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EXPERIMENTAL ARTICLES

Characterization of Twelve *Erwinia amylovora* Bacteriophages

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Abstract—*Erwinia amylovora* bacteriophages are of interest as fire blight control agents. This paper presents data on the biology and molecular genetic properties of 12 *E. amylovora* tailed bacteriophages. Genome sequences of seven of them were determined and the phages were identified as the representatives of *Caudoviricetes*; *Vequintavirinae*, *Ounavirinae* and *Autographiviridae* families. The bacteriophages studied were active against *E. amylovora*, *Pantoea agglomerans* and *Pantoea ananatis* strains. The myovirus Hena1 had the narrowest host range lysing 12% of the bacterial cultures tested, the remaining myoviruses—had broader host ranges (56%) for this collection. The phages showed different reactions to the presence of a chelating agent in the cultivation medium. A significant proportion of phage-resistant *E. amylovora* cells were found both in infected liquid cultures and in the experiments on the plates with the phage agar (more than 20% in case of exposure to podoviruses), which correlates with the data of limited number of previous investigations of the phenomenon for *E. amylovora* interactions with phages. High prevalence of resistant cells in the host cultures suggest that they are formed not due to spontaneous mutations but result from another phenotypic or genetic dissociation mechanism(s) that remains to be identified.

Keywords: fire blight, *Erwinia* bacteriophages, phage-resistance, *Autographiviridae*, *Caudoviricetes*, *Molineuxvirinae*, *Ounavirinae*, *Studiervirinae*

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E. amylovora bacteriophages have been of scientific and practical interest for more than half a century (Billing, 1960; Erskine, 1973; Gill et al., 2003; Schwarczinger et al., 2017). These viruses were studied as potential fire blight control agents (Nagy et al., 2012; Doffkay et al., 2015). The ability of phages to suppress the development of plant diseases was determined (Boulé et al., 2011; Nagy et al., 2015) and the experiments on phage engineering to increase the effectiveness of control were conducted (Born et al., 2017). Special attention was drawn to the carbohydrate-degrading enzymes of *E. amylovora* bacteriophages, in particular, EPS depolymerase (Sule et al., 2002; Born et al., 2014; Latka et al., 2017). To date, genetic data on *E. amylovora* bacteriophages were accumulated and a number of genome sequences was published (Dömötör et al., 2012; Lagonenko et al., 2015; Esplin et al., 2017; Knecht et al., 2018).

E. amylovora bacteriophages were isolated from the soil near fruit trees at the sites where fire blight was reported (Erskine, 1973; Schnabel et al., 2001; Gil et al., 2003; Boule et al., 2011; Yagubi et al., 2014; Buttimer et al., 2018; Akremi et al., 2020; Park et al., 2022), from the aerial parts of apple, pear, raspberry, quince, cherry, hawthorn both with and without disease symptoms (Ritchie and Klos, 1977; Gill et al., 2003; Schnabel and Jones, 2001; Muller et al., 2011b; Samoilova and Leclerque, 2014; Gasic et al., 2014; Lagonenko et al., 2015; Esplin et al., 2017; Schwarczinger et al., 2017; Sharma et al., 2018; Akremi et al., 2020), as well as from water samples (Gasic et al., 2014; Park et al., 2022).

Many bacteriophages isolated using *E. amylovora* strains are also capable of infecting bacteria of other species. There are closely related species (family *Erwiniaceae*): *Pantoea agglomerans* (Gill et al., 2003; Born et al., 2011; Boule et al., 2011; Muller et al., 2011b; Yagubiet al., 2014; Lagonenko et al., 2015; Schwarczinger et al., 2017; Buttimer et al., 2018; Sharma et al., 2019; Gayder et al., 2020), *Pantoea ananatis* (Born, 2011), *Pantoea vagans* (Born et al., 2011; Schwarczinger et al. 2017; Arens et al., 2018; Buttimer et al., 2018; Sharma et al., 2019), *Erwinia pyrifoliae* (Muller et al., 2011b; Park et al., 2018, 2022; Kim et al., 2020), *Erwinia billingiae* (Born et al., 2011; Muller et al., 2011b; Schwarczinger et al., 2017),

Erwinia persicina (Born et al., 2011; Schwarczinger et al., 2017), *Pantoea stewartii* (Muller et al., 2011b; Schwarczinger et al., 2017), *Erwinia rhapontici* (Schwarczinger et al., 2017), *Erwinia gerundensis* (Gayder et al., 2020), and *Serratia marcescens*, a representative of the *Yersiniaceae* family (Kim et al., 2020). In a bacteriophage susceptibility screening experiment using a large number of *E. amylovora* and *E. pyrifoliae* isolates, bacteriophages of a wide range of hosts were detected by qPCR. Thus, nine of the studied bacteriophages increased the number of their genomes by at least one order of magnitude in 88% of the 106 studied bacterial strains (Gayder et al., 2019).

Particles of isolated *E. amylovora* bacteriophages are represented by four morphotypes (Bradley, 1965): in addition to the large group of tailed bacteriophages A (Gill et al., 2003; Lehman, 2009; Born et al., 2011; Boule et al., 2011; Muller et al., 2011b; Lagonenko et al., 2015; Schwarczinger et al., 2017; Knecht et al., 2018; Gayder et al., 2019), B (based on attitude to siphoviruses) (Meczker et al., 2014), and C (Gill et al., 2003; Born et al., 2011; Boule et al., 2011; Muller et al., 2011b; Schwarczinger et al., 2017; Knecht et al., 2018; Park et al., 2018; Gayder et al., 2019) morphotypes, filamentous bacteriophages of the F morphotype were recently discovered (Akremi et al., 2020). Among the bacteriophages of *E. amylovora*, giant bacteriophages "jumbo" are described (Arens et al., 2018; Buttimer et al., 2018; Sharma et al., 2019; Kim et al., 2020). *E. amylovora* phage vB_EamM_Y3 possesses atypical 21 whisker-like structures along the surface of the contractile tail (Buttimer et al., 2018).

The isolated *E. amylovora* bacteriophages have a prevailing type of genomic nucleic acid, linear dsDNA (Born et al., 2011; Esplin et al., 2017; Meczker et al., 2014; Lagonenko et al., 2015; Muller et al., 2011a; Arens et al., 2018; Buttimer et al., 2018; Knecht et al., 2018; Sharma et al., 2018, 2019). However, filamentous *E. amylovora* bacteriophage PEar has a circular single-stranded DNA genome that is converted into a double-stranded replicative form in the host cell (Akremi et al., 2020).

E. amylovora bacteriophage genomes have various sizes and numbers of predicted ORFs. The genomes of the so-called giant jumbo bacteriophages *E. amylovora* range in size from 235 to 275 kb and the number of annotated ORFs ranges from 243 to 333 (Arens et al., 2018; Buttimer et al., 2018; Sharma et al., 2019). The tiny genomes of *E. amylovora* PEar bacteriophages range in size from 6608 to 6801 with predicted 10 or 11 ORFs (Akremi et al., 2020).

Bacteriophage genomes often have a modular organization represented by gene clusters specialized in functions. For example, the PEar bacteriophage genome contains the following modules: the replication module, the structural module, the assembly module and the regulator module. At the end of the genome, below gene IV, there is an intergenic region, a characteristic feature of filamentous phages. The IV gene encodes an integral outer membrane protein required for virion export (Akremi et al., 2020).

Such a modular mosaic can serve as a driving force for adaptation to new hosts during genetic recombination. The following feature is observed in the genomes of bacteriophages *E. amylovora* L1, M7, S6 and Y2, which have cluster organization. Most of the late genes have a significant level of homology between enterobacterial and these bacteriophages; however, they have gaps with a low level of similarity. These gap regions are located mainly in the region encoding tail proteins, suggesting that host specificity is associated with differences in the sequence of tail proteins (Born et al., 2011). However, bacteriophage genes are not always organized into discrete functional clusters, as in the bacteriophage *E. amylovora* PhiEaH1 (Meczker et al., 2014).

Various packaging mechanisms and features of the physical ends of the genomes were described for *E. amylovora* bacteriophages. Thus, the genomes of bacteriophages L1, M7, S6, and Y2 have direct terminal repeats (DTRs) ranging in size from 172 to 2.608 bp (Born et al., 2011). The genome of bacteriophage vB_EamP-S2 has DTRs (297 bp) and bacteriophage vB_EamM-Bue1 has a circularly permuted/terminally redundant genome (Knecht et al., 2018). For the bacteriophages *E. amylovora* RAY, MadMel, Desertfox, Bosolaphorus, Simmy50, and Mortimer, according to PhageTerm analysis and terminase homology, the "full head" packaging mechanism of viral DNA in the absence of the pac site was established (Sharma, 2019). Bacteriophage vB_EamM_Y3 has a circularly permuted genome with terminal repeats (Buttimer et al., 2018). The "full head" packing mechanism was proposed for the bacteriophages Joad and RisingSun (Arens et al., 2018).

The analysis of genomic data is of direct importance in the selection of bacteriophages for therapeutic purposes. To detect target groups of bacteriophages among several isolates, PCR of conservative molecular markers, for example, the sequence of the major capsid protein (Born et al., 2019) or the large subunit of the viral terminase (Samoilova and Leclerque, 2014) of bacteriophages *E. amylovora*, can be used. In addition, bacteriophages involved in horizontal gene transfer may be a reservoir of unwanted genes, such as toxins or bacterial virulence factors. Thus, analysis of the genomes of *E. amylovora* bacteriophages revealed a gene homologous to *amsF*, which is responsible for the biosynthesis of amylovoran by the bacterium *E. amylovora* (bacteriophage phiEaP-8) (Park et al., 2018). The *amsF* gene was also found in the genome sequence of the bacteriophage PhiEaH2 (Dömötör et al., 2012).

The tRNAs are encoded by many bacterial viruses and some of these phage tRNAs were found to have various sequence and structural "anomalies." For *E. amylovora* bacteriophages, nontrivial structures of

nucleic acids were also described. For example, using the tRNAscan-SE program, it was predicted that phiEa21-4 bacteriophage tRNAs have abnormal anticodon loops: instead of 7 bases, tRNAs have loops of 6 and 9 bases. Note that functional tRNAs with eight- and nine-basic anticodon loops were previously constructed, and wild-type tRNAs with nine-base anticodon loops were found in *Astasia longa* flagellates and the yeast *Schizosaccharomyces pombe* (Lehman et al., 2009).

Accumulated genomic data allow us to refine the classification and phylogenetic relationship among bacteriophages. However, the classification of viruses is not a trivial task because of constant rearrangements within the genomes of natural virus populations. A significant relationship was found between individual bacteriophages of *E. amylovora* and bacteriophages of genetically distant host bacteria, for example, *Pseudomonas*, *Vibrio*, *Salmonella*, *Serratia*, *Shigella* and *Dickeya* (Whichard et al., 2010; Lagonenko et al., 2015; Arens et al., 2018; Buttimer et al., 2018).

Despite considerable amount of data has been accumulated on *E. amylovora* phages, a large proportion of virus genes lack functional characteristics. For example, for two *Erwinia* bacteriophages Joad and RisingSun with genomes larger than 200 kb, BLASTP analysis of the putative proteome showed the presence of homologous proteins in the database for only 57% of the proteins of two bacteriophages, and 33% of them have an unknown function (Arens et al., 2018). Moreover, to date, there is a few studies of lysogeny in bacterial cultures of *E. amylovora* (Erskine, 1973; Roach et al., 2015) and problems of phage resistance of *E. amylovora* (Knecht et al., 2022).

Here we announce the data on the biology of 12 *E. amylovora* bacteriophages isolated in Belarus. We present information on the results of *E. amylovora* infection with the bacteriophage in the laboratory conditions, which is important for understanding the potential for use in plant protection. We have demonstrated a different ability of *E. amylovora* 1/79Sm to form phage-resistant variants, observed in several variants of experiments on studying the *E. amylovora* bacterial culture growth. For 7 bacteriophages, the sequences of their genomes can be found in the GenBank database. In this connection, the data in this article may be compared or extrapolated to previously known *E. amylovora* bacteriophages.

MATERIALS AND METHODS

Bacterial Strains and Bacteriophages and Their Cultivation

The study was conducted using both phytopathogenic and nonpathogenic bacterial strains. These include 12 *E. amylovora* strains: 1/79Sm (Germany, spontaneous streptomycin resistant mutant of 1/79, *Cotoneaster* sp., 1979) (Bellemann et al., 1994), E2, E3, E4, E5, L-3-1, L-3-2, L-3-5, L-3-6, L-3-8 (Belarus, isolates from *Malus* sp.; *Pyrus communis*, 2007—2009) (Lagonenko et al., 2011); 133/95 (Poland, *Cydonia* sp., 1985) (Lagonenko et al., 2011), D4 (mini-Tn5XylE transposon mutant of E2); 9 *Pantoea* sp. strains (Belarus, isolates from different plants, 1980—1981): *P. agglomerans* 194, 197, 198, 216, 219, 220 and 246, *Pantoea ananatis*, 208 and 245; 2 *E. coli* strains: B and DH5α; *Pectobacterium carotovorum* 14a (Belarus); *Pectobacterium atrosepticum* 36A (Belarus, isolated from *Solanum tuberosum*, 1978).

Cultivation of microorganisms was performed in lysogeny broth (LB) medium (10 g tryptone, 10 g NaCl and 5 g yeast extract) or on lysogeny broth agar (LA). LA was supplemented with 15 g L^{-1} of Bactoagar (Amresco) for the plates or with 7 g L^{-1} for the top soft agar layer of double agar overlay assay.

To study the bacteriophage host range and the phage ability to lyse the *E. amylovora* 1/79Sm bacterial culture, we also used Peptone Yeast Extract Broth (10 g peptone, 8.5 g NaCl and 5 g yeast extract) or Peptone Yeast Extract Agar supplemented with 15 g L^{-1} or 7 g L^{-1} of the American Bacteriological Agar (Industrias Roko S.A.).

Isolation of Bacteriophages

For bacteriophages isolation, we used enrichment cultures with *E. amylovora* 1/79Sm strain as described in (Besarab, 2022). Bacteriophages were purified by five sequential single plaque isolation.

Cryopreservation of Bacteriophages

The bacteriophage lysates were added to sterile Eppendorf 1.5 mL tubes with the supplementation of 10% glycerol as a cryoprotectant and placed into a low-temperature refrigerator at −70°C.

Assessment of Bacteriophage Sensitivity to Chloroform

To study the effect of chloroform on the viability of bacteriophages, an equal volume of chloroform was added to phage lysates with an average titer of 10^8–5×10^8 PFU mL^{-1} (myoviruses) or 10^7 PFU mL^{-1}(podoviruses) and left for 1 h with shaking at 28°C, after that the bacteriophage titration was performed.

Transmission Electron Microscopy (TEM) Analysis

TEM study of bacteriophages was conducted as described in (Kulikov, 2014). The average virion sizes were determined based on 6–14 measurements of the capsid diameter using 4–9 viral particles.

Host Range Determination

Host ranges were determined by a spot-test. A 10 μL drop of the phage lysate (10^7 PFU mL^{-1}) was spotted onto plates prepared with the bacterial strains. After overnight incubation at 28°C, we assessed the presence of lysis zones in the places where drops of bacteriophages were applied. Positive results were further verified by titration using a standard double agar overlay plaque assay.

The Study of Culture Growth upon Infection with Bacteriophages

To study the growth of the bacterial culture upon infection with bacteriophages in liquid nutrient medium, we analyzed the OD_{600} of *E. amylovora* 1/79Sm culture according to the methods (Nagy et al., 2014; Schwarczinger et al., 2011) with modifications. In eight test tubes with 5 mL of LB, 0.1 mL of the bacterial culture (OD_{600} = 1.0) was added. In seven of them, 0.1 mL of phage lysates of various titers (from 10^2 to 10^8 PFU mL^{-1}) was added. After 17 h of incubation, we measured the OD_{600} of the bacterial culture. The decrease in the growth of the bacterial culture was calculated by the difference between the mean values of OD_{600} of the control and OD_{600} of the bacteriophage-infected culture, divided by the mean value of OD_{600} in the control.

To calculate CFU and PFU, as well as the growth curve of a bacterial culture based on the measurement of OD_{620}, we prepared a suspension of *E. amylovora* 1/79Sm culture in the log phase (OD_{600} of culture 0.25), phage lysate (titer 10^8 PFU mL^{-1}) and fresh nutrient medium LB in a ratio of 10 : 3 : 27, mixed and incubated 20 h at 28°C. Graphing of bacterial culture growth was carried out using Thermo Scientific Multiskan FC photometer. For the study of culture growth upon infection with bacteriophages in the presence of sodium citrate, we used the LB nutrient medium without and with the addition of sodium citrate.

Study of Culture Growth upon Infection with Bacteriophages in the Presence of Sodium Citrate

The effect of citrate on the ability of bacteriophages to lyse a bacterial culture is judged by the titer of phage lysates, the number of negative colonies when inoculated on a medium with the addition of citrate, and the presence of lysis zones on the lawn of a bacterial culture in the presence of citrate (Gabrilovich, 1968). In this work, the effect of citrate on bacteriophage infection was judged by the bacterial culture growth rate, as well as by PFU and CFU counts in the bacterial culture mixed with phages as described above ("Study of culture growth upon infection with bacteriophages," the second paragraph).

Assessment of the Frequency of Occurrence of Phage-Resistant Variants in Bacterial Culture

To study the frequency of occurrence of phage-resistant variants in *E. amylovora* 1/79Sm bacterial culture upon infection with bacteriophages, the culture was inoculated in the logarithmic growth phase on two-layer agar plates, where the upper soft layer contained 5×10^8 PFU of bacteriophage, or also without a bacteriophage (control plates). After two-day incubation at 28°C, we counted the number of bacterial colonies on plates with bacteriophage and on control plates.

Bacteriophage DNA Sequencing and Analysis

Bacteriophage genomic DNA was isolated by phenol-chloroform extraction as described in (Sambrook and Russel, 2001). Genomic DNA was fragmented to an average size of 200–300 bp using the Covaris S220 system (Covaris, Woburn, Massachusetts, USA). The barcoded KAPA Shotgun Library Preparation Kit (KAPA Biosystems) was then used. Emulsion PCR was performed using the One Touch system (Life Technologies). Beads were prepared using One Touch 2 and Template Kit v2, and sequencing was performed using Ion Proton 200 Sequencing Kit v2 and P1 Ion chip. DNA was sequenced using an Ion Torrent Proton sequencer system (Applied Biosystems, USA) according to the manufacturer's instructions. The primary assembly was performed using Newbler version 2.9, resulting in a single contig for each bacteriophage. For the bacteriophage genomes announced here, the coverage was ×203 (Stepyanka) and ×84 (Roscha1).

Genome annotation was performed by Prokka software (Seemann, 2014) with further manual curation. Functional annotations were performed using NCBI BLASTx searches, and tRNA gene prediction was accomplished using tRNAscan-SE v. 2.0 (Lowe and Chan, 2016) and ARAGORN v1.2.41 (Laslett and Canback, 2004). Bacteriophage DNA termini and packaging mechanism were determined by PhageTerm (Garneau et al., 2017). Genetic and physical maps of the bacteriophage genome were generated using Proksee (Grant and Stothard, 2008). The automatic generation of gene cluster comparison figures was performed using clinker (Gilchrist and Chooi, 2020). Hena1 DNA isolation, sequencing and analysis were described in (Besarab et al., 2020).

PCR Detection of the Bacteriophages

For specific amplification of the bacteriophage genome sequences, PCR was performed using the primers shown in Table 1.

Amplification reactions were carried out using active precise control mode and the following temperature-time profile. Initial denaturation for 5 min at 95°C was followed by 32 cycles of denaturation at 95°C

Table 1. Primer sequences used for amplification of specific regions of the genomes of *E. amylovora* bacteriophages

Bacteriophage	Target gene	F 5'-3', annealing temperature	R 5'-3', annealing temperature	Specificity
Hena2	Major capsid protein	H2mcpFw: GCAAATGGCTCACGTTTCC 56°C	H2mcpRw: GTGAACTTACCGTTGTACTGACG 57°C	Hena2 (794 bp)
VyarbaL	Major capsid protein	McpFw: GGATTGCACCTTCGGCTAAG 57°C	McpRw: CGTATTGACTCAACCAGCGG 57°C	VyarbaL (959 bp)
Micant	Tail tubular protein B	MicTTPFw: CTTCAGTAGCCGCCATAACG 57°C	MicTTPRw: CACCACGTTAGACGCAGC 57°C	Micant (787 bp)
Loshitsa2	Tail tubular protein B	L2TTPFw: CTCTCCCGCTGGGATTAGC 57°C	MicTTPRw	Loshitsa2 (625 bp)
Stepyanka	Major capsid protein	StMcpFw: GACGCAGACCACCATGAC 56°C	StMcpRw: GCTGGTGAGAACCTCGATG 56°C	Stepyanka (775 bp)
Hena1	Major capsid protein	H1McpFw: CGCAACCCTGGAAATCGTTC 58°C	H1McpRw: CGATACCACGAGGCAGGAC 58°C	Hena1 (779 bp)
Roscha1	Major capsid protein	RosMcpFw: CCAACCTCTCAGACCTCTGTTC 57°C	H2mcpRw	Roscha1 (692 bp)

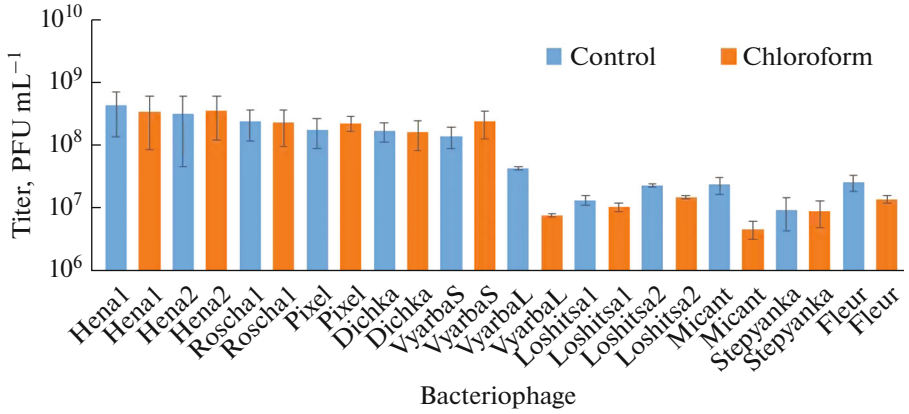

Fig. 1. Sensitivity of *E. amylovora* bacteriophages to chloroform. Note: Error bars reflect standard deviation.

for 30 s, annealing at a temperature determined for each pair of primers (Table 1) for 30 s, elongation at 72°C for 1 min, chain completion for 5 min at 72°C; cooling down to 12°C.

RESULTS AND DISCUSSION

Isolation of Bacteriophages from Natural Sources

We isolated *E. amylovora* bacteriophages from soil samples collected in 2017–2018 in Belarus. The site and source of bacteriophage isolation are presented in Table S1.

According to the plaque morphology, we splitted our isolates into four groups. Hena1 has punctate plaques; Hena2, Roscha1, Pixel, Dichka, VyarbaS—plaques ranging in size from punctate to 2 mm in diameter, with rough edges; Loshitsa1—2.5 mm plaques with a clear smooth edge; Loshitsa2, Micant, Stepyanka, Fleur, VyarbaL—3–3.5 mm plaques with a well-marked areole. Notably, the lysis zones for the listed bacteriophages on a lawn of *E. amylovora* 1/79Sm are turbid. This indicates incomplete lysis of the bacterial culture. In a series of subsequent experiments, we demonstrated the presence of viable bacterial cells resistant to the bacteriophage used for treatment (see the section "Study of the phage ability to lyse the *E. amylovora* 1/79Sm bacterial culture in liquid nutrient medium").

The Assessment of the Viability of Bacteriophages after Cryopreservation

Bacteriophages were cryopreserved at –70°C for long-term storage. After 5 days of storage, bacteriophages remained viable: the titer dropped within one order of magnitude for 5 myoviruses, and no statistically significant difference in titer was found for the rest of the bacteriophages (Fig. S1).

Bacteriophage Sensitivity to Chloroform

Chloroform is used to inhibit the growth of bacteria in solutions, however, the effect on bacteriophages is variable (Basdew and Laing, 2014; Cotton and Lockingen, 1963). Notably, 30% of bacteriophages are sensitive to chloroform (Łobocka et al., 2018). For this reason, we investigated the sensitivity of new bacteriophages to chloroform (Fig. 1).

We didn't observe a significant decrease in the phage titer after the chloroform treatment. For only 3 out of 12 phages (VyarbaL, Micant, and Fleur) a slight drop of the titer by less than one order of magnitude was observed after the chloroform treatment.

Thus, bacteriophages of the resulting collection retain their viability under laboratory conditions, which are important for scientific and industrial use.

Phage Particle Morphology

We examined all the bacteriophage isolates using TEM (Table 2 and Fig. 2). According to the data obtained, we found two Bradley (Ackermann and Eisenstark, 1974) morphotypes, A1 and C1, or myoviruses and podoviruses, in the bacteriophage collection, which are tailed bacteriophages with an isometric head. Tailed phages comprise one of the largest virus group, which dominate sequence and isolate collections; moreover, the isometric type of symmetry of the phage head is predominant (Ackermann, 1998; Kauffman et al., 2018; Luque, et al. 2020).

Tailed bacteriophages possess common principles of structural organization and it determines the presence of a set of semi-conserved genes responsible for virion architecture and assembly (capsid, portal, terminase, major tail and sheaths proteins) (Lopes et al., 2014). Great diversity in phage structure should be expected in the future study in the head-to-tail connection (Lopes et al., 2014) and host-binding structures such as tail fibers, tail spikes and tail tips (Nobrega et al., 2018).

Table 2. Morphology of *E. amylovora* bacteriophage particles

Bacteriophage	TEM	Bradley morphotype (Ackermann and Eisenstark, 1974)	Head, nm	Tail, nm
Hena1 (Besarab et al., 2020)	JEOL JEM-1400	A1	72.36 ± 5.38	126.28 ± 5.27
Hena2	JEOL JEM 2100	A1	69.51 ± 1.47	104.30 ± 2.01
Roscha1	JEOL JEM 2100	A1	69.19 ± 2.27	105.81 ± 3.52
Pixel	JEOL JEM 2100	A1	64.10 ± 3.45	106.67 ± 5.84
Dichka	JEOL JEM 2100	A1	67.35 ± 3.98	107.08 ± 2.50
VyarbaS	JEOL JEM 2100	A1	72.28 ± 1.86	106.96 ± 2.60
VyarbaL	JEOL JEM-1400	C1	55.91 ± 6.21	–
Loshitsa2 (Besarab et al., 2022)	JEOL JEM 2100	C1	59.80 ± 2.60	–
Micant (Besarab et al., 2022)	JEOL JEM 2100	C1	56.39 ± 2.69	–
Stepyanka	JEOL JEM 2100	C1	61.15 ± 1.87	–
Fleur	JEOL JEM 2100	C1	54.19 ± 2.38	–

Bacteriophage Host Range

The largest proportion of variable phage genes is involved in the adaptation of bacteriophages to diverse hosts (host recognition, DNA methylation) (Bellas et al., 2020). In this regard, screening for the host specificity of phage isolates will allow differentiation within the collection in addition to assessing the potential for plant pathogen control.

We studied the host ranges of new *E. amylovora* bacteriophages (Table 3). Bacteriophages differ in their specificity and, in addition to the host bacteria *E. amylovora*, lyse *P. agglomerans* and *P. ananatis* strains. This feature makes it possible to produce bacteriophages on nonpathogenic strains for practical application and research.

Five myoviruses demonstrated the highest *E. amylovora* sensitivity index (100% of studied strains were susceptible). The *E. amylovora* sensitivity index for podoviruses was up to 50%. Myovirus Hena1 had the lowest host range, and therefore was not used in further experiments on growth suppression of the *E. amylovora* bacterial culture.

Study of the Phage Ability to Lyse the E. amylovora 1/79Sm Bacterial Culture in Liquid Nutrient Medium

We investigated the ability to control the number of *E. amylovora* bacteria in a liquid medium for 11 bacteriophages capable of producing high titers of phage lysates. In the experiments, we observed two types of interactions between bacteriophages and bacterial cultures. The type of bacteriophage interaction correlated with the particle morphology of the phages in our collection.

Previously, we showed that upon infection with Hena2, Roscha1, Pixel, Dichka, and VyarbaS myoviruses, a higher titer of the introduced bacteriophage corresponded to a lower value of biomass accumulation (optical density), however, complete cessation of culture growth (visible lysis) of bacteria was not observed in any case. Thus, when the culture was infected (titer of phage lysate 10^8 PFU mL^{-1}), a decrease in the OD_{600} of the bacterial culture was an average of up to 90.1% (Besarab et al., 2021). In the experiment with podoviruses presented here, we observed no correlation between the dose of the introduced podoviruses and the value of biomass accumulation (optical density) of the bacterial culture (Fig. S2). When the culture was infected (titer of phage lysate 10^8 PFU mL^{-1}), a decrease in the OD_{600} of the bacterial culture was observed on average by 29.8% for VyarbaL bacteriophage, 52.1% for Loshitsa1, 44.8% for Loshitsa2, 45.5% for Micant, 33.0% for Stepyanka, 18.7% for Fleur.

Further, we investigated the growth of phage-infected cultures over time in plates with automatic registration of optical density using a plate reader-incubator and determined the concentrations of bacterial cells and bacteriophages in infected cultures after overnight incubation. We conducted bacterial culture and bacteriophage incubation in LB medium without additives and with the addition of sodium citrate. Citrate is a chelating agent that binds metal ions. The sensitivity of bacteriophages to the presence of citrate in the nutrient medium may indicate the need for divalent metal ions as adsorption cofactors (Gabrilovich, 1968). We analyzed the growth of bacterial culture with the addition of sodium citrate at concentrations of 0.1 and 1% (Figs. 3 and 4).

From plots of bacterial culture growth in a plate reader upon phage infection, we observed lower rates of bacterial culture growth in a medium without the addition of sodium citrate in the case of myoviruses. For podoviruses, the addition of sodium citrate to the medium did not affect the growth rate of the bacterial culture, and therefore the process of infection of bacteria by bacteriophages.

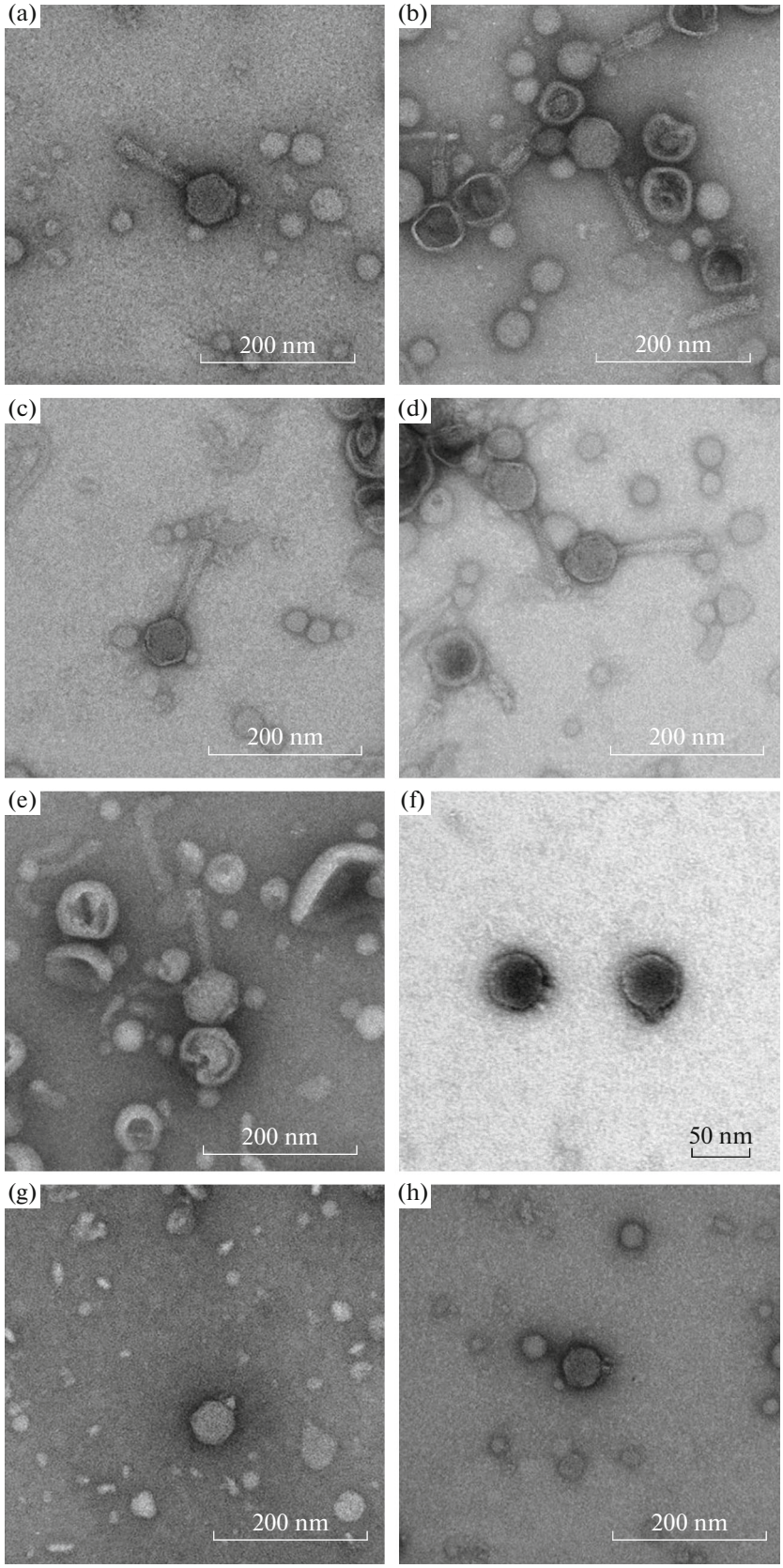

Fig. 2. The electron micrographs of *E. amylovora* bacteriophages: Hena2 (a), Roscha1 (b), Pixel (c), Dichka (d), VyarbaS (e), VyarbaL (f), Stepyanka (g), Fleur (h). Note: the micrographs of Hena1, Loshitsa2, Micant can be observed in (Besarab et al., 2020, 2022). TEM analysis revealed that Loshitsa1 is a podovirus (data not shown).

Table 3. *E. amylovora* bacteriophage host range

		Hena1*	Hena2	Rosch a1	Pixel	Dichka	VyarbaS	VyarbaL	Loshitsa1	Loshitsa2**	Micant**	Fleur	Stepyanka
E. amylovora	1/79Sm	+	+	+	+	+	+	+	+	+	+	+	+
	E2	–	+	+	+	+	+	–	–	–	–	–	–
	D4	–	+	+	+	+	+	–	–	–	–	–	–
	L-3-1	–	+	+	+	+	+	–	–	–	–	–	–
	L-3-2	–	+	+	+	+	+	–	–	–	–	–	–
	L-3-5	–	+	+	+	+	+	–	–	–	–	–	–
	L-3-6	+	+	+	+	+	+	+	+	+	+	+	+
	L-3-8	+	+	+	+	+	+	+	+	+	+	+	+
	E3	–	+	+	+	+	+	+	+	+	+	+	+
	E4	–	+	+	+	+	+	+	–	+	+	+	+
	E5	–	+	+	+	+	+	–	–	–	–	–	–
	133/95	–	+	+	+	+	+	+	+	+	+	+	+
E. coli	DH5α	–	–	–	–	–	–	–	–	–	–	–	–
	B	–	–	–	–	–	–	–	–	–	–	–	–
P. carotovorum	14a	–	–	–	–	–	–	–	–	–	–	–	–
P. atrosepticum	36A	–	–	–	–	–	–	–	–	–	–	–	–
P. agglomerans	194	–	–	–	–	–	–	–	–	–	–	–	–
	197	–	–	–	–	–	–	+	+	+	+	+	+
	198	–	–	–	–	–	–	–	–	–	–	–	–
	216	–	+	+	+	+	+	–	–	–	–	–	–
	219	–	–	–	–	–	–	–	–	–	–	–	–
	220	–	–	–	–	–	–	–	–	–	–	–	–
	246	–	–	–	–	–	–	–	–	–	–	–	–
P. ananatis	208	–	+	+	+	+	+	–	–	+	+	+	+
	245	–	–	–	–	–	–	+	–	+	+	+	+

* Partially cited from (Besarab et al., 2020). ** Cited from (Besarab et al., 2022).

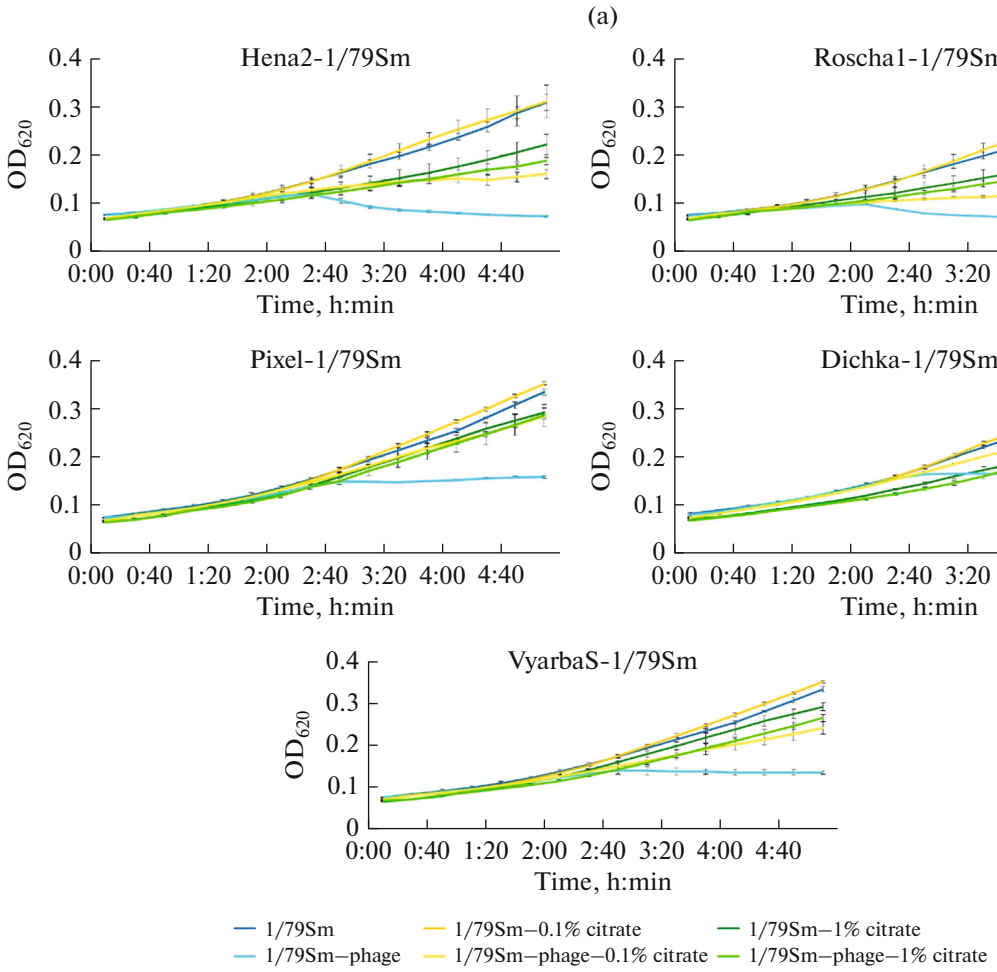

Fig. 3. Growth dynamics of the culture of *E. amylovora* strain 1/79Sm upon infection with bacteriophages: myoviruses (a), podoviruses (b). Note: Error bars reflect standard deviation. Quantitatively, the growth rate can be determined from the slope of the linear function as an approximation model of the bacterial culture growth curve.

The results also indicate a different nature of the distribution of the CFU and PFU numbers in overnight phage lysates, depending on whether the bacteriophage belongs to myoviruses or podoviruses. When infected with myoviruses (Fig. 4a), we observed almost equal (except for Pixel) decrease in the CFU number in LB medium without additives and with the addition of 0.1% citrate (by an average of 0.5–2 orders of magnitude, depending on the bacteriophage applied). However, the addition of the chelating agent sodium citrate in 1% concentration reduced the efficiency of infecting the *E. amylovora* 1/79Sm bacterial culture with bacteriophages. The decrease in the CFU number compared with the control averaged 2.61–4.68 times. Moreover, the bacteriophage titers in the medium with the addition of 1% sodium citrate were on average 25–173 times lower than those in the medium without additives. Upon infection with podoviruses, the CFU numbers in the control and phage-infected bacterial cultures after 20 h of incubation were actually equal, regardless of the composition of the medium (Fig. 4b). The bacteriophage titer in the medium with the addition of 1% citrate did not actually differ from the titer in the medium without additives (on average, the differences were 0.74–1.41 times). We can assume that the present myoviruses require divalent cations as the adsorption cofactors, whereas the podoviruses do not. Note that even closely related bacteriophages often have different sensitivity to environmental factors. The exposure to sodium citrate can lead to particle dissociation: Xp12 phage particles appeared to be decomposed in 3 mmol L^{-1} sodium citrate in Tris buffer at pH 7.5 at room temperature. In turn, divalent metal cations can stabilize the structure of phage particles and prevent their inactivation in different solutions (Jończyk et al., 2011; Whang, 1996). In addition, sodium citrate can participate in the regulation of processes in bacterial culture cells. In the *Escherichia coli* (STEC) and *stx*-phage system, when a bacterial culture was treated with 2.5% sodium citrate, significantly higher levels of Shiga toxin (Stx) production were observed compared

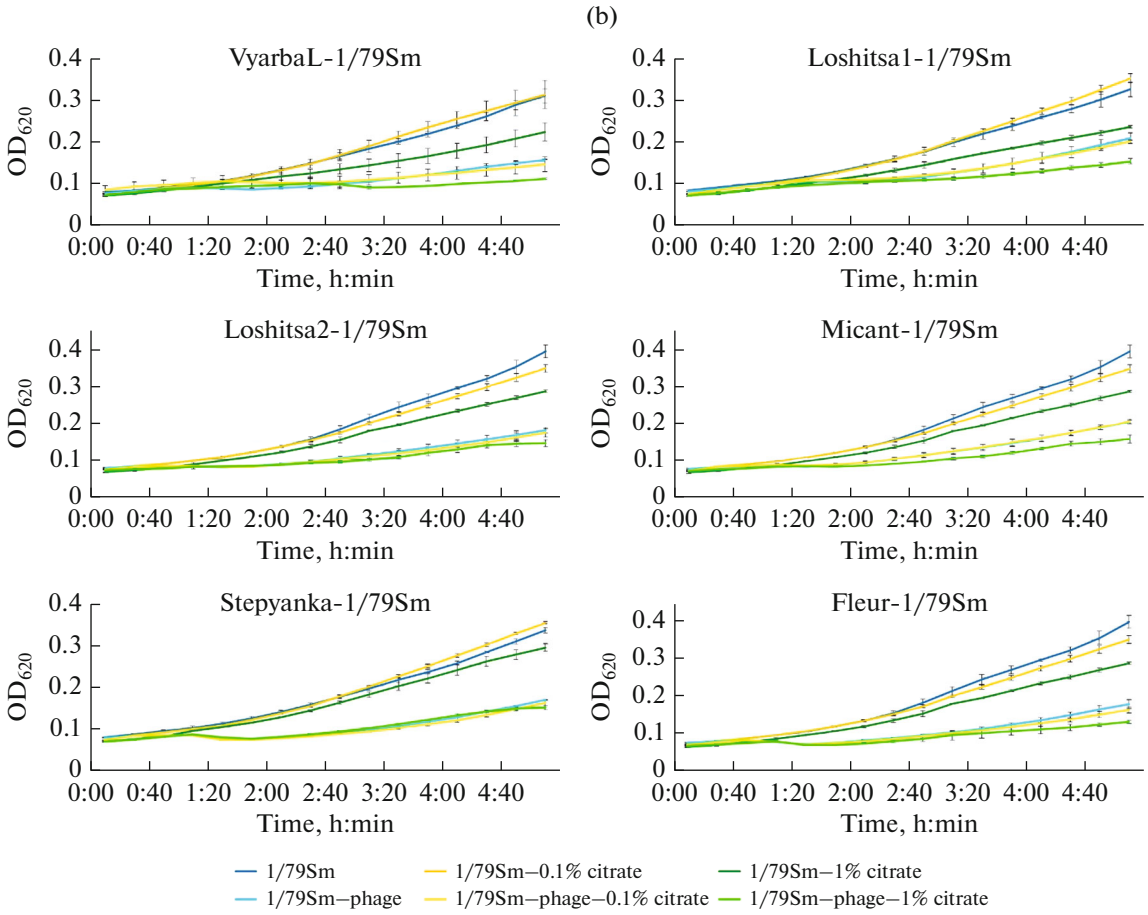

Fig. 3. (Contd.)

to the untreated cultures (Lenzi et al., 2016). We also compared the results of bacterial cell elimination using the presented bacteriophages with known data on *E. amylovora* bacteriophages. Literature data show a decrease in the *E. amylovora* CFU count in some cases by no more than an order of magnitude upon bacteriophage treatment of plant material (Müller et al., 2011b; Schwarczinger et al., 2011, 2017). When measuring the OD_{600} of *E. amylovora* Ea110 bacterial culture upon infection with 5 phages and their combinations at three concentrations, only in the case of one phage a decrease in OD_{600} up to 96 ± 4% was shown (Schnabel and Jones, 2001).

Literature data, as well as all three of our experiments with *E. amylovora* 1/79Sm strain indicate that the significant proportion of viable cells is frequently observed in phage-infected bacterial culture. At the next stage of the study, we demonstrated this phenomenon not only for planktonic cells, but also when inoculating a bacterial culture on phage agar (see the chapter "Characterization of the frequency of occurrence of phage-resistant variants in bacterial culture"). It is now obvious that the infectious ability of a bacteriophage needs to be studied under various environment conditions, or more precisely, when a bacterium is in various expression states (Ulrich et al., 2022).

Characterization of the Frequency of Occurrence of Phage-Resistant Variants in Bacterial Culture

For 11 bacteriophages with the highest sensitivity index of *E. amylovora* strains, we studied the frequency of occurrence of phage-resistant mutants upon infection of *E. amylovora* 1/79Sm. We established a high proportion of phage-resistant variants in the *E. amylovora* 1/79Sm bacterial culture: from 10^{-3}% (infection with Hena2, Roscha1, Pixel, Dichka, VyarbaS) (Besarab et al., 2021) to more than 20% (infection with the others) (Fig. 5).

The problem of bacterial adaptation to bacteriophages requires a comprehensive analysis. Resistance to the phage can be acquired both at the level of an individual cell (loss of phage receptor, masking of receptor, restriction–modification and CRISPR-Cas systems) and a community of cells when the infected cell prevents further spread of the infection (abortive infection) (Jurado et al., 2022). Moreover, acquired resistance in some cases has a fitness cost for bacteria,

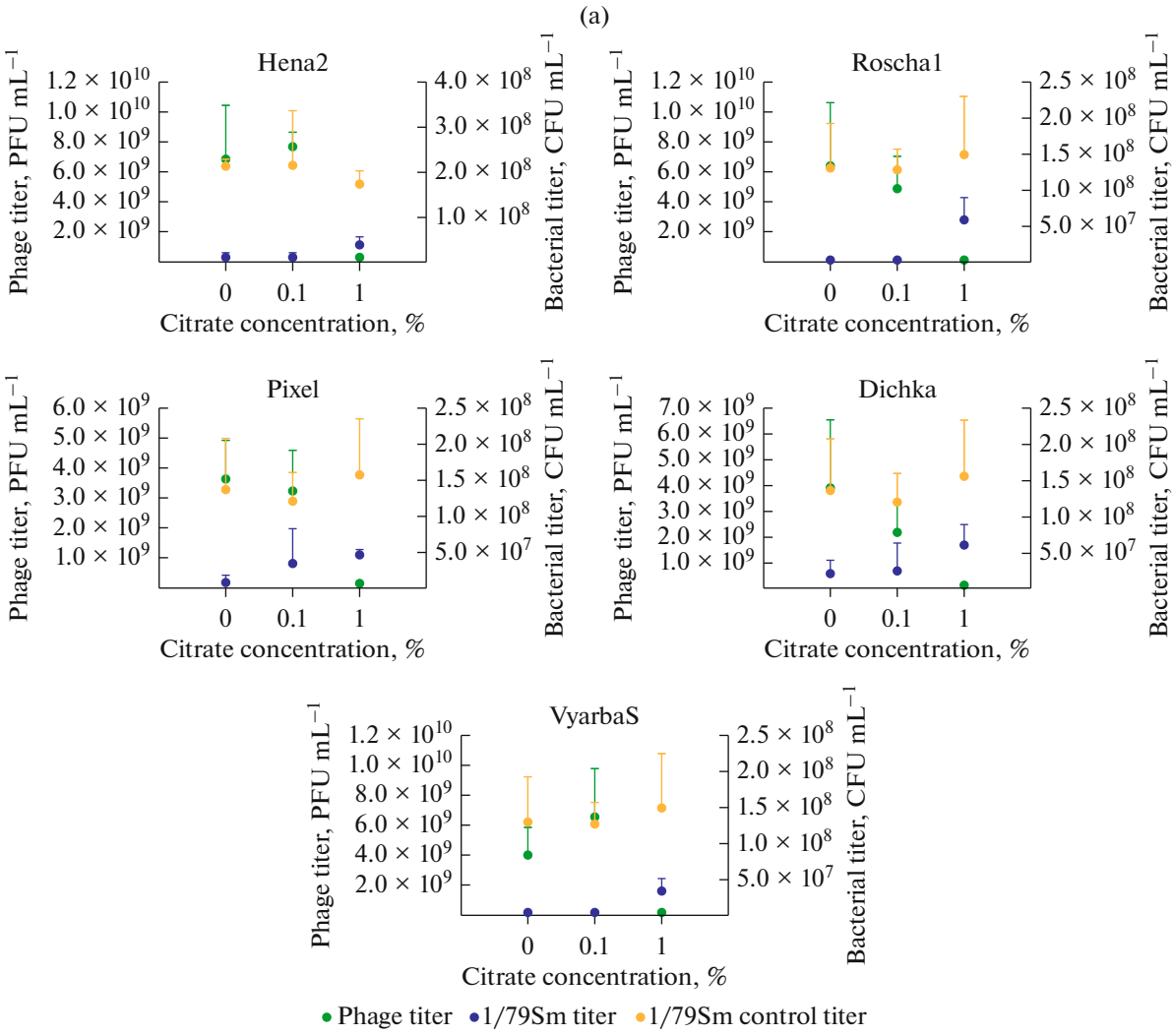

Fig. 4. The CFU and PFU numbers in the *E. amylovora* 1/79Sm bacterial culture upon infection with bacteriophages after 20 h incubation in LB medium without additives and with the addition of sodium citrate: myoviruses (a), podoviruses (b). Note: Error bars reflect standard deviation.

for example, when changes affect surface virulence factors, the bacterium may lose virulence (Oechslin, 2018; Laanto et al., 2020).

The observed "tuning" of resistance in bacteria appears interesting, and can be used to solve the problem of low efficiency of the bacteriophage for the control of highly adaptive bacteria. The experiment showed that depending on the phage infection pressure on the bacterial culture, different mechanisms of resistance took advantage of development. At low phage pressure, resistance was most often due to loss of the receptor, whereas at high phage pressure, CRISPR-Cas or transient resistance was detected (Rendueles et al., 2022). The sensitivity of bacteria to phages may depend on the environmental conditions and bacterial population density. It was demonstrated, that quorum sensing regulated the activity of CRISPR-Cas systems or downregulated the expression of the viral receptor porin (Jurado et al., 2022). Resistance to bacteriophage acquired by a bacterial cell is influenced not only by the combination of bacteriophages used, but also by their order of application. A certain order of phage exposure may entail a higher fitness cost for bacteria (Wright et al., 2019).

The above-mentioned transient adaptation of bacteria in the presence of a bacteriophage is a frequent occurrence (Jurado et al., 2022; Rendueles et al., 2022). A high proportion of the host cells resistant to six out of eleven phages tested in our experiment (Fig. 5) may indicate that some phase variation mechanism is driving spontaneous phenotypic dissociation in *E. amylovora* 1/79Sm strain. Noteworthy, that the proportion of the phage resistant bacteria was at the same level in all parallel cultures started from independent single colonies that excludes the possibility of accumulation of a mutant form in the cultures.

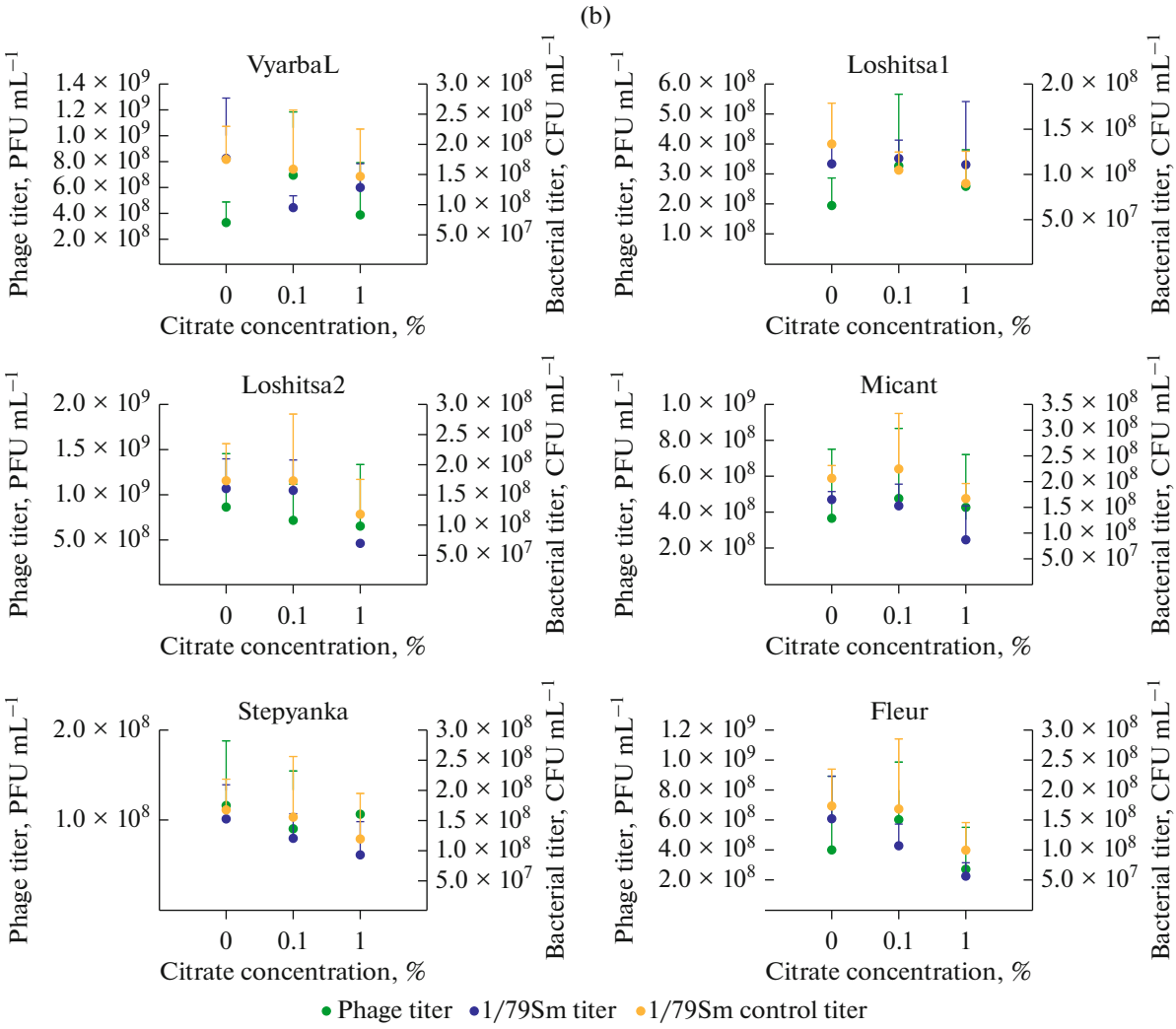

Fig. 4. (Contd.)

E. amylovora has undergone detailed study in connection with the quarantine status of the object and has a number of characteristic virulence factors, including those on the cell surface (amylovoran, levan, LPS) (Piqué et al., 2015). Studying key targets on the surface of *E. amylovora* may help in the selection and modification of lytic bacteriophages for fire blight control.

Genomic Characterization of E. amylovora Bacteriophages

We obtained complete genome sequences for seven bacteriophages from the presented collection of 12 bacteriophages (Fig. 6): Hena1 (NC_048828.1), Hena2 (OM522317.2), Roscha1 (ON706965.2), Loshitsa2 (OM513680.2), Micant (OM513679.2), VyarbaL (OM486945.2), and Stepyanka (ON715521.2). The genome sizes of the studied bacteriophages varied from 39314 to 148842 bp. The genome sequences of bacteriophages Hena1 (Besarab et al., 2020), Loshitsa2, and Micant (Besarab et al., 2022) did not have a high identity value with those available in GenBank. Bacteriophages Hena2, VyarbaL (Besarab et al., 2023), Roscha1, and Stepyanka shared a high degree of nucleotide identity with bacteriophages of various taxonomic groups. Here we present genome annotations of Roscha1 (member of *Caudoviricetes*, *Ounavirinae*) and Stepyanka (member of *Autographiviridae*, *Studiervirinae*) (Fig. 7).

A high degree of similarity at the genomic level was found for the pairs of bacteriophages Loshitsa2—Micant and Hena2—Roscha1 isolated in various geographical locations of Belarus. Between other bacteriophages only minor level of similarity was detected in some proteins (Fig. 6, Table 4). In most similarity groups, homologous gene products have a known common function.

For bacteriophages (except of Hena1) we used randomly fragmented next-generation sequencing (NGS) data and performed PhageTerm analysis to determine

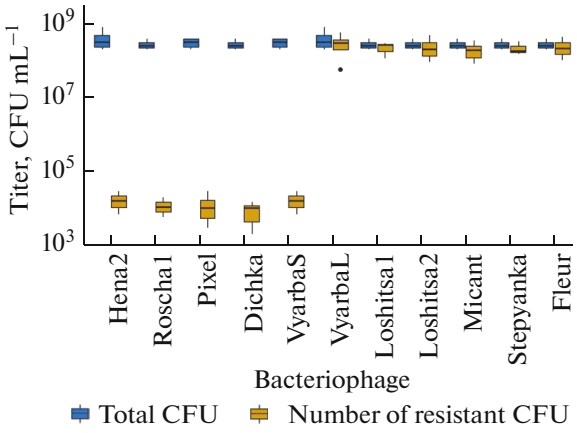

Fig. 5. The phage-resistant variants in *E. amylovora* 1/79Sm.

genome termini. Pvalues which correspond to highly reliable localization of the genome ends in the nucleotide sequence in PhageTerm method were 3.89e-42 (Roscha1, +strand) and 1.36e-87 (Stepyanka, +strand). The genomes of bacteriophages annotated here have sizes 39314 bp (Stepyanka) and 85137 bp (Roscha1) and, according to the PhageTerm Method, Stepyanka genome has 171 bp DTRs, Roscha1 genome—439 bp DTRs. Sequence similarity searches using blastn (taxid:10239, nucleotide collection (nr/nt)) showed that Stepyanka has 96.58% (coverage 97%) nucleotide sequence identity with *E. amylovora* bacteriophage vB_EamP-L1 (Born et al., 2011). The genome of vB_EamP-L1 had a length of 39282 bp with 172 bp DTRs. Bacteriophage vB_EamP-L1 was also active against three bacterial species among the investigated species: *E. amylovora*, *P. agglomerans* and *P. ananatis*. Bacteriophage vB_EamP-L1 in combination with other phages efficiently reduced viable counts below the detection limit (50 CFU mL^{-1}) after 1 h of incubation in *E. amylovora* control tests. These data may indicate that the bacteriophages presented in this work, despite the high incidence of phage-resistant variants in bacterial culture when they are used separately (78 ± 16% for Stepyanka), may be of interest as components of phage cocktails. Bacteriophage Roscha1 shared a nucleotide identity with *Enterobacter* phage phi63_307 (per. ident. 97.57%, coverage 99%), *Erwinia* phage phiEa21-4 (per. ident. 97.69%, coverage 98%) (Lehman, 2009), *Erwinia* phage phiEa104 (per. ident. 97.02%, coverage 98%) (Muller et al., 2011a), *Salmonella* phage ST-3 (per. ident. 97.56%, coverage 98%), and *Erwinia* phage SunLIRen (per. ident. 97.43%, coverage 98%). Bacteriophage phiEa21-4 was isolated in southern Ontario, Canada, demonstrated activity also against another plant pathogen—*E. pyrifoliae*. Bacteriophage phiEa104 was isolated in North America and had lytic activity besides *E. amylovora* and *P. agglomerans* strains against *E. pyrifoliae*, *E. billingiae*, *P. stewartii*. These data may favor a wider range of hosts for the bacteriophage Roscha1 we isolated. In work (Muller et al., 2011b) also noted that the presence or absence of the amylovoran capsule did not play a role in the susceptibility of bacterial strains to myoviruses, including phiEa104.

We designed primers to conserve regions of bacteriophage genomes—major capsid protein genes and tail tubular protein genes, to identify among the five unsequenced phages of our collection the viruses, related to the genomes sequenced by us. The PCR was positive for Pixel, Dichka, and VyarbaS DNA (Hena2 specific primers), Loshitsa1 DNA (Loshitsa2 specific primers), and Fleur DNA (Stepyanka specific primers) (Fig. 8).

PCR analysis did not reveal bacteriophages with a completely unknown DNA sequences. However, notable from the data obtained is a positive Loshitsa2 major capsid protein specific PCR for Loshitsa1, isolated in the same site as Loshitsa2, but having another phenotypic properties given in the current article. Thus, Loshitsa1 is of interest for further elucidation of the genetic background of bacteriophage biology.

CONCLUSIONS

Among the bacteriophages found on Belarus's territory, there were both new *E. amylovora* bacteriophages and the viruses closely related to bacteriophages from another continent isolated more than 10 years ago. Almost half of the resulting collection

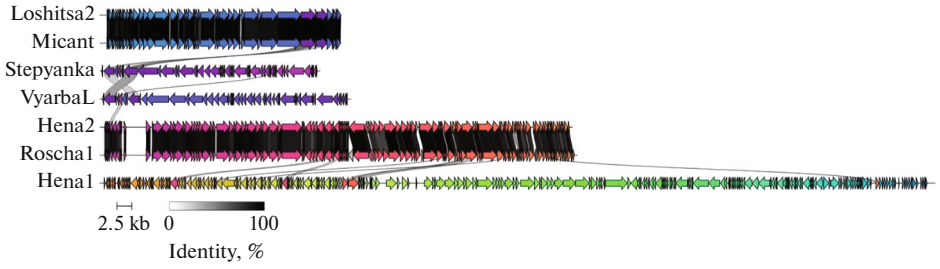

Fig. 6. Comparison of bacteriophage genomes by encoded proteins. Identity treshold used 30% of a.a. identity.

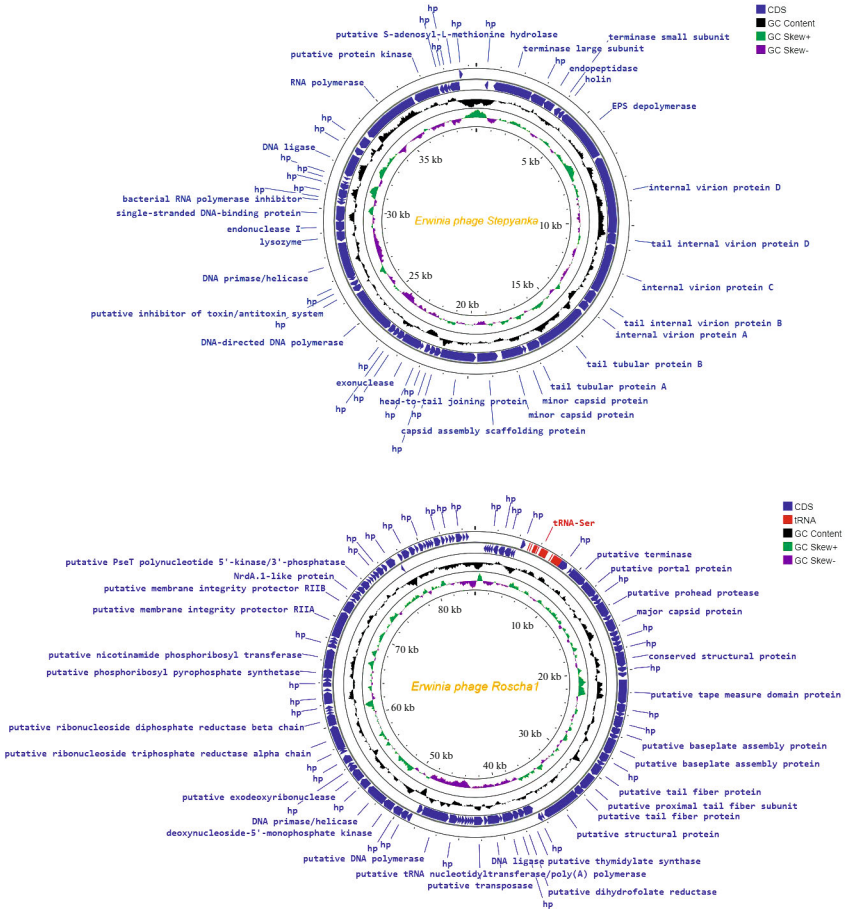

Fig. 7. Genomic maps of Stepyanka (a) and Roscha1 (b) phage genomes. Outer and inner lanes correspond to predicted genes on the forward and reverse strands, respectively (predicted genes are indicated in dark blue, tRNA genes are indicated in red). The two inner circles correspond to the GC plot and the GC skew, respectively. The short black lines at the top circles indicate the approximate position of the physical termini of the virion-encapsidated DNA. Circular presentation of the maps was chosen for compaction of the figure.

were myoviruses—*Kolesnikvirus*-like bacteriophages. The presented Roscha1 and Stepyanka genomes contain 58 and 44% genes with unknown functions, respectively. As noted earlier, *E. amylovora* bacteriophages contain a significant proportion of uncharacterized genes. However, most of the presented bacteriophage collection has related bacteriophages, which makes it possible to conduct a comparative analysis of phenotypes and genotypes in the future.

Bacteriophages differed in the host ranges; however, they were active against both strains of the plant pathogen and the potential carrier of bacteriophages in the biopreparation–bacteria of the *Pantoea* genus. As previously suggested (Jończyk et al., 2011), researchers should "know their phages" because of varied attitudes to physical and chemical factors according to the literature. *E. amylovora* bacteriophages remained viable under laboratory cultivation conditions, but demonstrated a different attitude towards the presence of a chelating agent in the medium—sodium citrate. In this paper, we present a primary study of the phage resistance of the *E. amylovora* bacterial culture in experiments on infection of *E. amylovora* 1/79Sm in a liquid nutrient medium and when seeded on phage agar. The data obtained allow us to raise the issue of the high frequency of occurrence of phage-resistant variants in the culture of *E. amylovora* bacteria (from 10^{-3}% to more than 20%) and to drive the attention of researchers to this problem. Understanding the mechanisms of formation of resistance to phage infection in *E. amylovora* cells may make it possible to improve already known and characterized *E. amylovora* bacteriophages, how it was previously performed using genetic engineering (Gibb, 2021). Note that in our study, myoviruses (except Hena1) were the most effective in suppressing the

Table 4. Homologous gene products of *E. amylovora* bacteriophages. Identity threshold 0.3

The homologous gene product group number	The gene product function and the source
1	EPS depolymerase (VyarbaL, Stepyanka), putative tail fiber protein/putative EPS depolymerase (Loshitsa2, Micant)
2	Terminase, large subunit (VyarbaL, Stepyanka), putative DNA maturase B (Loshitsa2, Micant)
3	hp (VyarbaL, Stepyanka)
4	hp (Hena1, Stepyanka)
5	SAR endolysin (VyarbaL, Hena2, Roscha1)
6	hp (Hena1, Hena2, Roscha1)
7	Nicotinamide phosphoribosyl transferase (Hena1, Hena2, Roscha1)
8	Ribose-phosphate pyrophosphokinase (Hena1), phosphoribosyl pyrophosphate synthetase (Hena2, Roscha1)
9	Lipoprotein (Hena1), transposase (Roscha1), hp (Hena2)
10	hp (Hena1, Roscha1)
11	Glutaredoxin (Hena1, Hena2, Roscha1)
12	tRNA nucleotidyltransferase (Hena1, Hena2), tRNA nucleotidyltransferase/poly(A) polymerase (Roscha1)

growth of *E. amylovora* bacterial culture (decrease in the CFU number after 20 h incubation with bacteriophages by an average of 0.5–2 orders of magnitude, lytic activity of bacteriophages against all tested *E. amylovora* strains) and can form the basis of antibacterial biopreparations.

SUPPLEMENTARY INFORMATION

The online version contains supplementary material available at https://doi.org/10.1134/S002626172460530X.

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The collection of bacterial strains used in the work was re-identified by mass spectrometry using the Bruker Daltonik MALDI Biotyper system in 2020 with the assistance

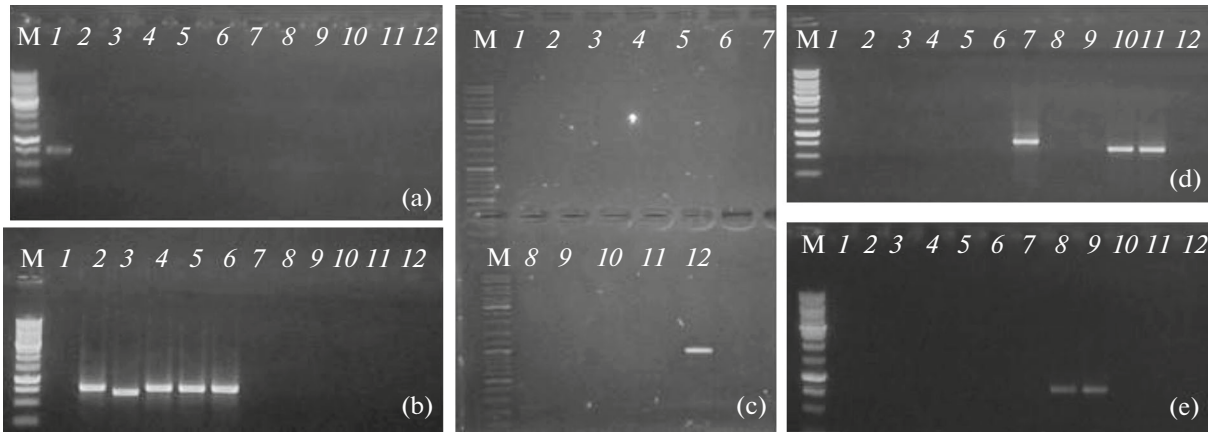

Fig. 8. The PCR detection of bacteriophage DNA using primers: H1Mcp (a), H2mcp and RosMcp (b), Mcp (c), MicTTP and L2TTP (d), StMcp (e). M: 100 bp DNA Ladder; *1*—Hena1, *2*—Hena2, *3*—Roscha1, *4*—Pixel, *5*—Dichka, *6*—VyarbaS, *7*—Micant, *8*—Fleur, *9*—Stepyanka, *10*—Loshitsa1, *11* —Loshitsa2, *12*—VyarbaL DNA.

of researcher P.Yu. Pechenov, the Biotechnological Center LLC "Green Lines" GC "SOYUZSNAB," Krasnogorsk, Russia.

AUTHOR CONTRIBUTION

All authors contributed to the study conception and design, and commented on previous versions of the manuscript. All authors have read and approved the final manuscript.

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ETHICS APPROVAL AND CONSENT TO PARTICIPATE

This work does not contain any studies involving human and animal subjects.

CONFLICT OF INTEREST

The authors of this work declare that they have no conflicts of interest.

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