Two novel *Erwinia amylovora* bacteriophages Loshitsa2 and Micant, isolated in Belarus

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Research Article

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Abstract

The complete genomes of new *Erwinia amylovora* bacteriophages, Loshitsa2 and Micant, are 43092 bp and 43028 bp, respectively, encode 51 putative proteins and have two tRNA genes. Comparative analysis with *Caudovirales* representatives suggests that Loshitsa2 and Micant bacteriophages are related to LIMElight bacteriophage within the family *Autographiviridae*, and could be proposed to share novel subfamily.

Introduction

Fire blight disease was first described in 1780 in the Hudson Valley, New York, and despite a long history of research of pathogen biology and strategies for controlling disease, the disease still causes concern around the world [1]. Fire blight affects a number of agricultural and ornamental plants belonging to the *Rosaceae* family. Causative agent of the disease *E. amylovora* was detected in hawthorn (*Crataegus*), cotoneaster (*Cotoneaster*), quince (*Cydonia*), loquat (*Eriobotrya*), mountain ash (*Sorbus*), pyracantha (*Pyracantha*), serviceberry (*Amelanchier*), photinia (*Photinia*), stranvaesia (*Stranvaesia*), apricot (*Prunus*), spirea (*Spirea*), raspberries and blackberries (*Rubus*). The disease is most severe on pear (*Pyrus*) and apple (*Malus*), leading to the death of trees [2, 3, 4]. The primary and especially vulnerable organ of fire bright infection is the flower [5], therefore prevention of blossom infection using spays of various antibacterial agents is considered as one of the possible measures in fire blight management. The number of chemicals available is limited, and traditionally fire blight chemical control methods include application of copper compounds and antibiotics [6]. In connection with phytotoxicity of some chemicals, for example, of copper compounds, and the spread of resistance, new tools of phytopathogen *E. amylovora* control are needed [7, 8, 9, 10].

E. amylovora bacteriophages are of interest as an alternative fire blight control agents [11]. Bacteriophages are highly specific and phage therapy already used successfully against bacterial infections in clinical practice [12, 13]. The advantage of bacteriophages for therapy is their natural origin, however, new isolates require detailed study in order to exclude lysogenic, pseudolysogenic and chronic phage infections. In addition, due to the narrow host range of bacteriophages, it is necessary to determine the etiology of pathogenic bacteria or to apply bacteriophage cocktails. Thus, for successful phage therapy, it is necessary to create collections of characterized bacteriophages.

Currently, the number of studied *E. amylovora* bacteriophages is rising rapidly. Bacteriophages of diverse morphotypes, variety of particle sizes and genome organization were revealed [14]. So-called “jumbo phages”, whose genomes are larger than 200 kbp, were described for *E. amylovora*, for example, vB_EamM_Y3, vB_EamM_Bosolaphorus, vB_EamM_Desertfox, vB_EamM_MadMel, and vB_EamM_Mortimer, vB_EamM_Asesino and vB_EamM_Wellington, EamM_Alexandra bacteriophages [15; 16]. Among the 60 *Erwiniaceae* phage genomes available in GenBank the smallest bacteriophage genome belonged to the *E. amylovora* bacteriophage ENT90–29564 bp [17]. It should be noted that genome studies are of direct importance in the creation of bacteriophage preparations, both to exclude selection of temperate bacteriophages or phage carrier of virulence factor genes and to quickly isolate new bacteriophages with the necessary properties [18, 19].

Previously, two *E. amylovora* bacteriophages were isolated in Belarus – Hena1 belonging to the Vequintavirinae subfamily (NCBI:txid2678601) and phiEa2809 of the Ackermannvirida*e* family (NCBI:txid1564096) [20, 21]. Here, we announce the genome sequences of two newly isolated *E. amylovora* podoviruses Loshitsa2 and Micant – novel representatives of the Autographiviridae family .

Materials And Methods

Bacterial strains and culture conditions

The study was conducted using both phytopathogenic and nonpathogenic bacterial strains. These include 12 *E. amylovora* strains: 1/79Sm (Germany, Spontaneous Sm-resistant mutant of 1/79, Cotoneaster sp., 1979) [22], 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); 133/95 (Poland, *Cydonia* sp., 1985 г.), 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).

The bacterial strains were cultured in lysogeny broth (LB) medium (10 g tryptone, 10 g sodium chloride (NaCl) and 5 g yeast extract) or on lysogeny broth agar (LA) plates at 28 °C. Growth in a liquid culture medium was performed with aeration. *E. amylovora* 1/79Sm strain was used as a host for bacteriophage isolation, propagation and characterization.

Bacteriophage isolation and purification

Bacteriophages were isolated using enrichment technique. Briefly, soil sample was resuspended in LB-medium with addition of 2% of the volume of *E. amylovora* 1/79Sm overnight culture. After overnight incubation at 28°C enrichment culture was centrifuged for 25 min at 6000×g, supernatant was spotted onto the surface of the double layer agar plates with the host bacteria in the top agar layer. Single plaques were picked and resuspended in LB. At least 5 passages of bacteriophage purification from a single plaque were carried out.

Determination of host range

For assessment of host range a spot test was performed. A 10 µl drop of the phage lysate (10^7 pfu/ml) was spotted onto plates prepared with the bacterial strains followed by overnight incubation at 28 °C. The positive results of the spot tests, observed clear zones on a bacterial lawn, were further verified by double agar overlay plaque assay to confirm plaque formation.

Morphology investigation by transmission electron microscopy (TEM)

TEM study of bacteriophages was performed as described in [23]. Phages were visualized with JEOL JEM-2100 200kV TEM, equipped with LaB6 electron gun and Gatan Ultrascan 1000XP 2k x 2k CCD detector. Images were taken at 40k indicated magnification with pixel size 0.25 nm and defocus between -1 and -2 mkm.

Bacteriophage DNA Extraction and Sequencing

Bacteriophage genomic DNA was isolated by phenol-chloroform extraction as described in [24] and sent to Genomic research and computational biology lab, FSCC of physico-chemical medicine, Federal Medical-Biological Agency, Moscow, Russian Federation for sequencing and assembly. DNA was sequenced using an Ion Torrent Proton sequencer system (Applied Biosystems, USA) with 224 (Loshitsa2) and 198 (Micant) coverage. Primary assembly was performed with Newbler version 2.9, resulting in single contigs.

Sequence analysis

Bacteriophage genome annotation was produced by Prokka [25] with further manual curation. Functional annotations were performed using NCBI BLASTx searches, tRNA genes prediction was accomplished using tRNAscan-SE v. 2.0 [26] and ARAGORN v1.2.41 [27]. Bacteriophage DNA termini and packaging mechanism were determined with PhageTerm [28]. Genetic and physical map of bacteriophage genome were generated using Proksee (CGView) [29]. Phylogenetic trees were constructed using “one click” at Phylogeny.fr [30] and the Virus Classification and Tree Building Online Resource (VICTOR) [31]. Automatic generation of gene cluster comparison figures was performed using clinker [32].

Nucleotide sequence accession number

The complete annotated genome sequences of *E. amylovora* bacteriophages Loshitsa2 and Micant have been deposited in GenBank under the accession numbers OM513680 and OM513679, respectively.

The complete genome of LIMElight was downloaded from the NCBI GenBank database (NC_019454.1).

Results And Discussion

Two *E. amylovora* bacteriophages were isolated from soil samples near apple trees in different districts of Minsk, Belarus on June (Loshitsa2) and July (Micant). Loshitsa2 and Micant produced 3-3.5 mm diameter plaques with translucent halo on double agar overlay plates (Fig. 1). Their plaques were turbid that was the hint of incomplete lysis. It may indicate the presence of lysogens or phage-resistant variants in bacterial culture.

The morphology of Loshitsa2 and Micant virion was revealed using TEM of the negatively stained samples. The bacteriophages had icosahedral heads measuring 59.80 ± 2.60 (Loshitsa2) and 56.39 ± 2.69 (Micant) and short non-contractile tails (Fig. 2). According to Bradley’s morphological groups of bacteriophages [33] two *E. amylovora* bacteriophages were classified as belonging to C type. It should be added that among the isolated *E. amylovora* bacteriophages representatives of three morphotypes are described in the literature: myoviruses, siphoviruses and podoviruses, with a capsid size of 53–143.2 nm [34, 14, 35]. Recently, there the first filamentous *E. amylovora* bacteriophages isolation was reported, the PEar viruses carrying a single-strand DNA genome between 6608 and 6801 nucleotides [36].

The host range analysis revealed that Loshitsa2 and Micant bacteriophages were polyvalent and able to infect strains of different genera (Table 1). Among sensitive bacteria there were strains of *E. amylovora, P. agglomerans* and *P. ananatis*. It should be noted that antagonistic relationships of *E. amylovora* and *Pantoea* species were described [37, 38]. *P. agglomerans* has a potential as biocontrol agent and can be used as a phage carrier [39, 40].

Table 1
The host range of two *E. amylovora* bacteriophages

Phages	Bacterial strains																				
	1/79Sm	E2	D4	L-3-1	L-3-2	L-3-5	L-3-6	L-3-8	E3	E4	E5	133/95	DH5α	B	14a	36A	194	197	198	208	216
Loshitsa2	+	-	-	-	-	-	-	+	+	+	-	+	-	-	-	-	-	+	-	+	-
Micant	+	-	-	-	-	-	-	+	+	+	-	+	-	-	-	-	-	+	-	-	-

The double-stranded DNA genomes of Loshitsa2 and Micant bacteriophages consists of 43092 bp and 43028 bp, respectively sharing 98.18% of nucleotide identity between them (coverage 99%). The GC% content is 54% for both bacteriophages. In total, 53 putative genes were predicted in each of two genomes, including 51 protein coding genes and 2 tRNA genes (Fig. 3). All Loshitsa2 and Micant genes have the same orientation. Among the genes with predicted function there were 8 putative structural genes: tail tubular protein A, tail tubular protein B, internal virion proteins, tail fiber protein with EPS depolymerase, head-tail connector and major capsid protein (MCP). Gene of viral scaffolding protein which is essential for phage capsid assembly is located upstream of the MCP gene. Putative genes of DNA maturase A and B crucial for DNA packaging follow the structural genes cluster. Two genes identified to be involved in host lysis by bacteriophages (holin and endolysin genes). There are 9 genes playing a vital role in DNA/RNA metabolism, replication and repair: ATP-dependent DNA-ligase, DNA primase, DNA helicase, DNA polymerase A, DNA polymerase II small subunit, DNA exonuclease, DNA endonuclease, nucleotide kinase, DNA-dependent RNA-polymerase.

Using PhageTerm analysis multiple preferred termini on the forward strand and unique termini on the reverse strand were predicted. Phage termini determined at 15277 (+ strand), 15534 (- strand) positions for Loshitsa2 and at 16880 (+ strand), 17138 (- strand) positions for Micant (Li's method data [28]). The headful mode of packaging PAC is concluded for both bacteriophages, when the terminase initiates packaging at a specific *pac* site of the phage concatemer, as described for P1 and P22 phages [28].

Bacteriophage genome sequences were queried against the viruses (taxid:10239) nucleotide collection (nr/nt) using blastn (carried out on 22/02/2022). Sequence similarity searches revealed that Loshitsa2 and Micant present 76.13% (coverage 38%) and 76.29% (coverage 40%) nucleotide sequence identity with *Pantoea* phage LIMElight [41], respectively. Bacteriophage LIMElight has also relatively small dsDNA genome of 44,546 bp, encoding 55 open reading frames. Nowadays bacteriophage LIMElight is classified in the family *Autographiviridae*, within the genus of the “Limelightvirus” (NCBI:txid881915). Interestingly, bacteriophage formed a small clear plaque of 1 mm in diameter on its host *P. agglomerans* strain GBBC 2043 and no plaques on *E. amylovora* strain GBBC 403. Primer-walking revealed that LIMElight genome has direct terminal repeats (DTRs) of 277 bp, suggesting other packaging mechanisms.

The novel *E. amylovora* bacteriophages also have limited similarity with phages of different host specificity. Bacteriophage Loshitsa2 has sequence similarity with *Erwinia* phage vB_EamP-L1 (coverage 2%, identity 81.19%, HQ728265.1), *Klebsiella* phages KMI6 (coverage 3%, identity 72.04%, MN101220.1), KMI5 (coverage 5%, identity 72.04%, MN101219.1) and KMI3 (coverage 5%, identity 72.04%, MN101217.1). Bacteriophage Micant has sequence similarity with *Erwinia* phage vB_EamP-L1 (coverage 2%, identity 81.28%, HQ728265.1), *Shigella* phage HRP29 (coverage 3%, identity 73.44%, NC_048174.1), *Enterobacter* phage ENC16 (coverage 3%, identity 71.61%, OL355133.1). *Erwinia* phage vB_EamP-L1 (NCBI:txid1051673), *Klebsiella* phages KMI6 (NCBI:txid2601617), KMI5 (NCBI:txid2601616), KMI3 (NCBI:txid2601614), *Shigella* phage HRP29 (NCBI:txid2530183) are also classified in the family Autographiviridae. *Enterobacter* phage ENC16 (NCBI:txid2906747) at the moment is of unclassified Caudovirales. Gene cluster comparison of listed bacteriophages is presented in Fig. 4.

Generated phylogenetic trees of the single phage proteins using “one click” at Phylogeny.fr (Fig. 5) and the VICTOR whole-genome sequence analysis placed bacteriophages Loshitsa2 and Micant in separate cluster closely related to LIMElight bacteriophage. Loshitsa2 and Micant bacteriophages should be classified in the family Autographiviridae and could be proposed to share novel subfamily with LIMElight bacteriophage.

Summing up, whole-genome sequence analysis revealed two novel bacteriophages - Loshitsa2 and Micant, isolated in Belarus using *E. amylovora* for its propagation. Bacteriophages have rather small genomes and share sequence similarity with *Pantoea* phage LIMElight. According to protein-based phylogeny and whole-bacteriophage genome sequence phylogeny Loshitsa2, Micant and LIMElight are proposed to form a new subfamily within the family Autographiviridae.

Declarations

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Conflict of Interest

The authors declares that they have no conflict of interest.

Ethical approval

This article does not contain any studies with human participants or animals performed by any of the authors.

Author Contributions

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

Competing Interests

The authors have no relevant financial or non-financial interests to disclose.

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Figures

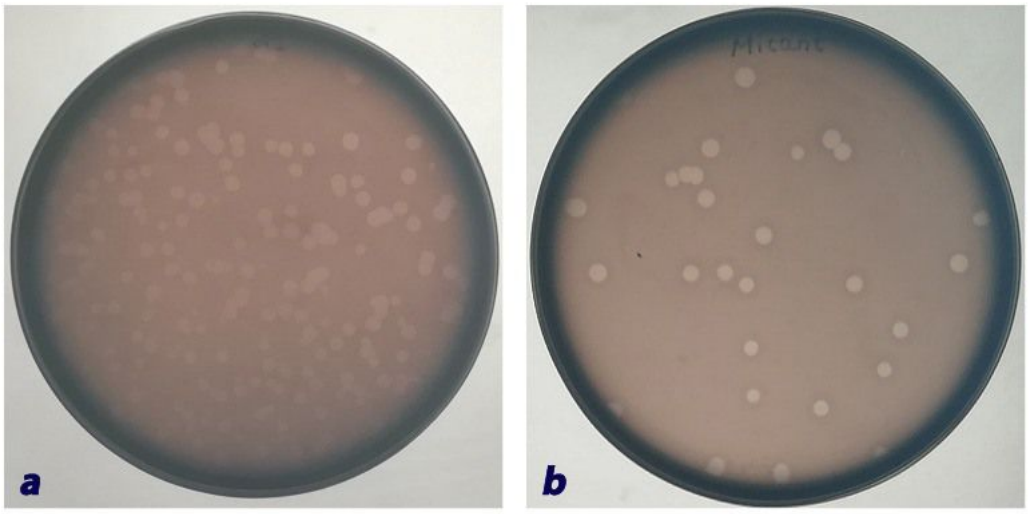

Figure 1

Bacteriophage plaque assay plates: Loshitsa2 (a) and Micant (b)

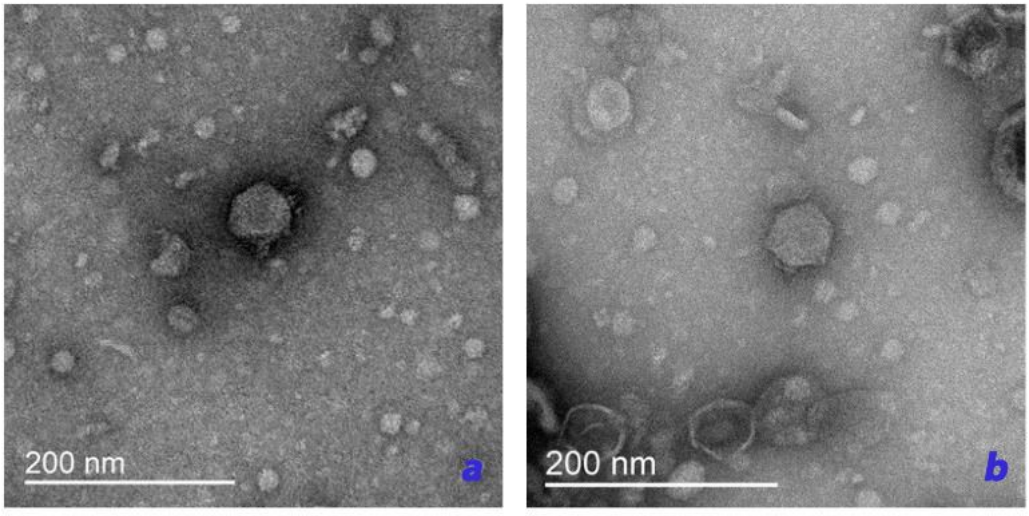

Figure 2

Electron microphotography of Loshitsa2 (a) and Micant (b) bacteriophage particles. Uranyl acetate staining. The bar indicates 200 nm.

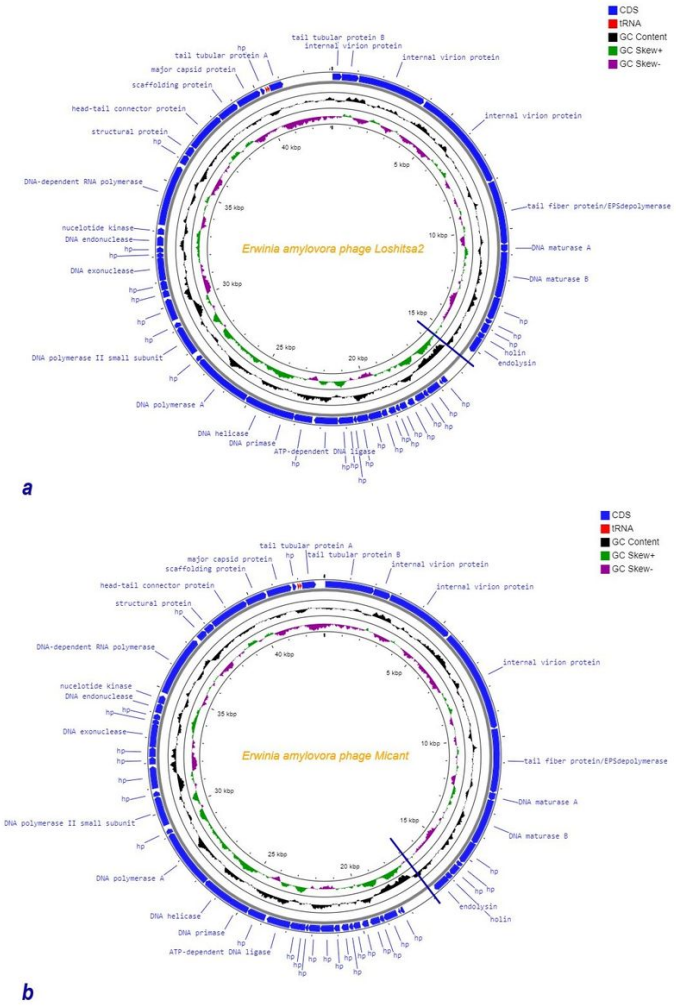

Figure 3

Genetic and physical map of phage Loshitsa2 genome (A) and phage Micant genome (B) prepared using Proksee. Outer lane corresponds to predicted genes on the forward strand (predicted genes are indicated in dark blue, tRNA genes are indicated in purple). Two inner circles correspond to GC plot and GC skew, respectively. Dark blue line depicts the approximate location of the physical termini of the virion-encapsidated phage genome

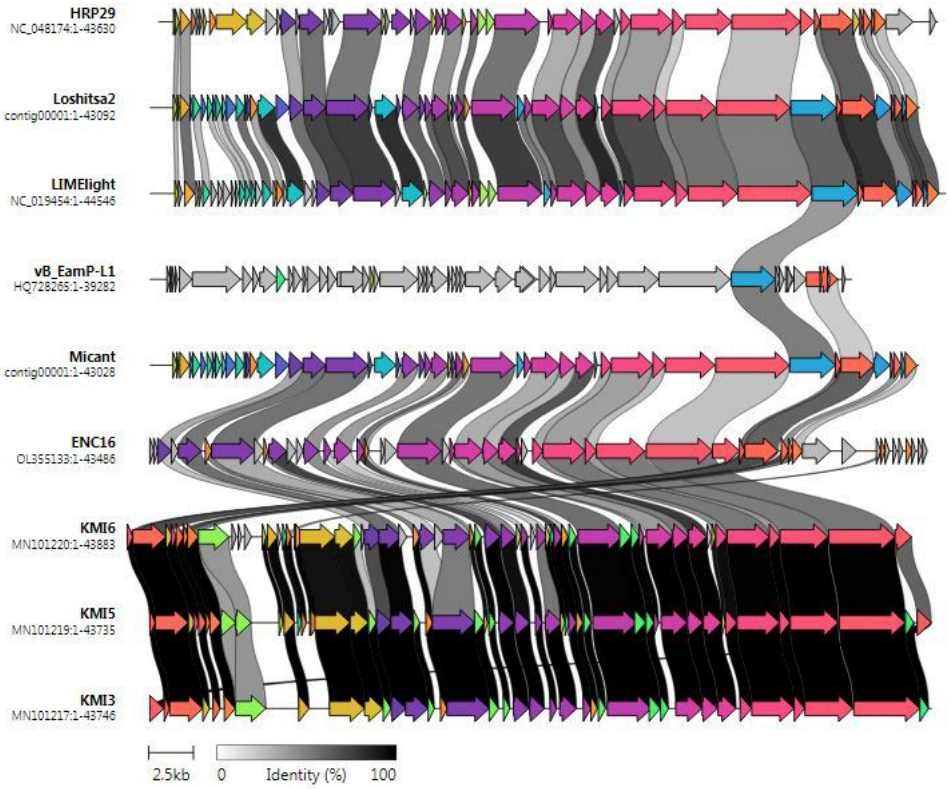

Figure 4

Gene cluster comparison of Loshitsa2, Micant and homologous bacteriophages whole genome sequences using clinker

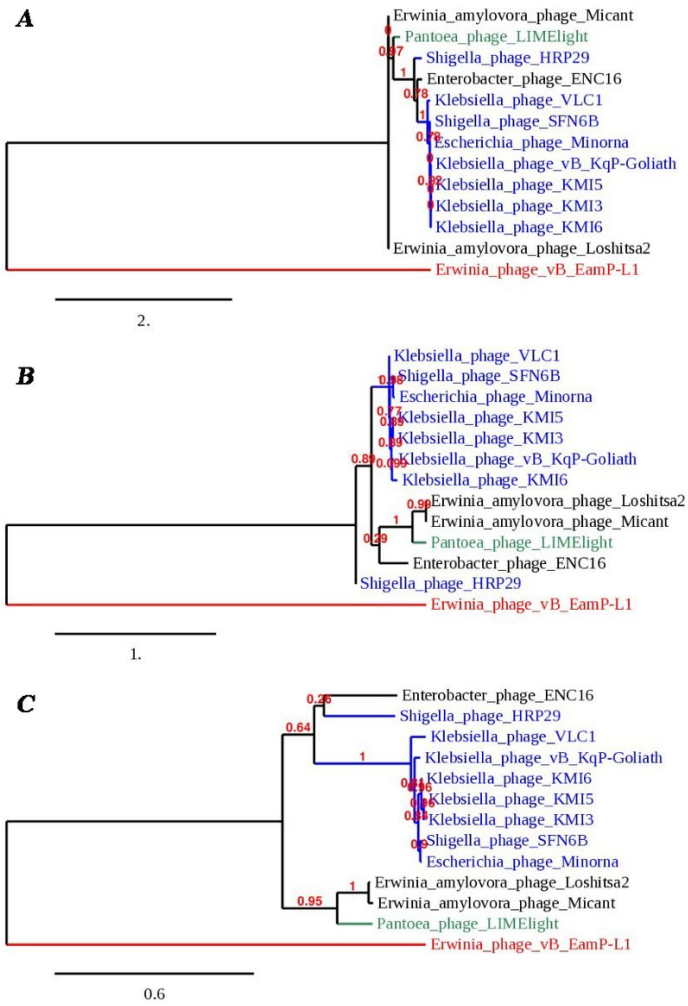

Figure 5

Phylogenetic analysis of major capsid proteins and homologous proteins (A), DNA polymerases (B) and RNA polymerases (C) of the phages which make up the *Autographiviridae* family. Red lines indicate the clade of "*Studiervirinae*"; blue lines, the "*Slopekvirinae*"; and green lines, the "*Limelightvirus*".

Supplementary Files

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