MASKED GENOTYPE 6 OF BOVINE LEUKEMIA VIRUS IS FOUND IN COLOMBIAN CATTLE

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Abstract

Background Viral diseases such as bovine leukosis are a major cause of health problems associated with economic losses in the livestock industry worldwide. The prevalence of bovine leukosis ranges between 4% and 90%, and this disease is considered endemic. The etiological agent is bovine leukemia virus (BLV). Ten BLV genotypes have been reported based on analysis of complete or partial envelope gene sequences, of which genotype 1 is the most prevalent. However, the genetic variability of BLV variants circulating in Colombia remains unexplored. Therefore, in the present study, we analyzed blood samples collected from 289 cows distributed in 75 farms across the country. PCR amplification of env, gag and tax gene segments was performed. The obtained amplicons were sequenced and then subjected to phylogenetic analyses. Results A total of 62% of the cows present at 92% of the farms were BLV-positive. Genotype 1 was exclusively detected by env and gag gene segments when analyzed using previously reported primers. However, tax gene analysis revealed circulation of genotype 6 variants, which were also detected based on env gene analysis with newly designed primers. These results indicate that current genotyping approaches based on partial env sequencing may bias BLV genetic variability approaches and underestimate the diversity of the detected BLV genotypes. Conclusions This report is the first molecular and epidemiological study of BLV conducted in Colombia and contributes to the global epidemiology of the virus; it also reinforces the great impact of BLV on the country's livestock and thus is a useful resource for farmers and government entities.

Background

Viruses are one of the main causes of health problems, of which bovine leukemia virus (BLV) is one of the five agents considered most significant for cattle. In Colombia reported a seroprevalence per animal of 42.7% [1], which agreed with the reported worldwide with a range between 4 and 90% [2-9]. The disease is characterized by asymptomatic passage in 70% of infected animals, with persistent lymphocytosis developing in 30% and leukemia in 5% [10-14].

BLV belongs to *Retroviridae* familyand Deltaretrovirus genus with two copies of a single-strand positive-RNA with a length of 8714 nucleotides. Its genome contains 8 open reading frames (ORFs) with 3 gene segments (*gag, pol*, *env*) encoding the structural proteins and enzymes necessary for viral replication, a *pX* region encoding the Tax and Rex auxiliary proteins, which perform regulatory functions, and two long terminal regions (LTRs) at the terminal ends of the genome [10-14]. The *gag* gene consists of 1183 nucleotides and encodes the capsid protein p24-CA, which is used frequently in the laboratory to determine the presence of the virus due to its high degree of conservation. The *env* gene has a length of 1547 nucleotides and encodes the envelope glycoproteins; which are composed of the extracellular portion (gp51) and the gp30 transmembrane region (gp30) [15]. gp51 is essential for recognition and entry of the virus into the host cell and is one of the most immunogenic viral proteins.

The *pX* region has 3304 nucleotides and encodes the Tax protein, which fulfills regulatory functions. This protein activates transcription in favor of viral gene expression and interacts with cellular transcription

factors. Mechanisms by which Tax induces cellular transformation include inhibition of DNA repair, blocking of apoptosis, acting as a transactivator of the LTRs and altering transcription regulation and cell translation. However, there is no evidence that mutations in Tax gene are related to the presence or development of leukemia in cattle by this virus or that this gene is related to the viral genotype.

Nowadays, 10 BLV genotypes have been reported in different regions of the world using restriction fragment length polymorphism (RFLP) techniques, sequencing of the complete viral genome, and partial or complete *env* sequencing. Genotype 1 is the most prevalent genotype worldwide. Since 1985, genotype 1 has been reported in 17 countries, mainly in the Americas, as well as in Europe, Australia and Asia. Similarly, genotypes 2, 3, 4 and 7 have also been reported in these regions. In contrast, genotypes 5, 6, 8, 9 and 10 have been reported with a lower prevalence and in more limited geographical regions. For instance, genotype 6 has been detected since 2007 in American and Asian countries [6-8, 16-30].

Currently, BLV detection and genotyping is based on a 444 bp segment amplification, as is recommended by the World Organization for Animal Health (OIE) for viral diagnosis [23]. Several studies have published their results based on what OIE suggests for genotyping, establishing genotype 1 as the most prevalent worldwide. However, topology of some trees shows different clusters in the same genotype, leading to the question if the amplification of the 444 bp segment of *env* region could efficiently identify all the current genotypes or instead, it mimics the presence of other circulating genotypes. Now, considering the current situation of BLV in Colombia, even if it was determined the seroprevalence earlier [1], there are few studies [31] in which the molecular epidemiology of the virus has been determined. The aim of this study was to identify the circulating genotype of BLV along the country in order to deep in the knowledge of the current situation of BLV and to reveal the possibility of masked genotypes, unable to identify with the primers suggested by OIE.

Results

Prevalence of BLV based on detection of the *gag* gene segment

In order to determine the prevalence of BLV in Colombia, we performed the *gag* gene amplification. The observed prevalence of BLV based on *gag* gene segment detection was 62% per animal and 92% per farm and was distributed per animal and per farm in the departments as follows: Cundinamarca 69 and 90%, Boyacá 71 and 94%, Antioquia 73 and 100%, Meta 85 and 100%, Nariño 14 and 75% and Cesar 17 and 75% (Fig. 1).

Determination of the viral genotype

To determine the circulating genotypes in Colombia, the *env* region of the BLV genome was amplified from the collected samples [23]. Amplification of this segment was achieved in 179 of the samples, although the corresponding sequence was only obtained from 100 of these samples. As shown in the phylogenetic tree in Figure 2, all of the Colombian samples included in this study were grouped within genotype 1 clade, with a support bootstrap value of 97%.

Due to the fact that all of the Colombian samples were grouped in the same clade (genotype 1) based on the *env* sequences analysis, we proceeded to perform the *gag* and *tax* gene analysis.

Phylogenetic analyses were initially done only for the *env* gene segment. The *gag* and *tax* sequences were analyzed after finding that all the Colombian samples were grouped in the same clade based on the *gag* sequences, which corresponded to genotype 1 (Fig. 3A). However, for *tax* region, four of the sequences clustered apart from genotype 1 clade, together with genotype 6 representatives (Fig. 3B). These four sequences seemed to have phylogenetic discordances between *tax* and *env* regions. Therefore, a new set of primers were designed considering currently genetic variability of BLV strains, as deduced by available sequences of all BLV genotypes reported in GenBank. With this new set of primers, a 750 bp region of the *env* gene was amplified*,* which was244 nt longer than the sequence amplified by the set of primers currently recommended for genotyping purposes [23]

Evidence of circulation of genotype 6

To analyze a phylogenetic discordance between *env* and *tax* regions, the 131 samples that were positive for *gag* gene were amplified with the new set of primers designed for *env* gene. The results showed that the four samples that were grouped within genotype 6 by *tax* gene also clustered together with genotype 6 strains when analyzed by this new *env* gene segment. Altogether, the analyses performed indicate the circulation of both BLV genotypes 1 and 6 in Colombia, with 127 out of 131 samples belonging to genotype 1, while the 4 remaining correspond to genotype 6 (Fig. 4).

Discussion

Molecular epidemiology studies of BLV worldwide an in this case in Colombia, makes it possible to identify circulating strains in specific regions to propose and build public policies aimed to control and, in the future, eradicate this virus which has been seen to produce problems not only in cattle but also has been reported as a possible risk factor involved in humans pathologies [32-35]. In the other hand, variability of genotypes as well as results of structural and functional properties of its envelope proteins [36] provide useful information for research focused on vaccines production with specific strains, that in the case of Colombia, will be appropriate to include conserved epitopes shared between genotypes 1, 6 and 3 according to the results obtained in this study and previously reported [31]. In addition, phylogenetic studies are important to understand the geographical distribution of the virus and thus, identify conserved and hypervariable regions including specific mutations that could be related with different levels of virulence or pathogenicity [6, 37-41].

The current detection of BLV circulating genotypes is suggested by the OIE by the use of primes reported by Fechner et al in 1996 [23], which were used initially in this study. However, when carrying out the phylogenetic analyzes, all the Colombian isolates were grouped in genotype 1, results that differ with the sequences obtained from other gene regions *(gag* and *tax)*. With the *tax* region, 4 of the positive samples of BLV were grouped in genotype 6, usually *tax* region is not used for genotyping since it is considered one of the most polymorphic regions of this retrovirus. Therefore, this suggests that the primers reported

by Fechner present disadvantages to differentiate genotypes of the virus reported after 1996, and thus, a new set of primers was designed in the current study. These primers were designed by bioinformatic analyzes including 69 sequences of the complete genome of the virus available in the GenBank representing the 10 genotypes reported up to December 2018, choosing a conserved region among all the genotypes in the *env* gene. As a result, a fragment of 750 bp was sequenced and was evidenced that indeed with the Fechner primers it was not possible to discriminate other genotypes, showing that genotypes 1 and 6 are simultaneously circulating in Colombia (Fig. 4).

In this study finding genotype 1 was expected because this is the most prevalent genotype worldwide [42]. However, evidence of G6 in Colombia is a novel finding. Overall, G6 has been reported in 4 Asian countries (Philippines, Thailand, Jordan, and India) and 5 South American countries (Argentina, Brazil, Bolivia, Peru and Paraguay) [24, 42]. Different genotypes distributed in the world and the emergence of new genotypes in specific areas as it was reported here, suggests the outcome of importing and exporting processes in the cattle industry, which contributes to increased viral prevalence rates and virus diversity [7, 18], that in the case of silent diseases such as enzootic bovine leukosis are transferred unnoticed within cattle in different regions, and thus, policies of control and diagnosis are necessary to be included worldwide.

One of the most relevant findings of this study was the detection of genotype 6 by *tax* region. So far, there has not been other studies using this ORF for genotyping as well as few available sequences for this region in databases that are representative of all viral genotypes that allow deeper studies in terms of comparing the relationship between *env* and *tax* genes with the purpose of genotyping. Further studies are needed to confirm its application for genotyping, but at least with the results obtained in this study could be suggested as a potential gene besides *env* region for genotyping.

On the other hand, besides of identifying the circulating genotypes, it is also important to discuss the prevalence reported in this study. It was found a 62% of positive animals and a 92% of farms, corresponding to one of the highest prevalence rates reported in Latin America [42]. In Colombia, the last report was published by Ortiz and coworkers [1] in which they obtained a lower seroprevalence rate (42% per animal and 67% by farm) compared with the current study, besides to be reported in the same cattle population.

One of the main differences between the two studies lies on the diagnostic method. Here, prevalence was determined by PCR tests, which detects directly fragments of the viral genome while Ortiz et al study used a ELISA commercial kit which detects antibodies in the host [1]. Even if in the retrovirus infections the presence of antibodies could be interpreted as presence of the virus, the sensitivity of ELISA kits might be lower than the detection level of a PCR test [43]. Now, in terms of antibodies production, it might be possible that the amount of antibodies in sera samples could be below the detection limits of ELISAs and thus, false negative samples could be reported, and especially with viruses with slow replication rate such as BLV, giving as a result low levels of antibodies in blood due to the immune response evasion [44].

Given the importance of cattle industry in the country and in Latin America, it would be relevant if governmental entities and policy-maker agencies consider including the enzootic bovine leukosis as an official control disease, facilitating the diagnosis to the cattle industry members to identify infected animals, and furthermore, to control the dissemination of the virus focused in the future on the promotion of eradication programs, and likewise, minimizing the impact that BLV has on cattle and eventually on humans who consume bovine-derived food products [37].

Studies like this one are very important for science and for One Health approaches, in which interfaces between animals-humans and ecosystems should be considered [45]. In the case of BLV, it has been shown that the virus not only is present in cattle but also in other species such as sheep, buffalo, goats, alpacas and humans [46-49]. Although is not clear yet the role that BLV might have in humans, evidence of its presence has been reported and is proposed as a potential risk factor for breast cancer development [32-34]. In addition, due to the fact that BLV is considered one of the main viral agents associated with economic impact in livestock production, which is distributed worldwide [42] and seems to have a zoonotic behavior, are enough arguments to continue in the research field of vaccines candidates and thus, to find strategies directed to control the viral infection by preventing risk factors within farms as well as the use of good livestock production practices for each specific region [1].

Conclusions

The epidemiological data provided here demonstrated a higher prevalence of BLV in Colombia compared with the seroprevalence previously reported. In addition, those findings contribute to the epidemiology of the virus identifying genotype 1 and 6 in Colombia. In addition, the new primers that were designed in this study will be available for further studies willing to amplify all the current circulating genotypes, as an update of the proposal of Fechner et al. Finally, although *tax* is not commonly used for genotyping, phylogenetic analyses showed that this gene does contribute to the genotype identification and may be useful for BLV genotyping as well.

Methods

Samples collection and DNA extraction

The number of samples to be studied was determined using the sampling formula to estimate a proportion with the WinEpiscope tool available online (http://www.winepi.net/). For the sample size (n) estimation was used the prevalence reported by Ortiz et al in 2016, with a 42%, giving as a result an *n* value of 289. Blood samples were taken from the coccygeal vein of healthy cows distributed all along the country in 6 different regions, between 2015 and 2016. Mononuclear cells were separated from blood samples using density gradient centrifugation with LymphoSep (MP®), after recovering the buffy coat, total DNA was extracted in order to look for proviral DNA with the High Pure PCR Template Preparation Kit (Roche®).

PCR sensitivity tests

Previously amplified amplicons belonging to the segments of *gag* (381 bp) and *tax* (396 bp) genes of BLV were purified with a PCR Wizard kit (Promega®) followed by the insertion of each of them into the pELMO vector (Ramos et al., 2017) and transforming them into *E. coli* TOP10 cells (Invitrogen®). The plasmid DNA was used as an amplification template in the sensitivity tests. Serial dilutions of each plasmid were performed with initial concentrations of 230 ng/µl and 411 ng/µl for *gag* and *tax*, respectively. Subsequently, the respective PCR assays were performed at each dilution to determine the detection limit of the technique corresponding to the maximum amplified dilution. For the *env* region, a segment of 801 bp was amplified as described above, but the sequence was cloned into the pEXP5-CT/TOPO (Invitrogen) vector with an initial concentration of 159 ng/µl.

Detection of BLV gene segments

As target, genes for this study were used primers directed to segments of the *gag, tax, and env* genes of the virus. PCR conditions and primers used are found on the table 1. For all the PCR assays described below, the PCR master mix (Roche®) was prepared according to the manufacturer's instructions. Plasmid DNA containing each gene segment (*gag, tax,* and *env*) was used as a positive control.

Table 1. Primers used for amplification*, sequencing* and construction of plasmid DNA† for the BLV *gag, tax* and *env* gene segments. NA (not applicable)

The presence of BLV was evaluated using a multiplex PCR targeted at amplifying the constitutive gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as an internal control and a 385 bp segment of the *gag* gene. Once positive samples for BLV were identified by *gag* gene amplification, PCR was carried out to detect a 373 bp segment of the *tax* region and another PCR was done in order to detect the circulating genotype with a segment of 530 bp of the *env* gene.

The amplicons obtained from the PCRs were purified with a High Pure PCR Product Purification kit (Roche®) according to the manufacturer's instructions. Subsequently, sequencing was performed by the Sanger sequencing service of Macrogen Korea. The sequences obtained in this study were deposited in the GenBank database with accession numbers MH041897 to MH042017, MH057402 to MH057465 and MH057466 to MH057532 for *env, gag* and *tax*, respectively.

Phylogenetic analysis

In order to identify the circulating genotype of BLV, it was compared the sequences obtained in this study with complete BLV genome sequences available in GenBank, 64 *gag* and 67 *tax* sequences from this study were compared with 16 sequences of different genotypes from GenBank. In the case of the *env* gene, 121 sequences from the Colombian strains were compared with 46 partial sequences, which included the 10 BLV genotypes described at the time.

The combined multiple alignment of all Colombian sequences was performed with ClustalW program implemented in Mega 7.

Once aligned, the best evolutionary model that described our sequence data was assessed using the "Find Model" interface in the Mega7 package based on the Akaike information criterion (AIC). Using this model, maximum likelihood trees were constructed using the MEGA 5.0 software [50]. As a measure of the robustness of each node, we employed the bootstrapping method (1000 pseudo-replicates). The phylogenetic trees obtained were edited using the FigTree program v1.4.1, which is available online (*http://tree.bio.ed.ac.uk/software/figtree/)*.

For the *env* sequences, phylogenetic inference was also performed by maximum likelihood analysis using the RAxML program. Phylogenetic tree inference using maximum likelihood/rapid bootstrapping was run on XSEDE (RAxML - HPC2 on XSEDE (8.2.10) (Stamatakis, 2014) with 1000 bootstrap replicates using the MrBayes program (Huelsenbeck and Ronquist, 2015). The phylogenetic inference was performed in a Bayesian framework with GTR +G, two runs and three chains.

List Of Abbreviations

BLV: bovine leukemia virus, ORFs: open reading frames, LTRs: long terminal regions, gp51: extracellular portion envelope protein, gp30: transmembrane region envelope protein, RFLP: restriction fragment length polymorphism, OIE: World Organization for Animal Health, GAPDH: glyceraldehyde-3-phosphate dehydrogenase, AIC: Akaike information criterion.

Declarations

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Consent for publication: Not applicable.

Competing interests: The authors declare that they have no competing interests.

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Table

Table 1. Primers used for amplification*, sequencing* and construction of plasmid DNA† for the BLV *gag, tax* and *env* gene segments. NA (not applicable)

ɟion	Primer sequence 5−3 (Forward and reverse)	PCR product (bp)	Annealing Temperature (°C)	Minimum detectable concentration (ng/ul)	Reference
*┼	AACACTACGACTTGCAATCC	385	59.3	2.3	Buehring, et al 2014
	GGTTCCTTAGGACTCCGTCG				
┼	CTTCGGGATCCATTACCTGA	373	56.5	0.042	
	GCTCGAAGGGGGAAAGTGAA				
*	CCCACAAGGGCGGCGCCGGTTT	509	62.8	$159x10^{-9}$	Fechner et al.,1997
	AACAACAACCTCTGGGAAGGGT				
*	TGTCCCTAGGAAAYCAAC	750	56	$159x10^{-4}$	Current study
	AGATTAACCAGGGAGATAGG				
┼	ATGAGATGCTCCCTGTCCCTAG	801	57.6	NA	Corredor et al., 2018
	ACGTCTGACCCGGGTAGG				

Figures

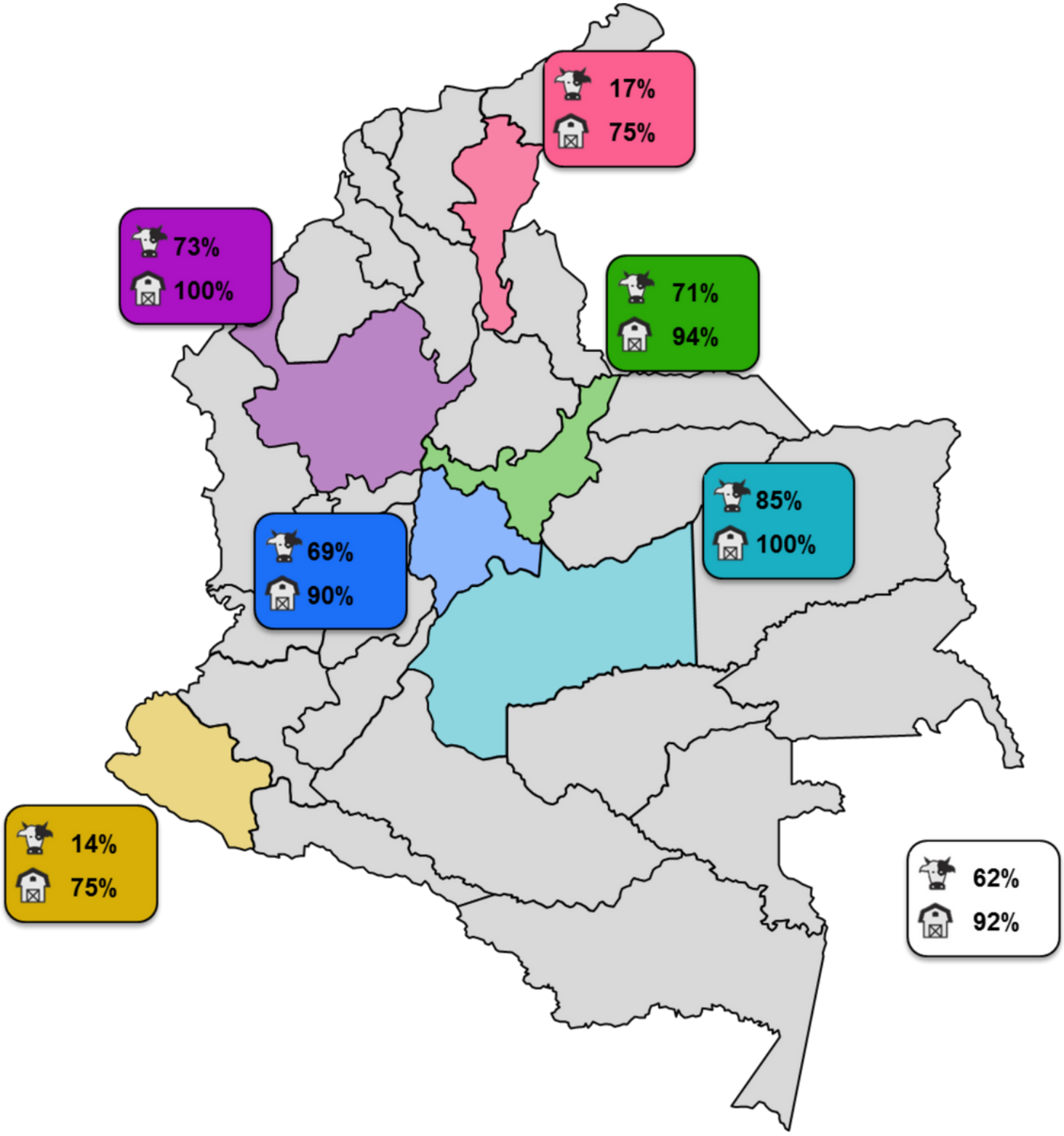

Figure 1

Prevalence of BLV by detection of the gag gene segment in the Colombian cattle population. Map of Colombia showing prevalence rates per animals and farms distributed in six regions of Colombia: Cundinamarca 69 and 90% (dark blue), Boyacá 71 and 94% (green), Antioquia 73 and 100% (purple), Meta 85 and 100% (turquoise), Nariño 14 and 75% (yellow) and Cesar 17 and 75% (pink), respectively. The overall prevalence of BLV in Colombia was 62% per animal and 92% per farm.

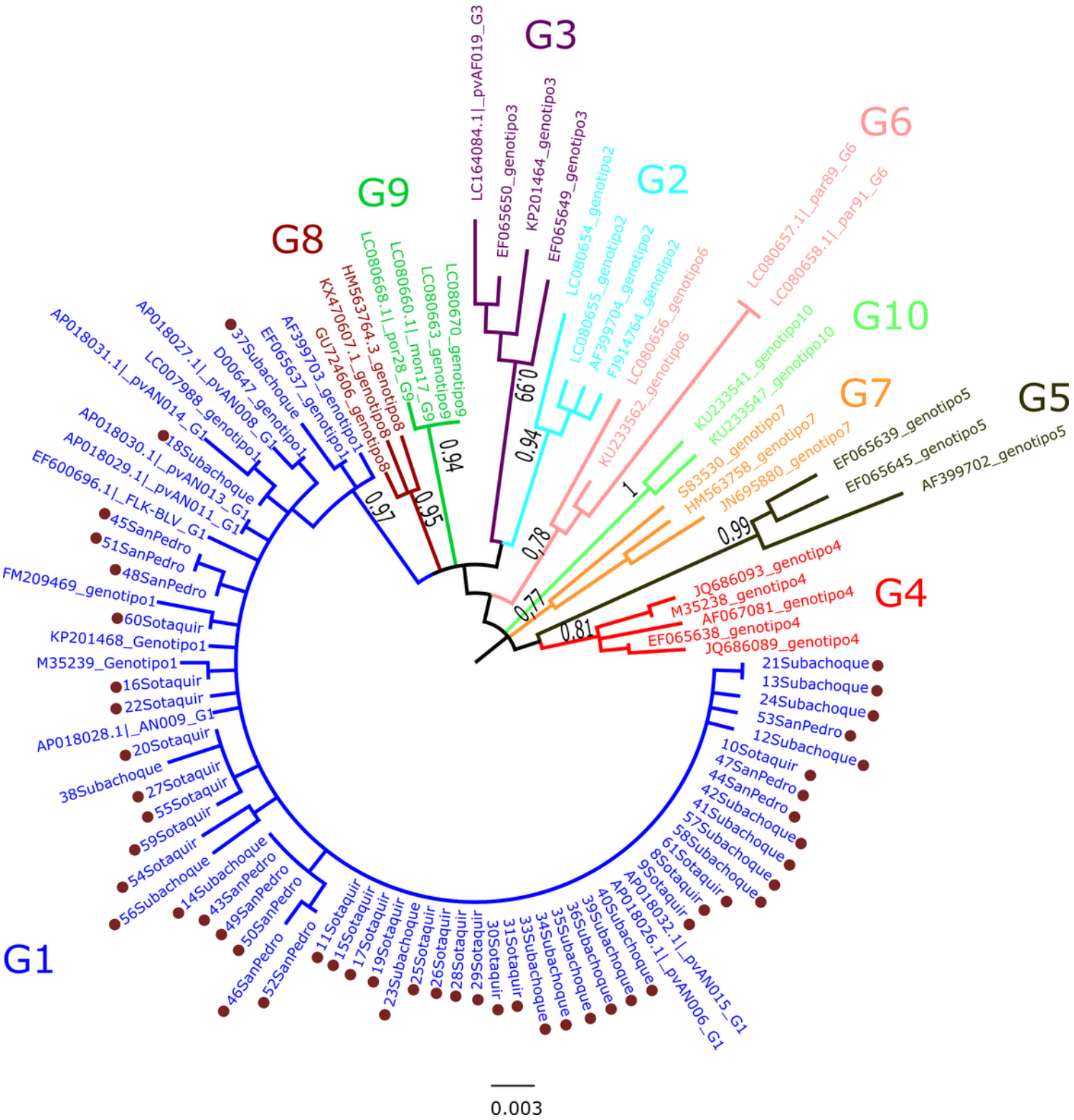

Figure 2

Maximum-likelihood phylogenetic tree analysis of the env gene of BLV circulating in Colombia. The ML method based on the General Time Reversible model maximum composite likelihood (MCL) approach was used, and the topology with a superior log likelihood value was selected. A discrete Gamma distribution was used to model evolutionary rate differences among sites (5 categories (+G, parameter = 0.3005)). The rate variation model allowed some sites to be evolutionarily invariable ([+I], 39.13% sites).

The analysis involved 100 nucleotide sequences. A total of 506 positions were included in the final dataset.

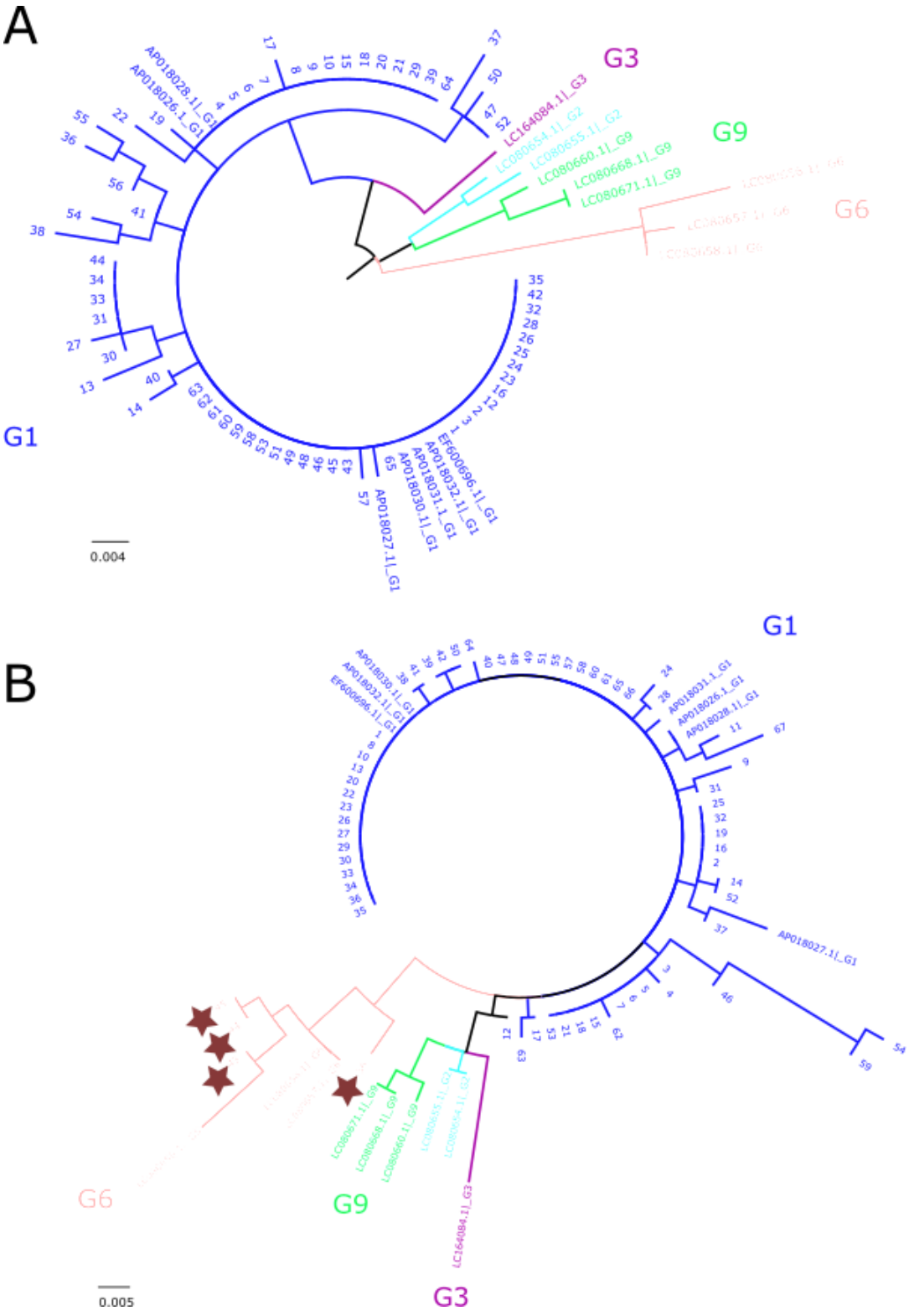

Figure 3

Maximum-likelihood phylogenetic tree analysis of the gag and tax genes of BLV circulating in Colombia. The evolutionary history was inferred using the ML method based on the General Time Reversible model. A discrete Gamma distribution was used to model evolutionary rate differences among sites (5

categories; (+G, parameter = 0.1621) for gag and (+G, parameter = 0.1749) for tax)). The rate variation model allowed some sites to be evolutionarily invariable ([+I], 43.30% sites for gag and [+I], 42.83% sites for tax). The analysis involved 80 and 83 nucleotide sequences for gag and tax. A total of 306 and 314 positions were included in the final dataset for gag and tax. Fig. 3A, phylogenetic tree of the gag gene segments. Note that all Colombian samples are grouped into genotype 1. Fig. 3B, phylogenetic tree constructed with the tax gene segments. Note that four Colombian samples are included in the genotype 6 cluster.

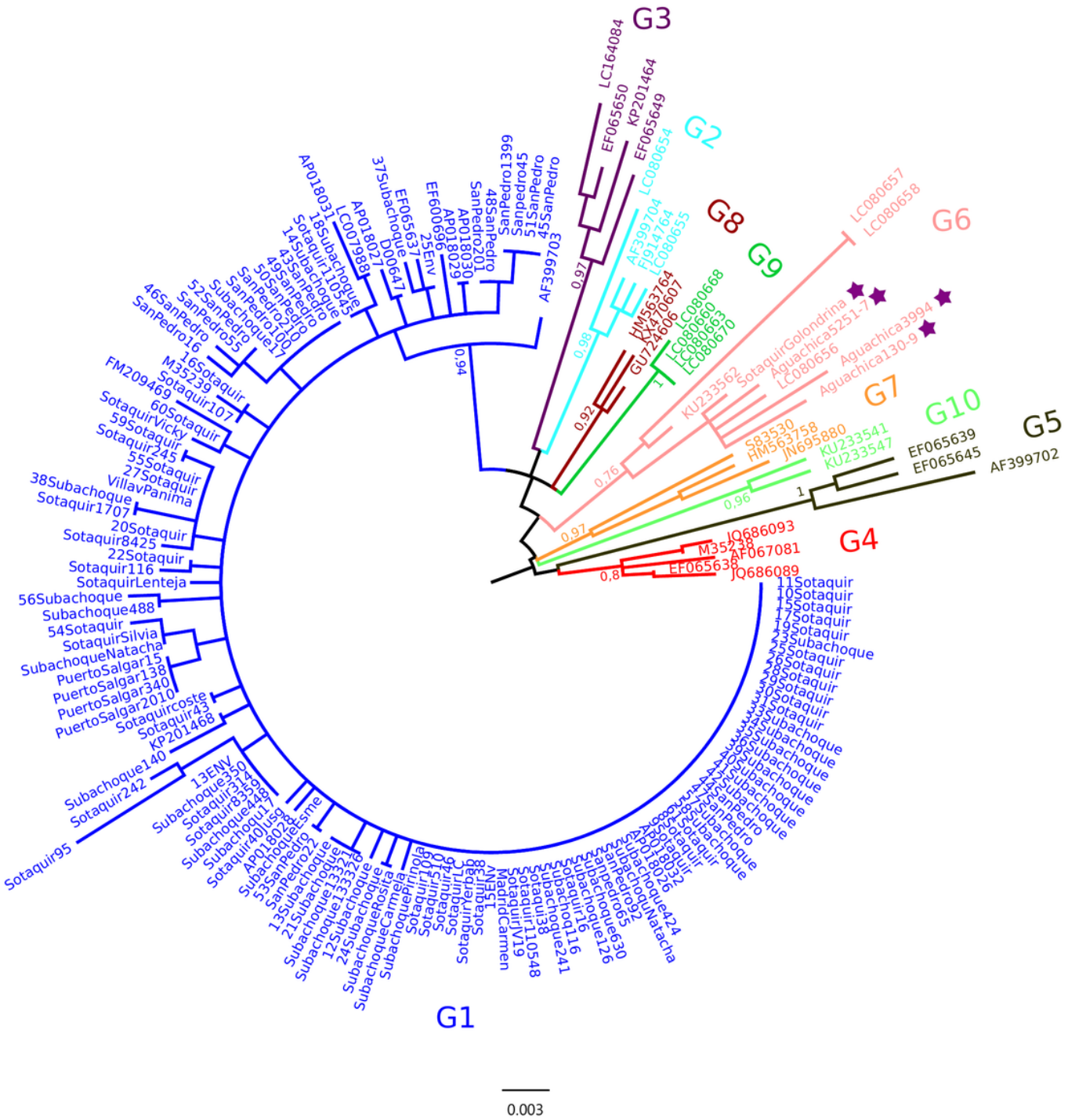

Figure 4

Maximum-likelihood phylogenetic tree analysis of the env gene of BLV circulating in Colombia. The evolutionary history was inferred using the ML method based on the Tamura-Nei model. A discrete Gamma distribution was used to model evolutionary rate differences among sites (5 categories (+G, parameter = 0.3588)). The analysis involved 168 nucleotide sequences.