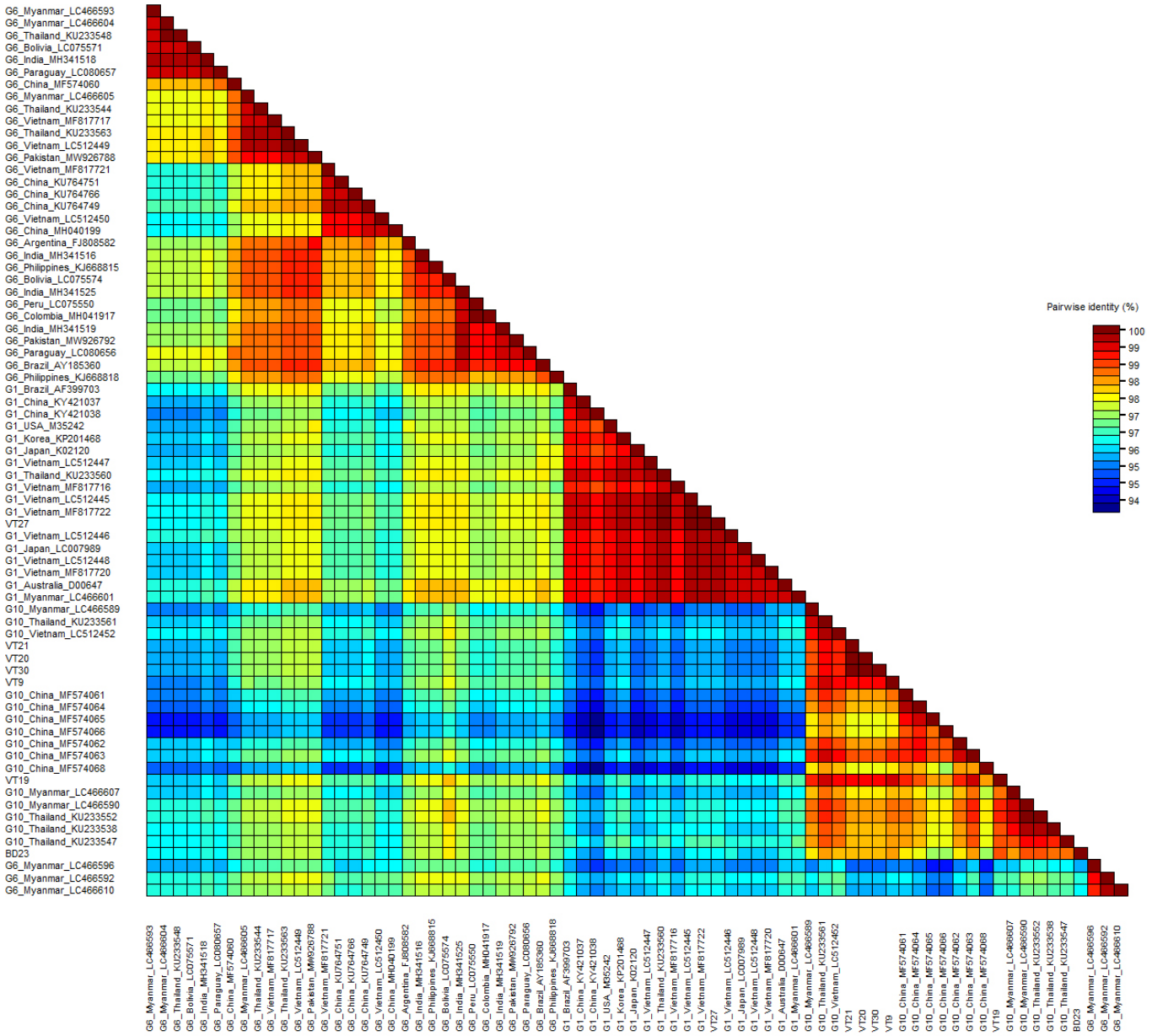


Fig. 3. Color-code matrix of pairwise identity scores generated by the alignment of 423 bp nucleotide sequences of *BLV-env-gp51* genotype 1, 6, and 10 strains isolated in Vietnam and representatives from other countries. Genotype (G).

POTENTIAL CONFLICTS OF INTEREST. The authors have nothing to disclose.

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