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Genetic identification of bovine leukaemia virus

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Abstract: Molecular genetic research methods make it possible to evaluate the genetic diversity of bovine leukemia virus (BLV) and are the most informative approaches to its genetic identification. Molecular genetic research methods work well for the phylogenetic analysis of sequenced nucleotide DNA sequences of the provirus, as well as for the polymerase chain reaction-restriction fragment length polymorphism analysis (PCR-RFLP) according to the phylogenetic classification of the pathogen. The purpose of the research was to study the scientific and methodological approaches to the genetic identification of bovine leukemia virus, integrated into the molecular monitoring of infection of cattle with BLV genotypes. The authors used PCR-RFLP-genotyping and comparative phylogenetic analysis of aligned nucleotide sequences of the *env* gene fragment of the BLV provirus isolates to detect the genotypic affiliation of the cattle from twenty-one livestock farms of the Republic of Tatarstan. As a result, isolates of four out of ten BLV genotypes were found in the Tatarstani cattle, namely genotypes 1, 4, 7, and 8. The research involved a comparative analysis of 505 nucleotide sequences of a fragment of the BLV *env* gene, including those deposited in GenBank NCBI. The analysis confirms the inconsistency of several earlier PCR-RFLP typing strategies with the current approach in assessing the genotypic diversity by phylogenetic analysis. The improved strategy of PCR-RFLP genotyping of BLV corresponds with its modern phylogenetic classification. The strategy makes it possible to identify all the known genotypes of the viral pathogen. Its validity has been proved by *in silico* modelling of restrictogrammes and a phylogenetic analysis of the *env* gene fragment of 57 reference isolates of ten BLV genotypes that generate 57 genotype-associated combinations of diagnostically significant PCR-RFLP profiles.

Keywords: Bovine leukaemia virus, BLV, cattle, gene, genotype, genetic identification, PCR, RFLP, sequencing

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INTRODUCTION

Enzootic Bovine Leukosis (EBL) is a chronic infectious disease of a tumorous nature. It causes significant economic damage to the dairy and beef cattle industry due to poor production, low quality, cattle mortality, and expensive epidemic prevention measures [1, 2].

Foodstuffs of infected animals can be dangerous to humans due to the harmful metabolites it contains. The causative agent affects all kinds of raw material (milk,

meat, by-products) and products, remaining a potential source of human infection [3–5].

Pasteurization of milk inactivates the virus but does not degrade its genome. The genetic material of the provirus maintains its integrity in canned meat [6]. Moreover, there are dairy products with partial pasteurization regime, e.g. classic cheeses, granulated cottage cheese, powdered milk with a low heating temperature, etc. The temperature processing parameters used in accordance with the regulatory and technical documentation cannot destroy harmful metabolites and, in some cases, do not kill the virus [7].

According to some researches, DNA of BLV provirus was found in epithelial cells of human mammary glands, including those of breast cancer patients. The hypothesis states that BLV may destabilize the host genome, thus leading to cancerous degeneration of cells [8–12].

Obtaining high-quality raw materials of animal origin is the most important challenge for meat and dairy industry. The challenge includes the development of functional and gerodietic foods [13–15].

According to the requirements of the Technical Regulations of the Customs Union "On safety of milk and dairy products" (TR CU 033/2013), BLV preventive measures and eradication activities are extremely important, given the significant prevalence of this incurable disease in the Russian Federation [16, 17].

An early genetic diagnosing of the pathogen is part of the system of anti-epizootic measures, followed by the removal of infected animals from the herd. Molecular genetic research methods make it possible to assess the genetic diversity of BLV [18]. This is the most informative approach to the gene identification of the virus. Molecular genetic research methods work well for the phylogenetic analysis of nucleotide DNA sequences of the provirus, as well as for PCR-RFLP analysis according to the phylogenetic classification of the pathogen [19].

The current phylogenetic classification of BLV includes ten genotypes. The first seven genotypes were described by Argentinean scientists in 2009 [20], while genotype 8 was described by researchers from Russia [21–23], Croatia [24], and a European team of scientists [25] in 2011–2013. Genotype 9 was investigated by a team of Argentinean, Chilean, and Japanese scientists in 2016 [26]. Genotype 10 was described by a team of researchers from Thailand and South Korea [27] in 2016.

The objective of the current research was to study the scientific and methodological approaches to the genetic identification of BLV integrated into the molecular monitoring of infection of cattle herds with BLV genotypes. The following tasks were set:

– to establish the genotypes of BLV isolates in Tearstain cattle;

– to define the types of BLV isolates with deciphered nucleotide sequences of the *env* gene fragment, depending on the chosen gene identification strategy;

– to improve the strategy of PCR-RFLP-genotyping of BLV and make it consistent with the modern phylogenetic BLV classification.

STAGY OBYECTS AND METHODS

The research involved a total of 179 blood samples from AGID-positive cows. The samples were provided by agricultural enterprises from 21 districts of the Republic of Tatarstan. The samples were genetically examined for BLV. The examination included a phylogenetic analysis of sequenced *env* gene fragment of the pathogen and a PCR-RFLP-genotyping consistent with the phylogenetic classification of the infectious agent.

To extract DNA from the whole conserved blood, we used a commercial PCR diagnostic kit, 'DNA-Sorb B', produced by the Central Research Institute of Epidemiology of the Federal Supervisory Service for Consumer Rights and Human Welfare, Ministry of Health of the Russian Federation.

Nested PCR with extracted samples of BLV proviral DNA was performed with external (*env*5032 and *env*5608) and internal (*env*5099 and *env*5521) primers initiating the generation of *env* gene 444 bp fragment of causative agent at the final stage of reaction [28].

Restriction endonucleases used in PCR-RFLP-genotyping of BLV were consistent with its phylogenetic classification: *Bst*YI (isoshizomer *Bst*X2I), *Hph*I (isoshizomer *Asu*HPI), *Hae*III, *Pvu*II, *Ssp*I. The NEBcutter v.2.0 web resource was used for PCR-RFLP modelling.

For the detection of the obtained results of PCR and PCR-RFLP analysis, 2.5% agarose gel horizontal electrophoresis was applied with a TBE buffer (pH 8.0) containing ethidium bromide. The electrophoregrammes were examined in a UV-transilluminator (λ = 310 nm). The sizes of the generated DNA fragments were compared with standard DNA molecular weight markers (SibEnzyme Ltd, Russia).

Sequencing of the PCR amplification products of the *env* gene fragment of detected BLV provirus isolates was performed on ABI PRISM 3100 Genetic Analyser (Applied Biosystems, USA) in the laboratory of Scientific and Technical Complex Sintol (Russia). Internal oligonucleotide primers *env*5099 and *env*5521 were used as sequencing. The sequenced fragments of the *env* gene of BLV provirus isolates were aligned with the corresponding nucleotide sequences of the reference BLV isolates from GenBank with the help of BLAST and MEGA-4 programmes. The last stage included a phylogenetic analysis.

RESULTS AND DISCUSSION

The study featured a PCR-RFLP-genotyping and a comparative phylogenetic analysis of the aligned sequences of the *env* gene fragment of BLV provirus isolates from 21 districts of the Republic of Tatarstan.

As a result, out of 179 identified isolates, ten isolates belonged to genotype 1; 106 isolates belonged to the cluster of genotype 4; 55 were characterized as genotype 7, and the remaining eight provirus isolates belonged to genotype 8 (Table 1).

According to the results obtained by PCR-RFLP-genotyping and phylogenetic analysis of sequenced *env* gene fragment, there are four out of ten currently known BLV genotypes in Tatarstan: 1, 4, 7, and 8.

Fig. 1 shows the genotypes of BLV isolated with the help of phylogenetic analysis of nucleotide sequences of *env* gene fragment.

An additional assessment of the heterogeneity of the reference BLV representatives for the *env* gene included an analysis of the intra- and intergenotypic heterogeneity of genotypes. The data in Table 2 indicate that it is impossible to use the 'heterogeneous' criterion for assessing the genetic diversity of BLV.

As part of the next task, BLV isolates with the decoded nucleotide sequences of the *env* gene fragment were identified according to the chosen genetic identification strategy. The degree of consistency of genotypic approaches was assessed by comparing the data of the *in silico* PCR-RFLP and the phylogenetic analyses.

A comparative analysis of 505 nucleotide sequences of the BLV *env* gene locus, including those deposited with GenBank NCBI, confirms the inconsistency of a number of earlier PCR-RFLP typing strategies [28–30] with the current approach in assessing the genotypic diversity by means of phylogenetic analysis.

Thus, the BLV isolates that belong to the Belgian subgroup according to D. Beier et al. (2001) [28], belong to genotype 4 according to the phylogenetic classification; Australian subgroup can be referred to genotypes 1, 3, 6, 8, or 9; Japanese subgroup – to genotypes 1, 6, or 7. In addition, the genotyping strategy [28] includes 11 additional unique combinations of PCR-RFLP profiles, conditionally identical to 11 unclassifiable BLV subgroups (Table 3).

Besides, BLV isolates that belong to genotype 1 and 7 according to the phylogenetic analysis, can be referred to Australian and Japanese subgroups, as well as to three unclassifiable subgroups, according to the strategy of D. Beier et al. (2001) [28]; genotypes 2, 5, and 10 belong to two unclassifiable subgroups; genotypes 3 and 8 – to Australian subgroup; genotype 4 – to the Belgian subgroup and four unclassifiable subgroups; the genotype 5 – to two unclassifiable subgroups; genotype 6 – to the Australian, Japanese and two unclassifiable subgroups; genotype 9 – to the Australian and one unclassifiable subset (Table 3).

BLV isolates that were genotyped according to M. Licursi et al. (2002) [29] as genotype 1 may belong to genotypes 1, 4, 6, or 7 according to the phylogenetic classification; genotype 3 – to genotypes 1, 6, or 7; genotype 5 – to genotypes 1, 3, 6, 7, or 9; genotype 6 – to genotypes 2, 4, 5, or 7 (Table 4).

For the genotyping strategy described in [29], there are 19 unique combinations of PCR-RFLP profiles that are conditionally identical to 19 unclassifiable BLV genotypes (Table 4).

Besides, BLV isolates that are genotyped according to phylogenetic analysis as genotype 1 may refer to 1, 3, 5, and three unclassifiable BLV genotypes according to the strategy of M. Licursi et al. (2002) [29]; genotype 2 belongs to genotype 6 and two unclassifiable genotypes; the genotype 3 – to genotype 5 and one unclassifiable genotype; genotype 4 – to genotypes 1 and 6 and five unclassifiable genotypes; genotype 5 – to genotype 6 and two unclassifiable genotypes; genotype 6 – to genotypes 1, 3, and 5 and three unclassifiable genotypes; genotype 7 – to genotypes 1, 3, and 6 and four unclassifiable genotypes; genotype 8 – to one unclassifiable genotype; genotype 9 – to genotype 5; genotype 10 – to three unclassifiable genotypes (Table 4).

It should be mentioned that, when analyzing *in silico* PCR-RFLP data from 505 BLV representatives, we found not a single nucleotide sequence of the *env* gene fragment that would belong to genotypes 2 and 4 according to M. Licursi et al. (2002) (Table 4). This fact did not make it possible to prove the actual existence of PCR-RFLP profiles indicated for these two BLV genotypes.

Table 1. Distribution of 179 genotyped samples of BLV provirus DNA according to 21 districts of the Republic of Tatarstan, Russian Federation

Districts		Number of analysed samples	BLV genotypes									
			1	2	3	4	5	6	7	8	9	10
1	Aznakaevsky	10	–	–	–	9	–	–	1	–	–	–
2	Al'keyevsky	13	–	–	–	5	–	–	8	–	–	–
3	Arsky	7	–	–	–	6	–	–	1	–	–	–
4	Buinsky	7	–	–	–	2	–	–	3	2	–	–
5	Vysogorsky	4	–	–	–	4	–	–	–	–	–	–
6	Drozhanovsky	12	–	–	–	4	–	–	7	1	–	–
7	Zainsky	8	–	–	–	7	–	–	–	1	–	–
8	Klaibitsky	7	–	–	–	7	–	–	–	–	–	–
9	Laishevsky	13	–	–	–	12	–	–	1	–	–	–
10	Leninogorsky	19	–	–	–	15	–	–	4	–	–	–
11	Mamadyshksy	10	10	–	–	–	–	–	–	–	–	–
12	Menzelinsky	6	–	–	–	6	–	–	–	–	–	–
13	Musl'umovsky	4	–	–	–	–	–	–	4	–	–	–
14	Nizhnekamensky	14	–	–	–	8	–	–	5	1	–	–
15	Pestrechinsky	1	–	–	–	1	–	–	–	–	–	–
16	Rybnoslobodsky	8	–	–	–	8	–	–	–	–	–	–
17	Sarmanovsky	2	–	–	–	2	–	–	–	–	–	–
18	Spassky	9	–	–	–	3	–	–	6	–	–	–
19	Tukaevsky	9	–	–	–	4	–	–	3	2	–	–
20	T'ulyachinsky	6	–	–	–	–	–	–	6	–	–	–
21	Chistopolsky	10	–	–	–	3	–	–	6	1	–	–
Total number of samples		179	10	–	–	106		–	55	8	–	–

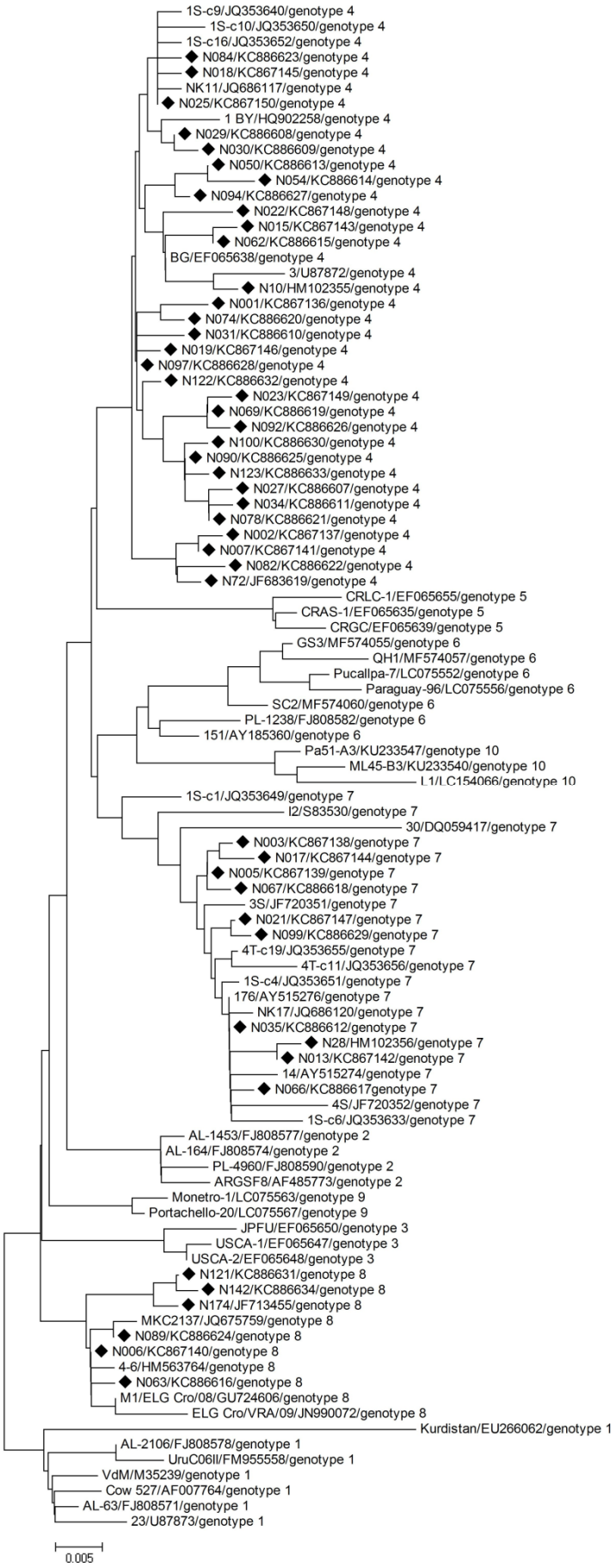

Fig. 1. Dendrogramme of 99 isolates of 10 BLV genotypes, based on a phylogenetic analysis of the *env gene* fragment [MEGA-4: algorithm NJ, 400 nt, 99 seq.] Legend: black diamond marks GenBank NCBI nucleotide sequences of the *env gene* fragment of BLV provirus isolates in the Republic of Tatarstan.

Table 2. Intra- and intergenotypic heterogeneity of reference BLV representatives according to *env* gene (% ratio)

PHYLOGENETIC CLASSIFICATION OF BLV										
GENOTYPE	1	2	3	4	5	6	7	8	9	10
1	0–5	3–6	3–7	3–7	3–7	3–7	3–8	2–6	3–6	4–7
2	3–6	0–1	3–4	2–4	4–5	3–5	3–5	2–4	3	4–6
3	3–7	3–4	0–2	3–5	4–5	3–5	3–6	3–4	3	4–6
4	3–7	2–4	3–5	0–3	3–5	2–5	2–6	2–4	2–4	3–5
5	3–7	4–5	4–5	3–5	0–2	4–6	3–5	4–5	4–5	5–6
6	3–7	3–5	3–5	2–5	4–6	0–4	3–6	2–5	3–5	3–5
7	3–8	3–5	3–6	2–6	3–5	3–6	0–4	2–5	3–5	4–6
8	2–6	2–4	3–4	2–4	4–5	2–5	2–5	0–2	2–3	3–5
9	3–6	3	3	2–4	4–5	3–5	3–5	2–3	0–1	4–5
10	4–7	4–6	4–6	3–5	5–6	3–5	4–6	3–5	4–5	0–2

Table 3. Comparison of *in silico* data for PCR-RFLP (typification according to D. Beier et al., 2001) and the phylogenetic analysis of the BLV *env* gene fragment

PCR-RFLP genotyping		PCR product (bp)	RFLP fragments (bp)			BLV genotypes										N
			*Pvu*II	*Bam*HI	*Bcl*I	1	2	3	4	5	6	7	8	9	10	
Subgroup	Belgian	444	280/164	444	225/219	–	–	–	142	–	–	–	–	–	–	142
	Australian	444	444	316/128	225/219	57	–	4	–	–	28	70	21	19	–	199
	Japanese	444	444	316/128	219/121/104	8	–	–	–	–	6	1	–	–	–	15
	?	444	444	444	225/219	43	–	–	1	–	–	2	–	3	17	66
	?	444	444	316/128	444	1	–	–	–	–	14	2	–	–	–	17
	?	444	444	316/128	225/191/28	1	–	–	–	–	–	–	–	–	–	1
	?	444	280/164	316/128	225/219	–	36	–	–	10	–	3	–	–	–	49
	?	444	280/164	316/128	444	–	1	–	–	–	–	–	–	–	–	1
	?	444	280/164	316/128	219/189/36	–	–	–	–	1	–	–	–	–	–	1
	?	444	280/164	444	444	–	–	–	1	–	–	–	–	–	–	1
	?	444	208/164	253/191	225/219	–	–	–	1	–	–	–	–	–	–	1
	?	444	280/164	444	219/121/104	–	–	–	4	–	–	–	–	–	–	4
	?	444	444	242/128/74	225/219	–	–	–	–	–	1	–	–	–	–	1
	?	444	444	444	444	–	–	–	–	–	–	–	–	–	7	7

Legend. N is the number of analysed BLV isolates with an established PCR-RFLP profile; "?" – an unclassifiable BLV taxon.

Table 4. Comparison of *in silico* data of PCR-RFLP (typification according to M. Licursi et al., 2002) and phylogenetic analysis of a fragment of the BLV *env* gene

PCR-RFLP genotyping		PCR product (bp)	RFLP fragments (bp)			BLV genotypes										N
			*Bcl*I	*Hae*III	*Pvu*II	1	2	3	4	5	6	7	8	9	10	
Genotype	1	444	225/219	198/94/87/32/27/6	444	98	–	–	1	–	28	68	–	–	–	195
	2	444	219/121/104	312/94/32/6	444	–	–	–	–	–	–	–	–	–	–	–
	3	444	219/121/104	285/94/32/27/6	444	8	–	–	–	–	6	1	–	–	–	15
	4	444	219/121/104	198/94/87/32/27/6	444	–	–	–	–	–	–	–	–	–	–	–
	5	444	225/219	285/94/32/27/6	444	1	–	3	–	–	1	1	–	22	–	28
	6	444	225/219	198/94/87/32/27/6	280/164	–	35	–	139	9	–	3	–	–	–	186
	?	444	444	198/94/87/32/27/6	444	1	–	–	–	–	12	–	–	–	7	20
	?	444	225/191/28	198/119/94/27/6	444	1	–	–	–	–	–	–	–	–	–	1
	?	444	225/219	312/94/32/6	444	1	–	–	–	–	–	–	–	–	–	1
	?	444	444	198/94/87/32/27/6	280.164	–	1	–	1	–	–	–	–	–	–	2
	?	444	219/189/36	198/94/87/32/27/6	280/164	–	–	–	–	1	–	–	–	–	–	1
	?	444	225/219	285/94/32/27/6	280/164	–	–	–	2	1	–	–	–	–	–	3
	?	444	444	198/87/49/45/32/27/6	444	–	–	–	–	–	–	1	–	–	–	1
	?	444	225/219	225/94/87/32/6	444	–	–	–	–	–	–	–	21		–	21
	?	444	225/219	285/94/32/21/6/6	444	–	–	1	–	–	–	–	–	–	–	1
	?	444	225/219	198/121/87/32/6	280/164	–	–	–	1	–	–	–	–	–	–	1
	?	444	225/219	198/119/94/27/6	280/164	–	–	–	1	–	–	–	–	–	–	1
	?	444	219/121/104	285/94/32/27/6	280/164	–	–	–	4	–	–	–	–	–	–	4
	?	444	225/219	198/87/49/45/32/27/6	444	–	–	–	–	–	–	2	–	–	–	2
	?	444	225/219	198/119/94/27/6	444	–	–	–	–	–	–	1	–	–	–	1
	?	444	444	198/121/87/32/6	444	–	–	–	–	–	1	1	–	–	–	2
	?	444	225/219	198/87/49/45/32/27/6	280/164	–	1	–	–	–	–	–	–	–	–	1
	?	444	444	198/94/81/32/21/6/6	444	–	–	–	–	–	1	–	–	–	–	1
	?	444	225/219	198/94/81/32/27/6/6	444	–	–	–	–	–	–	–	–	–	6	6
	?	444	225/219	279/94/32/27/6/6	444	–	–	–	–	–	–	–	–	–	11	11

Legend. N is the number of analysed BLV isolates with an established PCR-RFLP profile; "?" – an unclassifiable BLV taxon.

Table 5 compares the *in silico* data of PCR-RFLP according to the strategy by H. Fechner et al. (1997) [30] and the phylogenetic analysis of a fragment of the BLV *env* gene.

Thus, the BLV isolates identified according to H. Fechner et al. (1997) [30] as variant group A belong to genotype 4 according to the phylogenetic classification; variant group B – to genotypes 1, 6, or 7; variant group C – to genotypes 1, 3, 6, 7, or 9; variant group D – to genotypes 1, 4, or 7; variant group F – to genotypes 2, 5, or 7; variant group G – to genotype 1 (Table 5).

For this typing strategy [30], there are 17 unique combinations of PCR-RFLP profiles that are conditionally identical to 17 unclassifiable variant BLV groups (Table 5). Besides, BLV isolates genotyped by phylogenetic analysis as genotype 1 are characterized as variant groups B, C, D, G and three unclassifiable variant groups of BLV according to the strategy of H. Fechner et al. (1997) [30]; the genotype 2 belongs to variant group F and one unclassifiable variant group; genotype 3 – to variant group C; genotype 4 – to variant groups A, D and three unclassifiable variant groups; genotype 5 – to variant group F and two unclassifiable variant groups; genotype 6 – to variant groups B and C and four unclassifiable variant groups; genotype 7 – to variant groups B, C, D, F and two unclassifiable variant groups; genotype 8 – to variant group E; genotype 9 – to variant group C and one unclassifiable variant group; genotype 10 – to three unclassifiable variant groups of BLV (Table 5).

The priority task of the research was to improve the strategy of PCR-RFLP genotyping of BLV by making it consistent with the phylogenetic classification and taking into account the update information on the genetic diversity of the ten known BLV genotypes.

505 BLV isolates were generated during the analysis of restriction mappings of the *env* gene locus according to 5 restriction enzymes. The interpretation of their *env*-PCR-RFLP profiles actually reflects the strategy of PCR-RFLP genotyping of BLV in accordance with its phylogenetic classification. The data are represented in Table 6.

Table 5. Comparison of *in silico* data of PCR-RFLP (genotyping according to H. Fechner et al., 1997) and the phylogenetic analysis of a fragment of the BLV *env* gene

PCR–RFLP genotyping		PCR product (bp)	RFLP fragments (bp)					BLV genotypes										N
			*Bam*HI	*Bcl*I	*Bgl*I	*Hae*III	*Pvu*II	1	2	3	4	5	6	7	8	9	10	
Variant group	A	444	444	225/219	328/116	198/94/87/32/27/6 198/121/87/32/6	280/164	–	–	–	142	–	–	–	–	–	–	142
					444	198/119/94/27/6 285/94/32/27/6												
	B	444	316/128	219/121/104	328/116 444	285/94/32/27/6	444	8	–	–	–	–	6	1	–	–	–	15
	C	444	316/128	225/219	328/116	198/94/87/32/27/6 285/94/32/21/6/6 285/94/32/27/6	444	56	–	4	–	–	28	70	–	19	–	177
					444	198/87/49/45/32/27/6 198/119/94/27/6												
	D	444	444	225/219	328/116	198/94/87/32/27/6	444	42	–	–	1	–	–	2	–	–	–	45
	E	444	316/128	225/219	328/116	225/94/8732/6	444	–	–	–	–	–	–	–	21	–	–	21
	F	444	316/128	225/219	328/116	198/94/87/32/27/6	280/164	–	36	–	–	9	–	3	–	–	–	48
	G	444	316/128	225/219	444	312/94/32/6	444	1	–	–	–	–	–	–	–	–	–	1
	?	444	316/128	444	328/116	198/94/87/32/27/6	444	1	–	–	–	–	2	–	–	–	–	3
	?	444	316/128	225/191/28	328/116	198/119/94/27/6	444	1	–	–	–	–	–	–	–	–	–	1
	?	444	444	225/219	444	285/94/32/27/6	444	1	–	–	–	–	–	–	–	3	–	4
	?	444	316/128	444	328/116	198/94/87/32/27/6	280/164	–	1	–	–	–	–	–	–	–	–	1
	?	444	316/128	219/189/36	328/116	198/94/87/32/27/6	280/164	–	–	–	–	1	–	–	–	–	–	1
	?	444	316/128	225/219	444	285/94/32/27/6	280/164	–	–	–	–	1	–	–	–	–	–	1
	?	444	316/128	444	328/116	198/87/49/45/32/27/6	444	–	–	–	–	–	–	1	–	–	–	1
	?	444	444	444	328/116	198/94/87/32/27/6	280/164	–	–	–	1	–	–	–	–	–	–	1
	?	444	253/191	225/219	328/116	198/94/87/32/27/6	280/164	–	–	–	1	–	–	–	–	–	–	1
	?	444	444	219/121/104	328/116	285/94/32/27/6	280/164	–	–	–	4	–	–	–	–	–	–	4
	?	444	316/128	444	328/116	198/121/87/32/6	444	–	–	–	–	–	–	1	–	–	–	1
	?	444	316/128	444	444	198/94/87/32/27/6	444	–	–	–	–	–	11	–	–	–	–	11
	?	444	242/128/74	225/219	328/116	198/94/87/32/27/6	444	–	–	–	–	–	1	–	–	–	–	1
	?	444	316/128	444	444	198/121/87/32/6	444	–	–	–	–	–	1	–	–	–	–	1
	?	444	444	225/219	328/116	198/94/81/32/27/6/6	444	–	–	–	–	–	–	–	–	–	6	6
	?	444	444	444	328/116	198/94/81/32/27/6/6	444	–	–	–	–	–	–	–	–	–	7	7
	?	444	444	225/219	444	279/94/32/27/6/6	444	–	–	–	–	–	–	–	–	–	11	11

Legend. N is the number of analysed BLV isolates with an established PCR-RFLP profile; "?" – an unclassifiable BLV taxon.

Table 6. An improved strategy for PCR-RFLP-genotyping of BLV, consistent with the phylogenetic classification

G	BLV isolate	GenBank A/N	PCR product (bp)	RFLP fragments (bp)					C	N
				*Pvu*II	*Ssp*I	*Hph*I	*Hae*III	*Bst*YI		
1	AL–63	FJ808571	444	444	399/45	224/220	198/94/87/32/27/6	198/128/118	1	56
1	Cow 527	AF007764	444	444	399/45	224/220	285/94/32/27/6	198/128/118	2	8
1	23	U87873	444	444	399/45	224/220	312/94/32/6	198/128/118	3	1
1	AL–2106	FJ808578	444	444	399/45	224/220	198/94/87/32/27/6	246/198	4	42
1	UruC06II	FM955558	444	444	399/45	224/220	285/94/32/27/6	246/198	5	1
1	VdM	M35239	444	444	399/45	224/181/39	198/94/87/32/27/6	316/128	6	1
1	Kurdistan	EU266062	444	444	399/45	220/196/28	198/119/94/27/6	198/128/118	7	1
2	AL–164	FJ808574	444	280/164	399/45	224/220	198/94/87/32/27/6	198/128/118	8	34
2	PL–4960	FJ808590	444	280/164	399/45	224/220	198/87/49/45/32/27/6	198/128/118	9	1
2	ARGSF8	AF485773	444	280/164	399/45	444	198/94/87/32/27/6	198/128/118	10	1
2	AL–1453	FJ808577	444	280/164	444	224/220	198/94/87/32/27/6	198/128/118	11	1
3	USCA–1	EF065647	444	444	399/45	444	285/94/32/21/6/6	198/128/96/22	12	1
3	USCA–2	EF065648	444	444	399/45	444	285/94/32/27/6	198/128/96/22	13	2
3	JPFU	EF065650	444	444	399/45	444	285/94/32/27/6	198/128/118	14	1
4	BG	EF065638	444	280/164	399/45	224/220	198/94/87/32/27/6	444	15	115
4	3	U87872	444	444	399/45	224/220	198/94/87/32/27/6	444	16	1
4	1S–c16	JQ353652	444	280/164	399/45	444	198/94/87/32/27/6	444	17	16
4	N023	KC867149	444	280.164	399.45	224.220	198/94/87/32/27/6	253/191	18	1
4	1_BY	HQ902258	444	280/164	444	224/220	198/94/87/32/27/6	444	19	7
4	N034	KC886611	444	280/164	399/45	224/220	198/121/87/32/6	444	20	1
4	1S–c9	JQ353640	444	280/164	399/45	224/220	198/119/94/27/6	444	21	1
4	NK11	JQ686117	444	280/164	399/45	224/220	285/94/32/27/6	444	22	6
4	1S–c10	JQ353650	444	280/164	399/45	220/145/79	198/94/87/32/27/6	444	23	1
5	CRAS–1	EF065635	444	280/164	399/45	224/181/39	198/94/87/32/27/6	316/128	24	8
5	CRGC	EF065639	444	280/164	399/45	224/181/39	285/94/32/27/6	316/128	25	1
5	CRLC–1	EF065655	444	280/164	444	224/181/39	198/94/87/32/27/6	316/128	26	2
6	PL–1238	FJ808582	444	444	399/45	224/220	285/94/32/27/6	316/128	27	7
6	151	AY185360	444	444	399/45	224/220	198/94/87/32/27/6	316/128	28	27
6	GS3	MF574055	444	444	399/45	444	198/94/87/32/27/6	316/128	29	11
6	SC2	MF574060	444	444	399/45	224/220	198/94/87/32/27/6	242/128/74	30	1
6	QH1	MF574057	444	444	213/186/45	444	198/94/81/32/21/6/6	316/128	31	1
6	Pucallpa–7	LC075552	444	444	399/45	444	198/94/87/32/27/6	316/79/49	32	1
6	Paraguay–96	LC075556	444	444	399/45	444	198/121/87/32/6	316/128	33	1
7	N28	HM102356	444	444	444	224/137/83	198/94/87/32/27/6	294/128/22	34	7
7	176	AY515276	444	444	444	224/137/83	198/94/87/32/27/6	316/128	35	53
7	I2	S83530	444	444	444	224/220	285/94/32/27/6	316/128	36	1
7	14	AY515274	444	444	444	145/137/83/79	198/94/87/32/27/6	316/128	37	1
7	30	DQ059417	444	444	444	444	198/87/49/45/32/27/6	316/128	38	1
7	3S	JF720351	444	280/164	444	224/137/83	198/94/87/32/27/6	316/128	39	3
7	4T–c19	JQ353655	444	444	399/45	224/137/83	198/94/87/32/27/6	316/128	40	3
7	1S–c4	JQ353651	444	444	444	224/137/83	198/94/87/32/27/6	316/79/49	41	1
7	NK17	JQ686120	444	444	444	224/137/83	198/87/49/45/32/27/6	316/128	42	2
7	4S	JF720352	444	444	444	224/137/83	198/119/94/27/6	316/128	43	1
7	1S–c6	JQ353633	444	444	444	224/137/83	198/121/87/32/6	316/128	44	1
7	4T–c11	JQ353656	444	444	444	224/137/83	285/94/32/27/6	316/128	45	1
7	N067	KC886618	444	444	444	224/137/44/39	198/94/87/32/27/6	316/128	46	1
7	1S–c1	JQ353649	444	444	444	224/220	198/94/87/32/27/6	444	47	2
8	M1/ELG_Cro/08	GU724606	444	444	399/45	224/220	225/94/87/32/6	198/128/118	48	13
8	N174	JF713455	444	444	399/45	224/220	225/94/87/32/6	316/128	49	4
8	ELG_Cro/VRA/09	JN990072	444	444	444	224/220	225/94/87/32/6	198/128/118	50	2
8	4–6	HM563764	444	444	399/45	224/137/83	225/94/87/32/6	198/128/118	51	1
8	MKC2137	JQ675759	444	444	399/45	444	225/94/87/32/6	198/128/118	52	1
9	Monetro–1	LC075563	444	444	399/45	224/171/49	285/94/32/27/6	198/128/118	53	19
9	Portachello–20	LC075567	444	444	399/45	224/171/49	285/94/32/27/6	246/198	54	3
10	Pa51–A3	KU233547	444	444	399/45	224/220	198/94/81/32/27/6/6	444	55	12
10	ML45–B3	KU233540	444	444	399/45	224/220	279/94/32/27/6/6	444	56	11
10	L1	LC154066	444	444	444	224/220	198/94/81/32/27/6/6	444	57	1

Legend. G – genotype; C – combination; N – the number of analysed BLV isolates with the established combination of PCR-RFLP profiles.

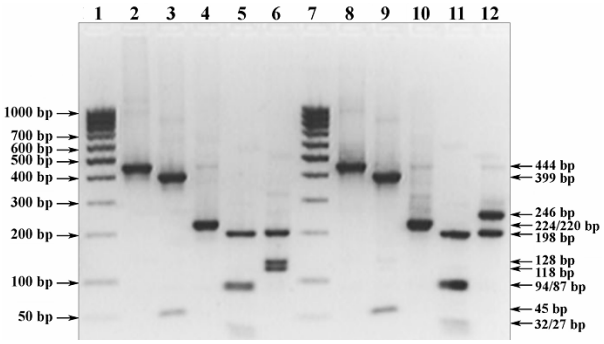

Fig. 2. Electrophoregramme of combinations of PCR-RFLP profiles (C1 and C4) of genotype 1 (an improved BLV genotyping strategy).

Legend. 1, 7) DNA markers 100 bp + 50 bp (SibEnzyme). 2–6) PCR-RFLP profile of BLV N-1 provirus isolate (C1, genotype 1): 2) *Pvu*II-RFLP (444 bp); 3) *Ssp*I-RFLP (399/45 bp); 4) *Hph*I-RFLP (224/220 bp); 5) *Hae*III-RFLP (198/94/87/32/27/6 bp); 6) *Bst*YI-RFLP (198/128/118 bp). 8–12) PCR-RFLP profile of BLV N-4 provirus isolate (C4, genotype 1): 8) *Pvu*II-RFLP (444 bp); 9) *Ssp*I-RFLP (399/45 bp); 10) *Hph*I-RFLP (224/220 bp); 11) *Hae*III-RFLP (198/94/87/32 / 27.6 bp); 12) *Bst*YI-RFLP (246/198 bp).

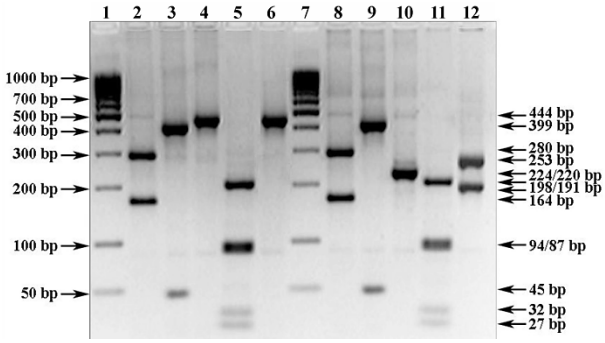

Fig. 3. Electrophoregramme of combinations of PCR-RFLP profiles (C17 and C18) of genotype 4 (improved BLV genotyping strategy).

Legend. 1, 7) DNA markers 100 bp + 50 bp (SibEnzyme). 2–6) PCR-RFLP-profile of the BLV N015 provirus isolate (C17, genotype 4): 2) *Pvu*II-RFLP (280/164 bp); 3) *Ssp*I-RFLP (399/45 bp); 4) *Hph*I-RFLP (444 bp); 5) *Hae*III-RFLP (198/94/87/32/27/6 bp); 6) *Bst*YI-RFLP (444 bp); 8–12) PCR-RFLP-profile of the BLV N023 provirus isolate (C18, genotype 8) *Pvu*II-RFLP (280/164 bp); 9) *Ssp*I-RFLP (399/45 bp); 10) *Hph*I-RFLP (224/220 bp); 11) *Hae*III-RFLP (198/94/87/32 / 27.6 bp); 12) *Bst*YI-RFLP (253/191 bp).

The PCR-RFLP-genotyping strategy of BLV, which we have improved, is consistent with its phylogenetic classification. The new classification makes it possible to identify all the ten currently known genotypes of the viral pathogen (Table 6).

It should be noted that genotype 1 is characterized by seven combinations of *env*-PCR-RFLP profiles (C 1-7), genotype 2 – by four combinations (C 8-11); genotype 3 – by three combinations (C 12-14); genotype 4 – by nine combinations (C15-23); genotype 5 – by three combinations (C 24-26); genotype 6 – by seven combinations (C27-33), genotype 7 – by fourteen combinations (C 34-47); genotype 8 – by five combinations (C 48-52); genotype 9 – by two combinations (C 53-54); genotype 10 – by 3 three combinations (C 55-57) (Table 6).

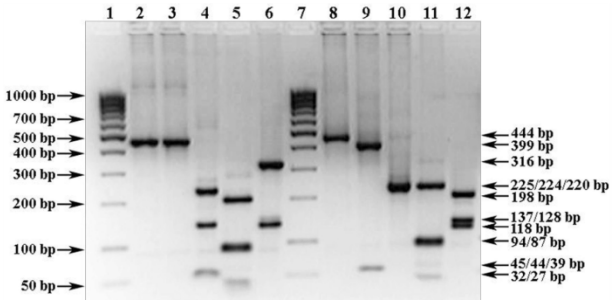

Fig. 4. Electrophoregramme of combinations of PCR-RFLP profiles (C46 and C48) of genotypes 7 and 8 (an improved BLV genotyping strategy).

Legend. 1, 7) DNA markers 100 bp + 50 bp (SibEnzyme). 2–6) PCR-RFLP-profile of the BLV N067 provirus isolate (C46, genotype 7): 2) *Pvu*II-RFLP (444 bp); 3) *Ssp*I-RFLP (444 bp); 4) *Hph*I-RFLP (224/137/44/39 bp); 5) *Hae*III-RFLP (198/94/87/32/27/6 bp); 6) *Bst*YI-RFLP (316/128 bp). 8–12) PCR-RFLP profile of the BLV N006 provirus isolate (C48, eighth genotype 8) *Pvu*II-RFLP (444 bp); 9) *Ssp*I-RFLP (399/45 bp); 10) *Hph*I-RFLP (224/220 bp); 11) *Hae*III-RFLP (225/94/87/32/6 bp); 12) *Bst*YI-RFLP (198/128/118 bp).

It should be emphasized that genotypes 8 and 9 can be easily identified even with the use of one restrictase, *Hae*III, generating RFLP fragments (225/94/87/32/6 bp) that are characteristic of genotype 8; *Hph*I – generating RFLP fragments (224/171/49 bp) that are characteristic of genotype 9. Representatives of genotypes 2 (*Bst*YI and *Pvu*II), 3 (*Hae*III and *Hph*I), and 5 (HphI and PvuII) can be identified with two restriction enzymes (Table 6).

Figs. 2–4 show illustrative examples of the implementation of the strategy of BLV PCR-RFLP-genotyping in accordance with its phylogenetic classification.

As one can see from the electrophoregramme in Fig. 2 (tracks 2–6), the PCR-RFLP profile of the BLV N-1 provirus isolate is identified as combination 1 (C1) of the *env*-PCR-RFLP profile of genotype 1, which includes at least 56 isolates deposited in the GenBank NCBI (Table 6).

The PCR-RFLP profile of the BLV N-4 provirus isolate (Fig. 2, tracks 8-12) characterizes combination 4 (C4) of the *env*-PCR-RFLP profile of genotype 1, with at least 42 identified representatives (Table 6).

The PCR-RFLP profile of the BLV N015 provirus isolate (Fig. 3, tracks 2–6) is identified as combination 17 (C17) of the *env*-PCR-RFLP profile of genotype 4. It includes at least 16 representatives (Table 6), two of which affect cattle in Tatarstan. According to GenBank NCBI, these nucleotide sequences of the *env gene* fragment are isolate N015 (GenBank A/N: KC867143) and isolate N062 (GenBank A/N: KC886615).

The PCR-RFLP profile of the BLV N023 provirus isolate (Fig. 3, tracks 8-12) is identified as combination 18 (C18) of the *env*-PCR-RFLP profile of genotype 4 (Table 6). Its nucleotide sequence of the *env* gene fragment from the GenBank NCBI is the only variant for the given combination (isolate N023, GenBank A/N: KC867149).

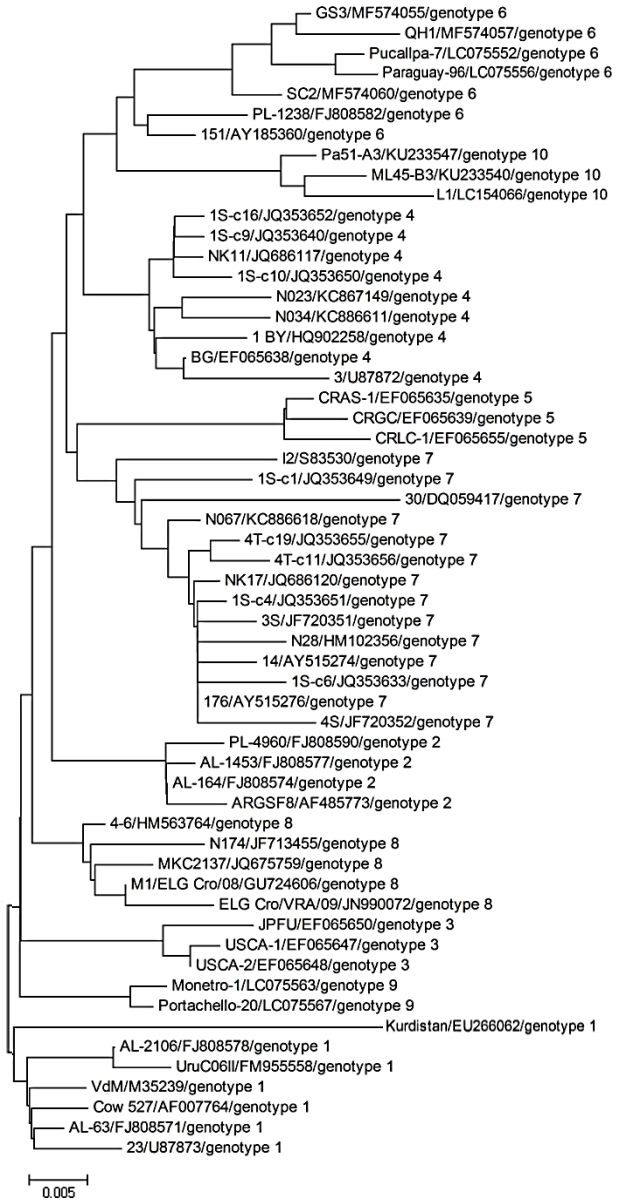

Fig. 5. Phylogramme of 57 reference isolates of the 10 currently known BLV genotypes, built on the basis of a phylogenetic analysis of the *env* gene fragment [MEGA-4: NJ algorithm, 400 nt, 57 seq.].

The PCR-RFLP profile of the BLV N067 provirus isolate (Fig. 4, tracks 2–6) characterizes itself as combination 46 (C46) of the *env*-PCR-RFLP profile of genotype 7 (Table 6). Its nucleotide sequence of the *env* gene fragment from GenBank NCBI is the only one for this combination (isolate N067, GenBank A/N: KC886618).

The PCR-RFLP profile of the BLV N006 provirus isolate (Fig. 4, tracks 8–12) belongs to combination 48 (C48) of the *env*-PCR-RFLP profile of genotype 8. It includes at least 13 representatives (Table 6), three of which affect cattle populations in Tatarstan. According to GenBank NCBI, their nucleotide sequences of the *env* gene fragment are isolate N063 (GenBank A/N: KC886616), isolate N006 (GenBank A/N: KC867140), and isolate N089 (GenBank A/N: KC886624).

The improved strategy of PCR-RFLP-genotyping corresponds with the modern phylogenetic classification of BLV and makes it possible to identify all its known genotypes. Its accuracy is based upon *in silico* modelling of restrictogrammes and the phylogenetic analysis of the *env* gene fragment of 57 reference isolates of the ten known BLV genes (Fig. 5). They produce 57 diagnostically significant genotype-associated combinations of PCR-RFLP profiles.

CONCLUSION

To determine the genotypes of BLV isolates obtained from Tatarstani cattle, we performed a phylogenetic analysis of the *env* gene fragment sequences and a PCR-RFLP analysis that corresponded with the phylogenetic classification of the infectious agent. The genotypic composition of 179 identified BLV isolates detected in cattle from livestock farms of 21 districts of the Republic of Tatarstan was represented by genotypes 1 (10 isolates), 4 (106 isolates), 7 (55 isolates), and 8 (8 isolates). Thus, we state the fact that four out of ten currently known BLV genotypes circulate on the territory of the Republic of Tatarstan, namely genotypes 1, 4, 7, and 8.

After that, we classified the BLV isolates with decoded nucleotide sequences of the *env* gene locus according to the chosen genetic identification strategy. Subsequently, we assessed the degree of consistency of genotypic approaches by comparing *in silico* PCR-RFLP data and the results of the phylogenetic analysis. We used 505 corresponding sequences, including those deposited in GenBank NCBI. As a result, we managed to prove that a number of previously used PCR-RFLP typing strategies were inconsistent with the current approach in assessing the genotypic diversity of BLV with the help of the phylogenetic analysis. The inconsistency of the three PCR-RFLP strategies for BLV typing with the modern phylogenetic classification is associated, among other things, with the on-going knowledge acquisition in the sphere of the genetic diversity of the ten known BLV genotypes.

During the final stage of the research, we improved the strategy of PCR-RFLP-genotyping of BLV to make it consistent with the phylogenetic classification. The new version takes into account the new data about the genetic diversity of BLV. It also includes an interpretation of the PCR-RFLP profiles of 505 BLV isolates. The interpretation resulted from a restriction mapping of the *env* gene fragment according to 5 restriction endonucleases. The improved strategy of PCR-RFLP-genotyping allows one to identify all the currently known BLV genotypes. The improved strategy owes its accuracy to *in silico* modelling of restrictogrammes and the phylogenetic analysis of the *env* gene of 57 reference isolates of ten BLV genes that generate 57 diagnostically significant genotype-associated combinations of PCR-RFLP profiles

CONFLICT OF INTEREST

The authors declare that there are no conflict of interest related to this article.

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