Biological characterization and genomic analysis of a novel bacteriophage, MopsHU1, infecting *Morganella psychrotolerans*

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Abstract

Morganella psychrotolerans is a histamine-producing bacterium that causes histamine poisoning. In this study, we isolated and characterized a novel phage, MopsHU1, that infects *M. psychrotolerans*. MopsHU1 is a podovirus with a limited host spectrum. Genomic analysis showed that MopsHU1 belongs to the family *Autographiviridae*, subfamily *Studiervirinae*, genus *Kayfunavirus*. Comparative analyses revealed that the MopsHU1 genome was similar to those of *Citrobacter* phage SH3 and *Cronobacter* phage Dev2. Moreover, the *Escherichia coli* phage K1F genome was also similar, except for its tailspike gene sequence. These results expand our understanding of the *Kayfunavirus* phages that infect *Morganella* spp..

Note: Nucleotide sequence data reported is available in the DDBJ/EMBL/GenBank database under the accession number LC799501.

Morganella psychrotolerans is a gram-negative bacterium belonging to the family *Morganellaceae*. This bacterium can grow at refrigerated temperatures and is involved in seafood histamine (scombroid) poisoning. This and some other gram-negative bacteria, such as *M. morganii*, *Raoultella planticola*, and *Photobacterium phosphoreum*, cause the accumulation of large amounts of histamine in seafood [1]: bacterial histidine decarboxylase converts free histidine in fish muscles into histamine via decarboxylation. Our previous studies showed that *M. psychrotolerans* may contaminate a variety of seafood [2] and has a strong histamine-producing ability [3]. In addition, the pathogenicity of this bacterium in rainbow trout has been reported [4]. This indicates that *M. psychrotolerans* is an important pathogen affecting human health and aquaculture.

Bacteriophages (phages) have potential as antibacterial agents in a variety of fields [5–7]. Some phages infecting *M. morganii* have been isolated and characterized [8–11]. Among them, *M. morganii* phage ΦMV-1 also showed infectivity against *M. psychrotolerans* JCM 16473^T; however, its efficiency of plating (EOP) on this *M. psychrotolerans* strain was lower than that on *M. morganii* strains [10]. This indicates that the primary host of ΦMV-1 is *M. morganii* and that there is little information on the phages infecting *M. psychrotolerans*. In this study, we isolated and characterized MopsHU1, a phage that infects *M. psychrotolerans*, to further understand the phages that infect this bacterium.

M. psychrotolerans was incubated in tryptic soy broth (TSB; BD Biosciences, Franklin Lakes, NJ, USA) at 25 °C for 18 h. A mixture of *M. psychrotolerans* Mps.1 and Mps.8 [2] was used for the isolation of MopsHU1 from the skin of a retail chicken. The phage titer was determined by the double agar overlay method using *M. psychrotolerans* Mps.8. Fresh culture and phage suspension (100 µL each) were inoculated to 4 mL of 0.5% molten soft agar. The mixture was poured onto tryptic soy agar (TSA; BD Biosciences) plates. After solidification of the soft agar, the plate was incubated at 25 °C for 1 d, and the formed plaque was isolated. The phage MopsHU1 was propagated using *M. psychrotolerans* Mps.8 and concentrated by polyethylene glycol precipitation. The concentrated phage was purified by CsCl density gradient centrifugation (100,000 × *g*, 3 h) and dialyzed against SM buffer (100 mM NaCl, 8 mM $MgSO_4$, 50 mM Tris-HCl, and 0.01% gelatin; pH 7.5). Purified phages were used for further experiments.

MopsHU1 formed clear plaques with halos on the lawn of *M. psychrotolerans* Mps.8 (Fig.

1A). To analyze the host range of MopsHU1, spot testing assays were conducted using 38 strains of *M. psychrotolerans* (Mps.1–Mps.37 and JCM 16473^T), 8 strains of *M. morganii* subsp. *morganii* (NBRC 3848^T, NBRC 3168, ATCC 25829, JNBP_03126, JNBP_03128, JNBP_03133, JNBP_03145, and JNBP_03147), and 1 strain of *M. morganii* subsp. *sibonii* (JCM 16939^T). MopsHU1 formed clear plaques on *M. psychrotolerans* Mps.8 only, and all other *M. psychrotolerans* and *M. morganii* strains were insensitive to this virus. This indicates that MopsHU1 infection is strictly specific.

The morphology of MopsHU1 was examined by transmission electron microscopy (TEM; microscope JEM-1011; JEOL, Tokyo, Japan) after negative staining with 2.5% samarium triacetate [12]. TEM revealed that MopsHU1 is a podovirus composed of a head and a short tail (Fig. 1B), 57 ± 2.7 nm and 12 ± 1.9 nm long, respectively.

Adsorption and one-step growth curves were obtained using an *M. psychrotolerans* Mps.8 culture that was incubated at 25 °C until OD_{600} reached 0.2. For the adsorption assay, the fresh culture was centrifuged (10,000 × *g*, 2 min, 4 °C) and washed with fresh TSB preincubated at 25 °C. An aliquot (50 µL) of the MopsHU1 suspension was inoculated into 5 mL of the bacterial culture to obtain 1×10^5 PFU/mL of phage titer. The inoculum was incubated at 25 °C, and 100 µL of this inoculum was mixed with SM buffer supplemented with chloroform. The number of free phages was determined using the double agar overlay method, as described above. The adsorption assay revealed that MopsHU1 could rapidly adsorb to host cells (Fig. 1C) at an adsorption rate constant of $3.44 \times 10^{-9} \pm 3.28 \times 10^{-10}$.

For the one-step growth curve analysis, 10 mL of the fresh bacterial culture (OD_{600} = 0.2) was inoculated with 100 µL of the MopsHU1 suspension for a concentration of 1×10^5 PFU/mL. After incubation at 25 °C for 5 min, phages adsorbed to cells were collected by centrifugation (10,000 × *g*, 2 min, 4 °C), and the bacterial pellet was suspended in 10 mL of fresh TSB preincubated at 25 °C. The culture was incubated at 25 °C, and 100 µL of the sample was collected. The phage titer was determined using the double agar overlay method, as described above. The phage titer of a sample treated with chloroform was also determined. From the one-step growth curves, the eclipse and latent periods of MopsHU1 were estimated to be at 10 and 20 min, respectively, whereas a burst size of 70 PFU per infected cell was determined (Fig. 1D).

Genome sequence analysis of MopsHU1 was performed after extracting the genetic material of the virus by the method of Jakočiūnė and Moodley [13] with minor modifications. Briefly, to remove bacterial DNA and RNA, 400 µL of the MopsHU1 suspension (approximately 10^{12} PFU/mL) was treated with DNase I and RNase A (Nippon gene, Tokyo, Japan) at 37 °C for 30 min, and 20 µL of 500 mM EDTA was added. Next, to digest the phage capsid, 20 µL of proteinase K (Qiagen, Hilden, Germany) was added, and the mixture was incubated at 56 °C for 1 h. Then, MopsHU1 DNA was extracted and purified using a DNeasy Blood & Tissue kit (Qiagen), and sequenced using a NovaSeq 6000 system (Illumina, San Diego, CA, USA). Quality trimming was conducted using fastp [14], and *de novo* assembly was conducted using SPAdes [15]. Genome termini were analyzed by inspection using read mapping and PhageTerm [16]. The MopsHU1 genome was automatically annotated using RAST [17], and the results were inspected using GeneMarkS [18] and blastp [19]. tRNAs were searched using tRNAscan-SE 2.0 [20]. The overall genome identity between MopsHU1 and its most related phage was calculated by multiplying the query coverage by the identity percentages using blastn [21]. Intergenomic similarities between *Studiervirinae* phages were calculated using VIRIDIC [22], and a comparative analysis was conducted using GenomeMatcher [23].

The results indicated that the MopsHU1 genome is 39,061 bp in length, with 51.2% GC content, and has 195 bp of short direct repeats in both genome termini. In addition, 48 ORFs were predicted and the functions of 27 of these ORFs were estimated. tRNAs were not detected, and genes associated with lysogeny, antibiotic resistance, and toxins were not found, either. These results suggest that MopsHU1 is a virulent phage. In addition, MopsHU1 encodes a DNA-dependent RNA polymerase gene characteristic of *Autographiviridae* phages. A blastn search indicated that *Citrobacter* phage SH3 was the most closely related phage to MopsHU1, with an overall genome identity of 89.7% (identity 93.40%, query cover 96%). This suggests that MopsHU1 belongs to the same genus as SH3 (family *Autographiviridae*, subfamily *Studiervirinae*, genus *Kayfunavirus*). Accordingly, VIRIDIC analyses also indicated that the intergenomic similarities between MopsHU1 and other *Kayfunavirus* phages were high, and that SH3 was the most similar phage (89.6%) (Fig. 2). Notably, this sequence similarity was lower than the species threshold but higher than the genus threshold [24], indicating that MopsHU1 is a new member of the genus *Kayfunavirus*, infecting *M. psychrotolerans*. MopsHU1 genome did not show

high similarity to those of *M. morganii* phages belonging to the genus *Minipunavirus*, MP2 [8] and MmP1 [11], confirming that MopsHU1 belongs to a different genus (Fig. 2).

The MopsHU1 genome was compared with those of other *Kayfunavirus* phages (Fig. 3). Overall, the MopsHU1 genome was similar to those of *Citrobacter* phage SH3 (KU687349), *Cronobacter* phage Dev2 (NC_023558), and *Escherichia* phage K1F (NC_007636), confirming that they all belong to the same genus. The amino acid sequence of the tailspike of MopsHU1 (ORF42) was similar to those of the tail fibers of *Citrobacter* phage SH3 and *Cronobacter* phage Dev2, although the host bacteria of these phages are different. The host range of *Cronobacter* phage Dev2 was limited to three *C. turicensis* serotype CT O:1 strains and one *C. malonaticus* serotype CMa O:2 strain, among 54 *Cronobacter* strains, and the tail fiber of Dev2 was estimated to bind the LPS O-antigen [25]. The tailspike protein of *E. coli* phage K1F, which has endosialidase activity, showed no sequence similarity to the corresponding MopsHU1 sequence, except for its N-terminal region (Fig. 3).

We predicted that ORF42 of MopsHU1 encodes a receptor-binding protein that may be a tailspike protein because CD-Search analysis [26] revealed that the product of this gene possesses a SGNH_hydrolase domain. In general, tailspike proteins exhibit enzymatic activities useful for interacting with bacterial surfaces [27]. Homologous genes of SH3 and Dev2 have been annotated as tail fibers, and a SGNH_hydrolase domain has been detected in the predicted products of the corresponding tail fiber genes [25, 28]. This domain is found in a diverse family of lipases and esterases, and in some phage proteins. The *Schitoviridae* phage G7C tailspike protein, containing a SGNH_hydrolase domain, has been revealed to function as an esterase that removes the O-acetyl group from *E. coli* 4s LPS [29]. The narrow host range and the presence of the SGNH_hydrolase domain in the tailspike protein of MopsHU1 suggest that LPS is involved in host cell adsorption of MopsHU1.

In this study, we characterized MopsHU1, a novel *M. psychrotolerans* phage. This phage is a new member of the genus *Kayfunavirus*. The genome sequence and biological characteristics of MopsHU1 will enhance our knowledge of *Kayfunavirus* phages that infect *Morganella* spp.

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Author contributions

All authors contributed to the study conception. The study design, data acquisition, and analysis were performed by Shogo Yamaki. The draft of the manuscript was written by all authors. All authors read and approved the final manuscript.

Conflicts of Interest

The authors declare no conflicts of interest.

Data availability

The data on this study are available from the corresponding author on reasonable request.

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239 Figure legends

240

241 Fig. 1. Biological characterization of MopsHU1. (A) Plaque morphology. (B) TEM images of MopsHU1.
242 (C) Adsorption of MopsHU1 on *M. psychrotolerans* Mps.8. The free phage proportion was calculated
243 as the phage titer at each time (P) divided by the phage titer at 0 min (P_0). Bacterial cell-free TSB was
244 used for the determination of P_0. (D) One-step growth curve of MopsHU1. Triangle and circle symbols
245 indicate phage titers with (△) or without (○) chloroform treatment. (C) and (D), experiments were done
246 in triplicate; error bars represent standard deviations.

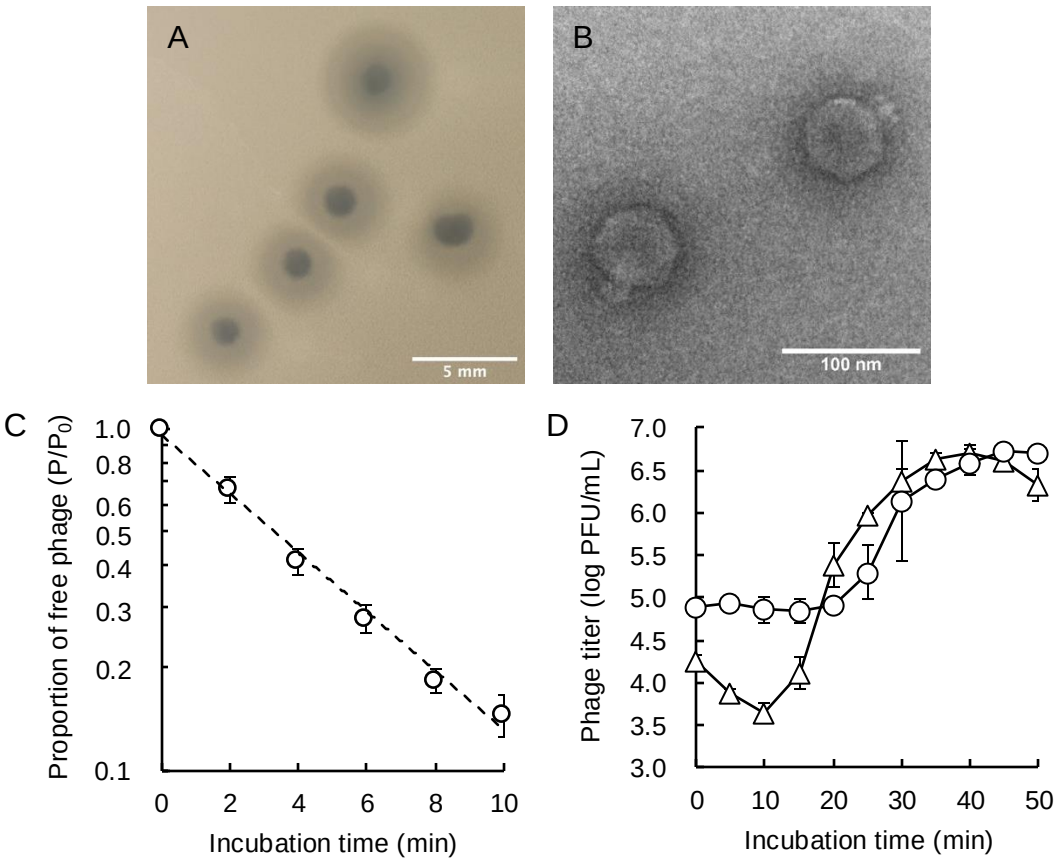
247

248 Fig. 2. Intergenomic similarities among phages belonging to the subfamily *Studiervirinae*, family
249 *Autographiviridae*. VIRIDIC was used for calculating intergenomic similarities and generating a
250 heatmap [22]. The right half of the heatmap shows intergenomic similarity values (%) for each phage
251 pair. The left half of the heatmap shows three alignment indicator values: the aligned genome fraction
252 for the phages found in each row (top) or column (bottom), and the genome length ratio for each phage
253 pair (center).

254

255 Fig. 3. Comparative analysis between the MopsHU1 genome with those of related phages belonging to
256 the genus *Kayfunavirus* (*Citrobacter* phage SH3, *Cronobacter* phage Dev2, and *Escherichia* phage K1F).
257 The tail fiber/tailspike genes are shown as blue ORFs. The analysis was conducted using
258 GenomeMatcher [23]; the alignment length and identity score thresholds were set at 20% and 30% for
259 visualization.

Fig. 1


A
5 mm
B
100 nm
C
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2
4
6
8
10
Incubation time (min)
D
Phage titer (log PFU/mL)
7.0
6.5
6.0
5.5
5.0
4.5
4.0
3.5
3.0
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20
30
40
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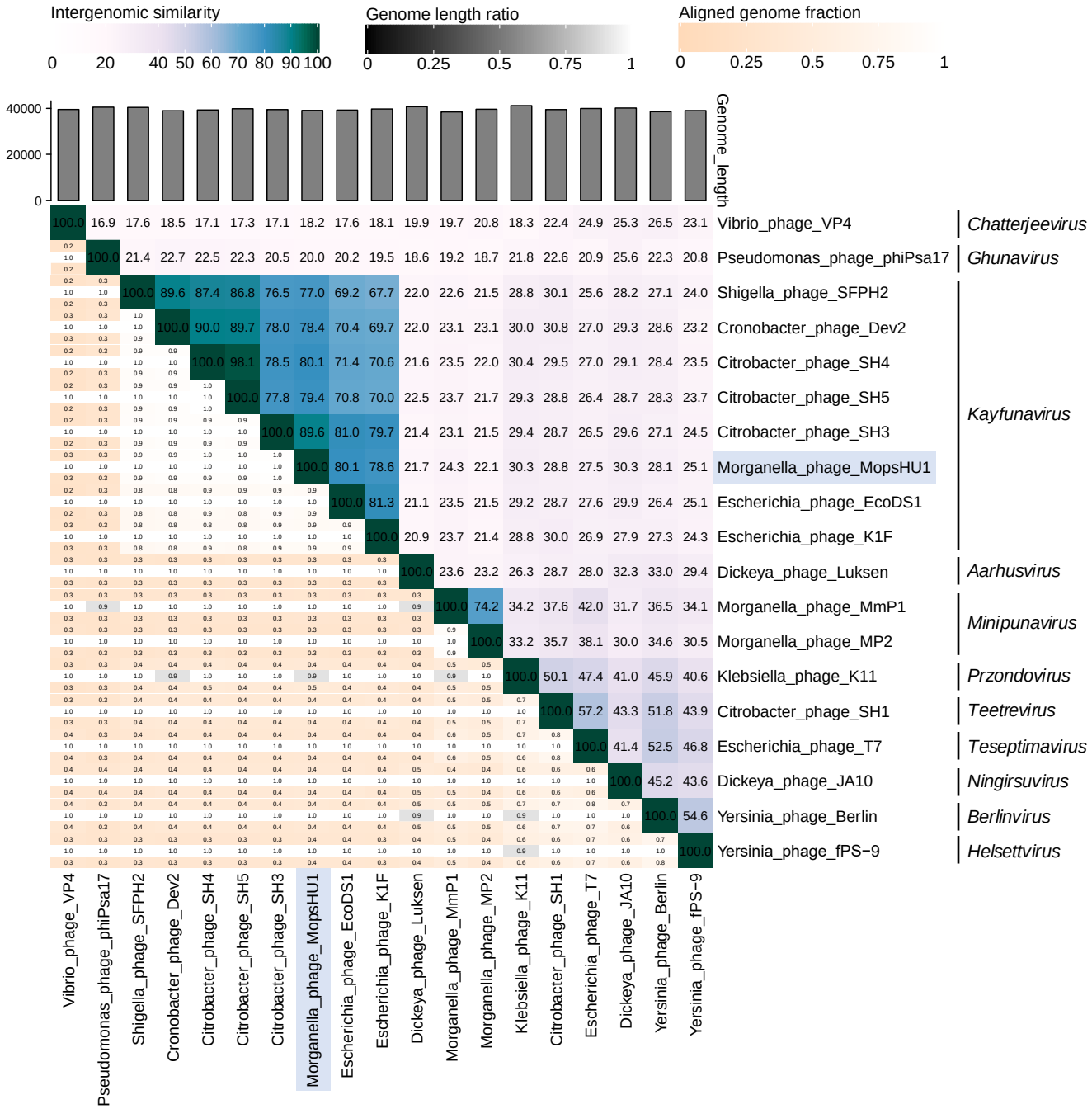
Incubation time (min)

Fig. 2


Intergenomic similarity
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Genome length ratio
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Aligned genome fraction
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Genome_length
Vibrio_phage_VP4
Pseudomonas_phage_phiPsa17
Shigella_phage_SFPH2
Cronobacter_phage_Dev2
Citrobacter_phage_SH4
Citrobacter_phage_SH5
Citrobacter_phage_SH3
Morganella_phage_MopsHU1
Escherichia_phage_EcoDS1
Escherichia_phage_K1F
Dickeya_phage_Luksen
Morganella_phage_MmP1
Morganella_phage_MP2
Klebsiella_phage_K11
Citrobacter_phage_SH1
Escherichia_phage_T7
Dickeya_phage_JA10
Yersinia_phage_Berlin
Yersinia_phage_fPS−9
Chatterjeevirus
Ghunavirus
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Ningirsuvirus
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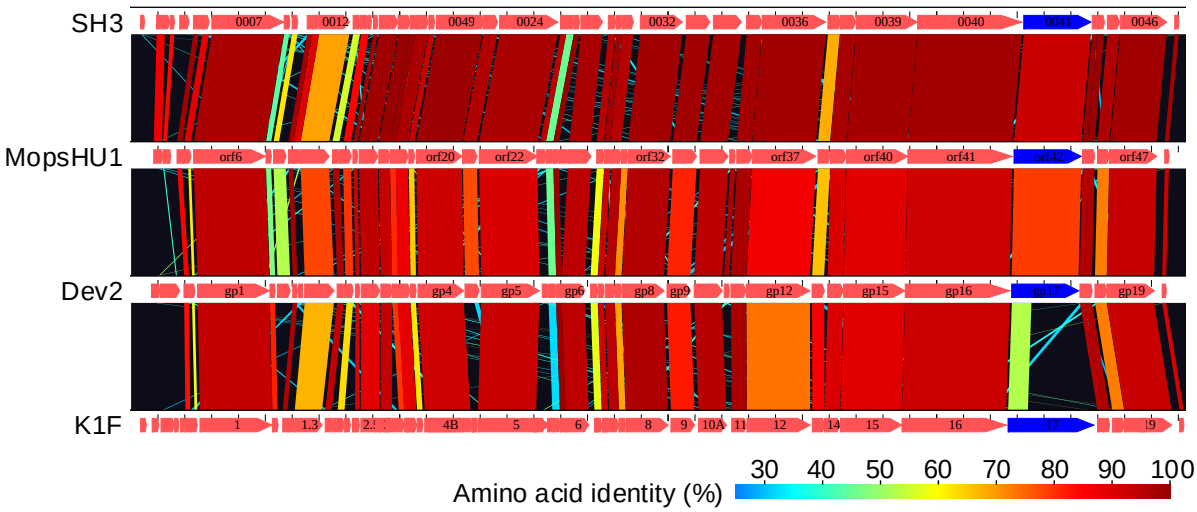
Helsettvirus

Fig. 3


SH3
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Dev2
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gp12
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gp17
gp19
K1F
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1.3
4B
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80
90
100
Amino acid identity (%)