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EXPERIMENTAL WORKS

Phylogeography of *Yersinia pestis* Vole Strains Isolated from Natural Foci of the Caucasus and South Caucasus

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Abstract—Comparative analysis of 57 strains of *Y. pestis* subsp. *microtus* bv. caucasica was carried out using molecular typing. The results obtained indicate the presence of three independent phylogenetic groups and indicate the advisability of isolation of the Leninakan mountain mesofocus from the Transcaucasian highland focus into an independent focus, as well as inclusion of part of the Pre-Araks low-mountain focus as the mesofocus along with the Pre-Sevan mountain and Zangezur–Karabakh mountain mesofoci into the Transcaucasian highland plague focus. It is shown that the strains circulating in the East Caucasus highland focus of plague are the most ancient branch of the caucasica biovar, and possibly of the entire phylogenetic tree of *Y. pestis*.

Keywords: MLVA25-typing, CRISP-typing, VNTR, *lcrV*, *aspA*, *Yersinia pestis*, phylogeography

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The natural plague foci of vole type, the Transcaucasian highland (including Leninakan mountain (04), Pre-Sevan mountain (05), and Zangezur–Karabakh mountain (06)) and East Caucasian mountain (39), were discovered in 1958 and 1977, respectively. Features of the phenotypes of vole strains of *Y. pestis* that circulate there made it possible to isolate them into the subspecies *caucasica*. In addition, similar strains of plague microbe were isolated on the territory of the Pre-Sevan low-mountain focus of gerbil type (07) that border the Transcaucasian highland focus [4]. More recently, Chinese, French, and Russian scientists [3, 12] proposed combining the *Y. pestis* strains of minority subspecies (*altaica*, *caucasica*, *hissarica*, *ulegeica*, *tallasica*, *xillingolensis*, *ginghaiensis*, and *angola*) into a new subspecies, *microtus*, giving them the status of biovars. The proposed terminology is already used in works by German, Mongolian, and French researchers [11]. In this work, we will use this version of intraspecific classification of the causative agent of plague.

Phenotypic methods of differentiation of the causative agent of plague are based on the ability to ferment various substrates, nutritional needs, and virulence to different animal species. Although phenotypic methods allow distinguishing subspecies and biovars of *Y. pestis*, it is impossible to carry out typing at the level of strains with their help. In addition, the instability of the manifestation of phenotypic features and their dependence on experimental conditions, as well as the presence of atypical strains, greatly complicate interpretation of results [1]. In this regard, recently, a decisive role in the typing of the plague microbe is played by molecular-biological methods [13]. With the help of special computer programs, the results of genotyping of strains of one species can be represented as a phylogenetic tree, while clusters of similar genotypes have a distinct geographic location in many species [8]. Previously, we used VNTR-typing of 25 loci (MLVA25) to study the diversity of strains of *Y. pestis* from the natural plague foci on the territory of China with the involvement of a small number of strains circulating in natural foci of CIS [12]. The caucasica biovar was presented in the cited paper by only seven strains from the Transcaucasian highland focus.

This publication describes MLVA25-typing of a set of 57 strains of *Y. pestis* isolated in the natural foci of a vole type of the Caucasus and Transcaucasian. In addition, CRISP-typing of the strains studied was carried out and the nucleotide sequences of genes *aspA* and *lcrV* were determined, while the geographic distribution of phylogenetic groups identified was assessed and division of the enzootic territories into foci was proposed in accordance with phylogeographic data.

MATERIALS AND METHODS

Bacterial strains. We used 57 strains of *Y. pestis* subsp. *microtus* bv. caucasica: 14 strains from Leninakan mountain focus, 7 from the Pre-Sevan mountain focus, 9 from the Zangezur–Karabakh mountain focus, 9 from the Pre-Araks low-mountain focus, and

18 from the East Caucasus mountain plague focus, as well as 2 strains of *Y. pestis* subsp. *pestis* bv. mediaevalis of the Pre-Araks low-mountain plague focus. The strains were isolated in 1958–1996. The bacteria were grown on Hottinger agar supplemented with 1% hemolyzed blood for 48 h at 28°C. The genomic DNA was isolated according to the MU 1.3.1794-03 Organization of Work upon Studies by PCR of Material Infected with Bacteria of I–II Pathogenicity Groups methodological guidelines.

Molecular typing. The primer sequences, the conditions of the polymerase chain reaction (PCR), and registration of the results of MLVA25-typing [12], CRISP-typing [10], as well as sequencing of the genes *lcrV* [7] and *aspA* [9], have been described in detail previously. The published whole genome sequences of the strains *Y. pestis* Pestoides F and *Yershina pseudotuberculosis* IP32953 (access numbers in the GenBank/EMBL/DDBJ: AF167309 and NC_006153.2, respectively) have been used for molecular typing *in silico*.

Analysis of results. The results obtained were introduced into the Bionumerics 5.1 database program. In order to construct dendrograms, we used the Neighbor-Joining method with a categorical factor (the *Y. pseudotuberculosis* IP32953 strain was used as a root species). The titles and numbers of the natural foci of plague are designated in accordance with [4, 6].

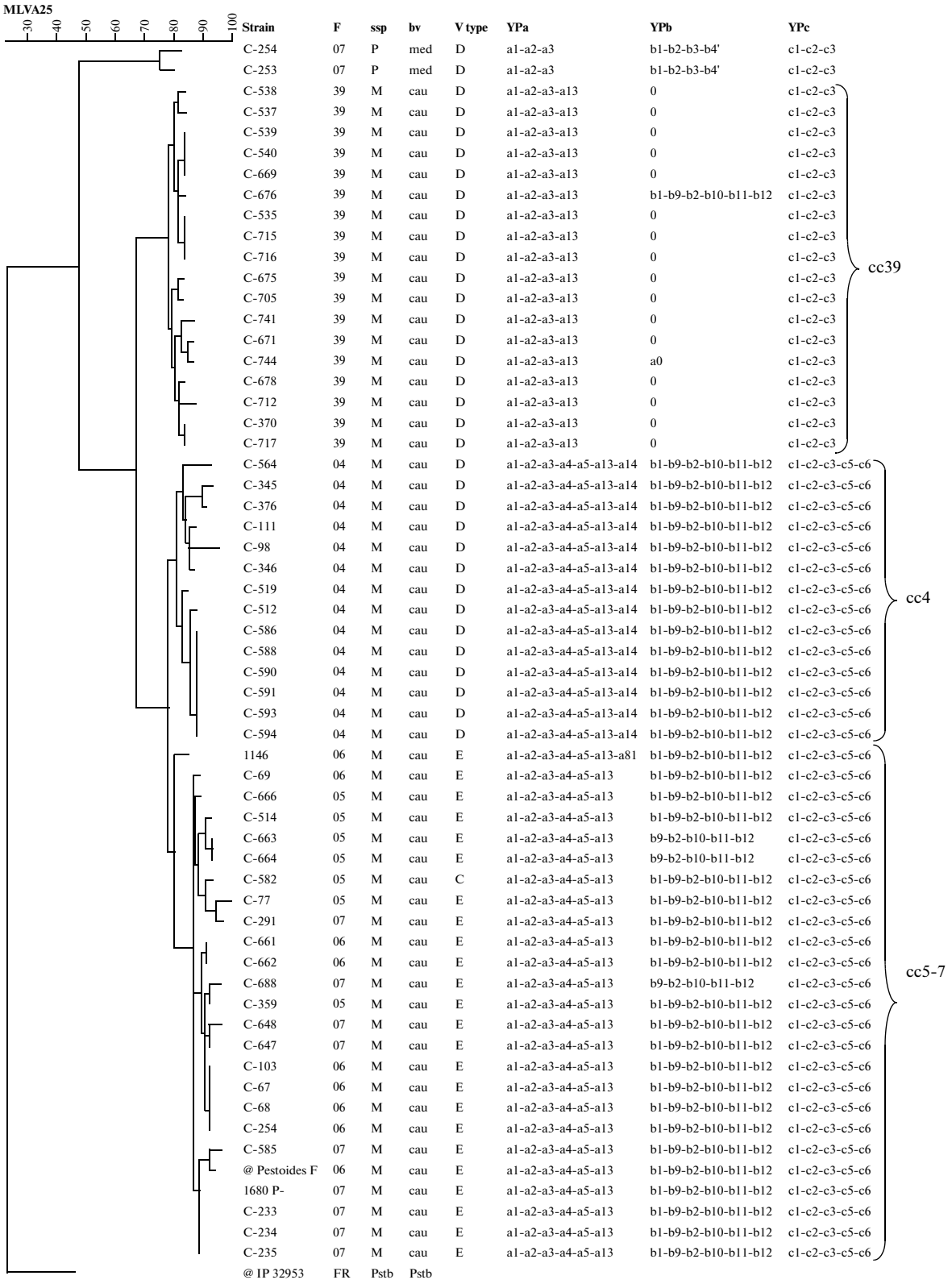
RESULTS AND DISCUSSION

The strains of *Y. pestis* bv. caucasica that we investigated using MLVA25-typing and that were isolated in the Caucasus region formed a single gene cluster (see figure) that preserves its autonomy on the dendrogram, which includes data on MLVA25-types of more than 600 isolates belonging to different intraspecific groups of the plague microbe [3]. The increase in the number of isolates from the natural plague foci 04–07 from 7 described in our previous publication [12] to 39 and inclusion in the study of 18 strains from the 39 focus allowed revealing three independent branches (clonal clusters) in the composition of the bv. caucasica cluster: cc39, cc4, and cc5-7 (see figure) that correspond to the natural foci 39 (Dagestan) and 04 (Armenia and Georgia) and group of foci 05–07 (Azerbaijan and Armenia). The distribution of strains in clonal clusters and subclusters included in their composition did not depend on the time of allocation of the *Y. pestis* cultures in the natural foci. All the isolates from foci 04 and 39 were included only in the composition of respective autonomous and relatively homogeneous clonal clusters cc4 (population 0.PE2b, according to Morelli G. et al., [14]) and cc39 (so far, nobody has conducted SNP-typing of the strains of this group), which may indicate stabilization of biocenotic ties between the populations of warm-blooded animals, insect vectors, and parasite–pathogens and the absence of drift on these territories of the strains of *Y. pestis* from other natural foci of plague. Clonal cluster cc5-7 (population 0.PE2a, according to G. Morelli et al., [14]) is more heterogeneous—its composition includes isolates from two mesofoci 05 and 06 (as part of the Transcaucasian highland natural plague focus) and the adjacent territory of focus 07. It is likely that, during abundance outbreaks of warm-blooded carriers from foci 05–07, their ecological niches overlap, with drift of the strains into the surrounding areas occurring.

For the validation of clustering and subtyping of the studied isolates in the composition of the cluster by the method MLVA25, we used assessment of polymorphism of the nucleotide sequences of the genes *aspA* and *lcrV*.

It is known that, in contrast to *Y. pseudotuberculosis*, the amino acid sequence of aspartase of the strains *Y. pestis* subsp. *pestis* is characterized by the replacement Val363 → Leu363 (GTG → TTG), and for the seven previously studied *Y. pestis* subsp. *microtus* bv. caucasica strains from the natural foci unspecified in pub_lications the substitution was Val363 → Ser363 (GTG → TCG) [2, 9]. The sequencing that we carried out of the gene *aspA* from 56 isolates that were isolated in all the natural foci where the strains of biovar caucasica circulate and the analysis of the nucleotide sequence of the gene *aspA* from the strain Pestoides F confirmed the replacement of GTG → TCG and suggested that it occurred before the isolation of *Y. pestis* subsp. *microtus* bv. caucasica into a separate genetic cluster, as it is characteristic of all three phylogenetic groups within the specified biovar.

It is known that the vole strains are characterized by polymorphism of the amino acid sequence of V antigen [7]. We conducted sequencing of the gene *lcrV* from 18 strains isolated in focus 39 and 14 strains from focus 04, which has shown that all the studied representatives of these phylogenetic groups synthesize V antigen of type D (Lys18, Lys72, Cys273, and Ser324-Gly325-Lys326), which is characteristic for all the strains of the main subspecies (see figure). At the same time, in 38 out of 39 tested strains from group of foci 05–07, we identified type E of the amino acid sequence of V antigen (Lys18, Lys72, Cys273, and Arg324), and in one, C-582, Type C (Asn18, Arg72, Ser273, and Arg324). Taking into account that the Vantigen of type D is closest in amino acid sequence to the same protein of the ancestor of *Y. pestis*, *Y. pseudotuberculosis*, and a total contrast to the classical type D for types E and C, the replacement of the three C-end amino acids (Ser324-Gly325-Lys326) by one (Arg324) is the result of the 16-bp deletion of the 3'-end of the gene *lcrV* caused by the two direct repeats (ATGACACG) [5, 15], we can assume that the youngest branch of the biovar caucasica is a phylogenetic group that is common to Pre-Sevan mountain (05) and the midmountain steppe part of the Pre-Araks low-mountain (07) natural plague focus and the most



Strain	F	ssp	bv	V type	YPa	YPb	YPc
C-254	07	P	med	D	a1-a2-a3	b1-b2-b3-b4'	c1-c2-c3
C-253	07	P	med	D	a1-a2-a3	b1-b2-b3-b4'	c1-c2-c3
C-538	39	M	cau	D	a1-a2-a3-a13	0	c1-c2-c3
C-537	39	M	cau	D	a1-a2-a3-a13	0	c1-c2-c3
C-539	39	M	cau	D	a1-a2-a3-a13	0	c1-c2-c3
C-540	39	M	cau	D	a1-a2-a3-a13	0	c1-c2-c3
C-669	39	M	cau	D	a1-a2-a3-a13	0	c1-c2-c3
C-676	39	M	cau	D	a1-a2-a3-a13	b1-b9-b2-b10-b11-b12	c1-c2-c3
C-535	39	M	cau	D	a1-a2-a3-a13	0	c1-c2-c3
C-715	39	M	cau	D	a1-a2-a3-a13	0	c1-c2-c3
C-716	39	M	cau	D	a1-a2-a3-a13	0	c1-c2-c3
C-675	39	M	cau	D	a1-a2-a3-a13	0	c1-c2-c3
C-705	39	M	cau	D	a1-a2-a3-a13	0	c1-c2-c3
C-741	39	M	cau	D	a1-a2-a3-a13	0	c1-c2-c3
C-671	39	M	cau	D	a1-a2-a3-a13	0	c1-c2-c3
C-744	39	M	cau	D	a1-a2-a3-a13	a0	c1-c2-c3
C-678	39	M	cau	D	a1-a2-a3-a13	0	c1-c2-c3
C-712	39	M	cau	D	a1-a2-a3-a13	0	c1-c2-c3
C-370	39	M	cau	D	a1-a2-a3-a13	0	c1-c2-c3
C-717	39	M	cau	D	a1-a2-a3-a13	0	c1-c2-c3
C-564	04	M	cau	D	a1-a2-a3-a4-a5-a13-a14	b1-b9-b2-b10-b11-b12	c1-c2-c3-c5-c6
C-345	04	M	cau	D	a1-a2-a3-a4-a5-a13-a14	b1-b9-b2-b10-b11-b12	c1-c2-c3-c5-c6
C-376	04	M	cau	D	a1-a2-a3-a4-a5-a13-a14	b1-b9-b2-b10-b11-b12	c1-c2-c3-c5-c6
C-111	04	M	cau	D	a1-a2-a3-a4-a5-a13-a14	b1-b9-b2-b10-b11-b12	c1-c2-c3-c5-c6
C-98	04	M	cau	D	a1-a2-a3-a4-a5-a13-a14	b1-b9-b2-b10-b11-b12	c1-c2-c3-c5-c6
C-346	04	M	cau	D	a1-a2-a3-a4-a5-a13-a14	b1-b9-b2-b10-b11-b12	c1-c2-c3-c5-c6
C-519	04	M	cau	D	a1-a2-a3-a4-a5-a13-a14	b1-b9-b2-b10-b11-b12	c1-c2-c3-c5-c6
C-512	04	M	cau	D	a1-a2-a3-a4-a5-a13-a14	b1-b9-b2-b10-b11-b12	c1-c2-c3-c5-c6
C-586	04	M	cau	D	a1-a2-a3-a4-a5-a13-a14	b1-b9-b2-b10-b11-b12	c1-c2-c3-c5-c6
C-588	04	M	cau	D	a1-a2-a3-a4-a5-a13-a14	b1-b9-b2-b10-b11-b12	c1-c2-c3-c5-c6
C-590	04	M	cau	D	a1-a2-a3-a4-a5-a13-a14	b1-b9-b2-b10-b11-b12	c1-c2-c3-c5-c6
C-591	04	M	cau	D	a1-a2-a3-a4-a5-a13-a14	b1-b9-b2-b10-b11-b12	c1-c2-c3-c5-c6
C-593	04	M	cau	D	a1-a2-a3-a4-a5-a13-a14	b1-b9-b2-b10-b11-b12	c1-c2-c3-c5-c6
C-594	04	M	cau	D	a1-a2-a3-a4-a5-a13-a14	b1-b9-b2-b10-b11-b12	c1-c2-c3-c5-c6
1146	06	M	cau	E	a1-a2-a3-a4-a5-a13-a81	b1-b9-b2-b10-b11-b12	c1-c2-c3-c5-c6
C-69	06	M	cau	E	a1-a2-a3-a4-a5-a13	b1-b9-b2-b10-b11-b12	c1-c2-c3-c5-c6
C-666	05	M	cau	E	a1-a2-a3-a4-a5-a13	b1-b9-b2-b10-b11-b12	c1-c2-c3-c5-c6
C-514	05	M	cau	E	a1-a2-a3-a4-a5-a13	b1-b9-b2-b10-b11-b12	c1-c2-c3-c5-c6
C-663	05	M	cau	E	a1-a2-a3-a4-a5-a13	b9-b2-b10-b11-b12	c1-c2-c3-c5-c6
C-664	05	M	cau	E	a1-a2-a3-a4-a5-a13	b9-b2-b10-b11-b12	c1-c2-c3-c5-c6
C-582	05	M	cau	C	a1-a2-a3-a4-a5-a13	b1-b9-b2-b10-b11-b12	c1-c2-c3-c5-c6
C-77	05	M	cau	E	a1-a2-a3-a4-a5-a13	b1-b9-b2-b10-b11-b12	c1-c2-c3-c5-c6
C-291	07	M	cau	E	a1-a2-a3-a4-a5-a13	b1-b9-b2-b10-b11-b12	c1-c2-c3-c5-c6
C-661	06	M	cau	E	a1-a2-a3-a4-a5-a13	b1-b9-b2-b10-b11-b12	c1-c2-c3-c5-c6
C-662	06	M	cau	E	a1-a2-a3-a4-a5-a13	b1-b9-b2-b10-b11-b12	c1-c2-c3-c5-c6
C-688	07	M	cau	E	a1-a2-a3-a4-a5-a13	b9-b2-b10-b11-b12	c1-c2-c3-c5-c6
C-359	05	M	cau	E	a1-a2-a3-a4-a5-a13	b1-b9-b2-b10-b11-b12	c1-c2-c3-c5-c6
C-648	07	M	cau	E	a1-a2-a3-a4-a5-a13	b1-b9-b2-b10-b11-b12	c1-c2-c3-c5-c6
C-647	07	M	cau	E	a1-a2-a3-a4-a5-a13	b1-b9-b2-b10-b11-b12	c1-c2-c3-c5-c6
C-103	06	M	cau	E	a1-a2-a3-a4-a5-a13	b1-b9-b2-b10-b11-b12	c1-c2-c3-c5-c6
C-67	06	M	cau	E	a1-a2-a3-a4-a5-a13	b1-b9-b2-b10-b11-b12	c1-c2-c3-c5-c6
C-68	06	M	cau	E	a1-a2-a3-a4-a5-a13	b1-b9-b2-b10-b11-b12	c1-c2-c3-c5-c6
C-254	06	M	cau	E	a1-a2-a3-a4-a5-a13	b1-b9-b2-b10-b11-b12	c1-c2-c3-c5-c6
C-585	07	M	cau	E	a1-a2-a3-a4-a5-a13	b1-b9-b2-b10-b11-b12	c1-c2-c3-c5-c6
@ Pestoides F	06	M	cau	E	a1-a2-a3-a4-a5-a13	b1-b9-b2-b10-b11-b12	c1-c2-c3-c5-c6
1680 P-	07	M	cau	E	a1-a2-a3-a4-a5-a13	b1-b9-b2-b10-b11-b12	c1-c2-c3-c5-c6
C-233	07	M	cau	E	a1-a2-a3-a4-a5-a13	b1-b9-b2-b10-b11-b12	c1-c2-c3-c5-c6
C-234	07	M	cau	E	a1-a2-a3-a4-a5-a13	b1-b9-b2-b10-b11-b12	c1-c2-c3-c5-c6
C-235	07	M	cau	E	a1-a2-a3-a4-a5-a13	b1-b9-b2-b10-b11-b12	c1-c2-c3-c5-c6
@ IP 32953	FR	Pstb	Pstb				

NJ-dendrogram of MLVA25-types of strains of *Y. pestis* bv. caucasica

Strain—strain name; @—strain was genotyped *in silico*; F—natural focus number; ssp—subspecies (P—*pestis*, M—*microtus*); bv—biovar (med—mediaevalis, cau—caucasica); V type—type of V antigen [6]; YPa (a1-a2-a3, etc.), YPb (b1-b-b3, etc.), and YPc (c1-c2-c3, etc.)—names (profiles of spacers) of CRISP loci [10].

ancient is the population of strains that circulate in the East Caucasus highland focus (39) and that diverge, according to the data of MLVA25-typing, from clonal clusters cc4 and cc5-7 until the last division.

Summarizing the results obtained in the early stages of the research, we may note that MLVA25-typing, in conjunction with sequencing of the genes *lcrV* and *aspA*, allowed revealing three branches within the cluster bv. caucasica. These branches correspond to populations of *Y. pestis* that circulate in natural foci 39 (Dagestan, the most ancient) and 04 (Armenia and Georgia) and group of foci 05–07 (Azerbaijan and Armenia, the youngest). These data suggest that mesofoci 05 and 06 and the adjacent part of focus 07 can indeed be regarded as a single natural plague focus—the Transcaucasian highland focus—and the Leninakan mountain focus must be provided with an independent status, but a final decision on the change of the status of the latter requires verification of the results of MLVA25-typing and determination of the nucleotide sequence of the gene *lcrV* by other molecular typing methods (SNP-, CRISP-typing, etc.).

The correctness of the division of the strains bv. caucasica into clonal clusters cc4, cc5-7, and cc39 was further tested using CRISP-typing, which includes in the analysis more than 130 marker–spacers [10]. As can be seen from the figure, the spacer profiles characteristic of the strains studied completely confirmed the clusterization carried out using MLVA-25 typing. The spacer profiles of the locus YPa allowed distinguishing four possible clonal clusters: cc4, cc5-7, cc39, and strains *Y. pestis* subsp. *pestis* bv. mediaevalis from plague focus 07. The spacer profiles of the locus YPb coincided only in bacteria from clonal clusters cc4 and cc5-7. The spacer profiles of the locus YPc provided separation of all the investigated strains only into two groups: clonal clusters cc4 and cc5-7, *Y. pestis* subsp. *pestis* bv. mediaevalis and cluster cc39.

In cluster cc5–7, strain 1146 stands alone. According to MLVA25-typing, it is part of group cc5-7, but is not clustered with other subclusters of the group, and has a unique profile of spacers of the locus YPa (the set of spacers a1-a2-a3-a4-a5 has spacer a81 added to it, which to date has been identified only in one strain). It should be noted the strain line used in our work was used for more than 30 years as the test culture for identification of pesticin production [1, 4, 6], subjecting it to constant reseeding on artificial nutrient media. At the same time, the other strains that we studied were obtained from collections of microorganisms of antiplague institutes, where during storage they were subjected to a minimum number of reseedings and, therefore, are closest to the original natural isolates.

Thus, the results of using MLVA25- and CRISP-typing in conjunction with assessment of polymorphism of the genes *aspA* and *lcrV* suggest that strains of *Y. pestis*, which circulate in the East Caucasus highland focus, are the most ancient branch of the caucasica biovar and possibly of the entire phylogenetic tree of *Y. pestis*. In our view, the results obtained are sufficient ground for isolation of the Leninakan mesofocus from the composition of the Transcaucasian into an independent one, as well as inclusion of part of the Pre-Araks low-mountain focus in the status of a mesofocus along with the Pre-Sevan and Zangezur–Karabakh mountain mesofoci in the consist of the Transcaucasian highland focus.

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