



Comparison of the genomes of *Vibrio* phages pVco-5 and pVco-7: implications for phage-host interactions

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Received: 20 April 2025 / Accepted: 27 June 2025 / Published online: 27 August 2025
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Abstract

Oyster hatcheries suffer significant mortality caused by members of the bacterial genus *Vibrio*, which infect *Crassostrea gigas* larvae. Bacteriophages present a potential alternative to antibiotics, with N4-like phages showing promise against vibriosis. We investigated the adsorption properties and determined the complete genome sequence of phage pVco-7 and performed phylogenetic and comparative analysis with other N4-like *Vibrio* phages to gain insights into phage resistance mechanisms and infection strategies. The phage has a linear dsDNA genome with 126 genes and a typical N4-like organization. Tail fiber variations were identified that could potentially influence host recognition, providing insights into how phages of the genus *Vicoquintavirus* interact with their host as well as their evolutionary relationships. These findings will contribute to the development effective biocontrol strategies against vibriosis in commercially important bivalve aquaculture.

Keywords Vibriosis · *Vibrio coralliilyticus* · Bacteriophage · Phage resistance · N4-like phage · *Crassostrea gigas*

Handling Editor T. K. Frey

Nucleotide sequence data reported here are available in the GenBank database under the accession number PP107878.

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Introduction

Marine bivalves, like fish, crustaceans, and seaweed, are very important in aquaculture. According to the Food and Agriculture Organization (FAO) of the United Nations, the global production of cultured bivalves was 16.71 million tons in 2021, resulting in revenue of USD 28.55 billion [1]. Oysters account for 39.9% of total bivalve production and are the most popular and widely consumed bivalves worldwide [1]. To ensure a stable supply of seeds of various bivalves, shellfish with high fishery value are produced in hatcheries. During this process, mass mortality of bivalve larvae occurs due to infection by various pathogens, including *Vibrio coralliilyticus* [2–4].

V. coralliilyticus causes tissue lysis and loss of coral reefs, and it infects and kills fish and bivalve larvae [5–7]. Damage caused by *V. coralliilyticus* infections results in significant losses in marine bivalve hatcheries, and antibiotics, probiotics, and bacteriophages are applied to prevent *V. coralliilyticus* infection [8–11]. Phages have been proposed as an alternative to antibiotics [12, 13] and have been applied to prevent vibriosis in marine bivalve larvae [9, 14, 15]. A combination of various phages applied as a cocktail has become the preferred approach to reduce therapeutic failure and prevent the emergence of phage resistance [16–18].

A deep understanding of the interactions between phages and their host bacteria could lead to an optimized strategy against vibriosis in the aquaculture industry.

In the present study, we sequenced the genome of *Vibrio* phage pVco-7 and conducted a comprehensive analysis of its genomic features. We compared these features with those of its close relative, pVco-5, with the aim of understanding the emergence of phage resistance and the infection strategy of members of the genus *Vicoquintavirus*.

Materials and methods

Isolation of bacteriophages

Phages infecting *V. coralliilyticus* were isolated as described previously [9, 10]. Bacterial cultures in the exponential growth phase were mixed with environmental samples in equal proportions. The resulting coculture was incubated at 27°C for 24 h to enrich the phage population. The culture was then centrifuged and serially diluted from 10^0 to 10^{-7} and applied onto bacterial lawns to verify the presence of enriched phages. The samples of the resulting plaques were filter sterilized (0.2 µm) to eliminate any remaining bacteria. A conventional double-layer agar assay was performed to confirm the presence of phage plaques. Single phages were obtained by five cycles of plaque isolation.

Propagation and purification of bacteriophages

Phages were propagated as described previously [19]. Overnight bacterial cultures were inoculated with phages at a multiplicity of infection (MOI) of 0.01 and cultured at 27°C for 24 h. After centrifugation at 10,000 g for 20 minutes, NaCl was added to the supernatant to a final concentration of 0.5 M. The mixture was centrifuged again, followed by the addition of polyethylene glycol 8000 to precipitate phage virions. CsCl gradient ultracentrifugation was performed at 50,000 g for 3 h using a Type 70 Ti-fixed-angle titanium rotor (Beckman Coulter, CA, USA). The bluish-white band containing phage particles was collected and subjected to dialysis (7,000 MWCO membrane; Thermo Fisher Scientific, MA, USA). Purified phages were stored at 4°C for further use.

Transmission electron microscopy

Transmission electron microscopy was performed as described previously [20]. Purified phage samples were spotted onto a copper grid, and after 1 min, the excess sample was removed and 2% uranyl acetate was applied to negatively stain the phages on the grid. After removing

the remaining solution, the grids were air-dried and examined using a Talos L120C transmission electron microscope (FEI, OR, USA) operating at 120 kV for morphological analysis of the phages. Three biological replicates were used for measuring phage dimensions.

Adsorption assay

The adsorption kinetics of the N4-like *Vibrio* phages pVco-5 and pVco-7 were evaluated as described previously [13]. Wild-type *V. coralliilyticus* 58 and the pVco-7-resistant mutant R7 were used as host strains to quantify unadsorbed free phages. A bacterial suspension (1.5×10^7 CFU/mL) was infected with phage at a multiplicity of infection of 0.001 and incubated at 27°C. At 0, 5, 10, 15, 20, and 30 minutes postinfection, samples were collected and centrifuged at 12,000 g for 5 minutes. Unadsorbed phages in the resulting supernatants were quantified using the double-layer agar method, employing wild-type *V. coralliilyticus* 58 as the host.

DNA isolation

Genomic DNA was extracted from *Vibrio* phage pVco-7 as described previously [21]. Ten units of RNase A and DNase I was added to 1 mL of phage suspension, which was then incubated at 37°C for 1 h. The reaction was stopped using 0.5 M ethylenediaminetetraacetic acid (EDTA), and proteinase K was used to digest viral proteins. Phenol, chloroform, and isoamyl alcohol were then added in a 25:24:1 ratio, followed by centrifugation at 12,000 g for 10 min at 4°C. Ice-cold absolute ethanol was then added, and the suspension was centrifuged again. The resulting pellet was washed twice with 70% ethanol and resuspended in nuclease-free water. The genomic DNA preparation was stored at −20°C.

DNA sequencing and genomic analysis

Genomic DNA of pVco-7 was sequenced using an Illumina HiSeq system (Illumina, San Diego, CA, USA) at Macrogen (Seoul, Republic of Korea), and the sequence was assembled using SPAdes v.3.12 [22]. Open reading frames were predicted using Prokka v.1.12b, GenMarkS, RAST, BLAST, and HHpred [23–26]. tRNAs were identified using ARAGORN and tRNAscan-SE v.2.0 [27, 28]. Whole-genome-sequence dot plots and phylogenetic trees were generated using Gepard (word size, 10) and Virus Classification and Tree Building Online Resource (VICTOR) [29, 30]. A comparative analysis was performed using the Coregenes 5.0 average nucleotide identity calculator (<https://www.ezbiocloud.net/tools/ani>) and Clinker with default settings [31–33].

Nucleotide sequence accession number

The complete genome sequence of *Vibrio* phage pVco-7 was deposited in the GenBank database under the accession number PP107878.

Results and discussion

Biological characteristics of vicoquintaviruses

Previously, we investigated the potential applicability of phage pVco-7 for control of *Vibrio* infections in oyster larvae, both alone and in combination with the *Vibrio* phages pVco-5 and pVco-14 [10], and we reported that pVco-7 showed biological characteristics similar to those of pVco-5, including virion and plaque morphology, stability against environmental stresses (pH and temperature), bactericidal properties, and host range (including plating

efficiency). There were no discernible morphological differences observed between the two N4-like phages (Fig. 1). Interestingly, the only notable difference observed was the protective effect against vibriosis in bivalve larvae, which correlated with variations in the rate of emergence of bacteriophage resistant mutants. This may be attributed to the higher contact frequency (i.e., adsorption efficiency) of pVco-7, compared to its close relative pVco-5, which could increase the likelihood of the emergence of resistant mutants (Fig. 2A) [34]. One mutant exhibiting resistance to pVco-7, designated R7, showed no adsorption of pVco-7, whereas pVco-5 maintained adsorption capability comparable to that of wild-type *V. coralliilyticus* (Fig. 2B). This suggests that resistance to pVco-7 in R7 is likely to involve a change in a bacterial surface receptor that is essential for phage binding [35]. To further explore potential differences in phage-host interactions, we compared the genomes of pVco-7 and pVco-5, focusing on genes potentially involved in host recognition.

Fig. 1 Transmission electron microscopic image showing the *Vibrio* phage pVco-5 (A) and pVco-7 (B). Bar, 50 nm

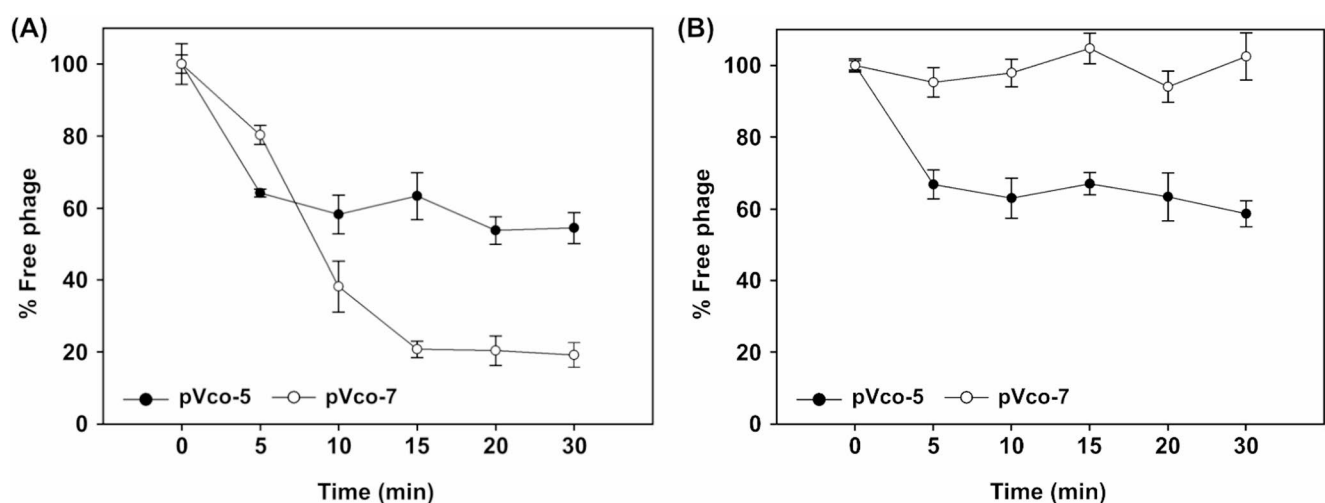
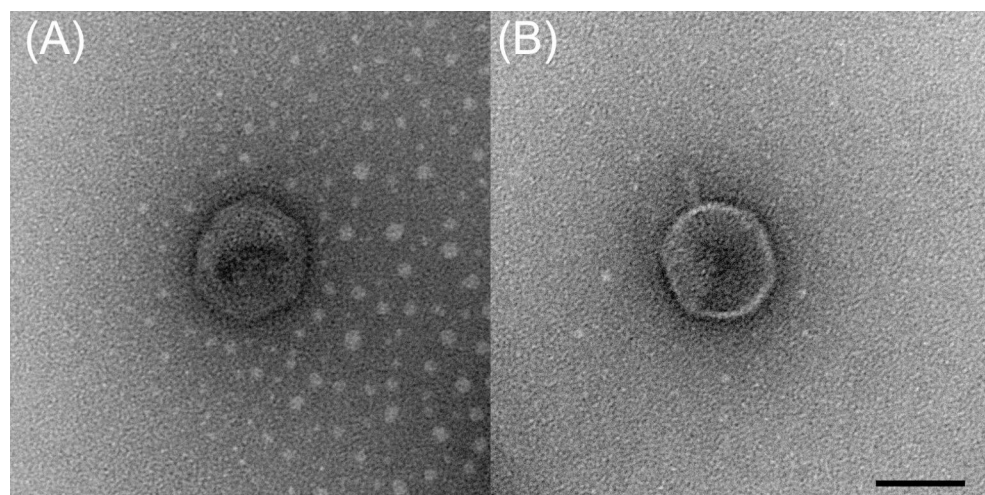


Fig. 2 Adsorption efficiency of *Vibrio* phages pVco-5 and pVco-7 on wild-type *V. coralliilyticus* (A) and pVco-7-resistant mutant R7 (B)

Genomic organization of vicoquintaviruses

A total of 16,051,880 sequence reads were processed, generating 1,621,239,880 base pairs (bp) of sequence data and resulting in the assembly of single contigs consisting of double-stranded DNA. Assembly of the raw reads showed that the pVco-7 genome is a linear DNA molecule consisting of 75,034 base pairs with a GC content of 38.7% and containing 126 genes (Fig. 3). Its features are similar to those of its closest relative, pVco-5, a member of the genus *Vicoquintavirus* that contains 127 genes in its 75,034-bp linear double-stranded DNA (38.7% GC content) [21]. A summary of a comparison of the genomic features of pVco-7 and pVco-5 is provided in Table 1. The genome of N4-like viruses consists of three main parts: the early and middle genes occupy the left half of the genome, whereas the late-phase genes are located in the right half, with the virion-associated RNA polymerase (vRNAP) acting as a boundary, positioned in the opposite orientation [36]. Interestingly, the genome arrangement of vicoquintaviruses displays a unique organization reminiscent of the N4-like structure. A gene cluster orthologous to the virion structural protein is encoded in the middle of the middle genes, leading to the division of the genome into four regions. The first right-oriented region (nt 1–25,897) includes 82 predicted open reading frames (ORFs) that are similar to those of the early and middle genes of N4 phages, encoding endonuclease VII, RNAPI, and RNAPII. Early transcription of N4-like viruses is initiated by vRNAP, which is injected with phage genomic DNA [37]. This process is prolonged with the aid of RNAPI and RNAPII for transcription of the middle genes [38]. An enzyme involved in host cell lysis, N-acetylmuramoyl-L-alanine amidase, is encoded in this region. The second region, spanning nt 26,043–32,235, containing ORFs that are transcribed in the leftward direction, encoding gp83, gp84, gp85, and gp86. Although their functions are difficult to predict, this region represents a unique genomic cluster found in phages of the genus *Vicoquintavirus* and their close relative, phage PhilmVa-1. Homology searches using the Prokaryotic Virus Remote Homologous Groups (PHROGs) database [39] suggested that these genes are likely to encode proteins that are orthologous to phage structural proteins such as tail proteins. Interestingly, gp86 is highly conserved in T5-like *Vibrio* phages such as Chloris, JAVIER, and Wyman (which share an N-terminal ortholog), vB_VpM-pA2SJ1, Ceto, and pVp-1 (with a C-terminal

Table 1 Genomic features of pVco-7 and pVco-5

	pVco-7	pVco-5
Accession number	PP107878.1	KY612839.1
Genome size	75,034 bp	74,325 bp
GC content	38.7%	38.72%
ORFs	127	125
- Structure & packaging	10	10
- Nucleotide metabolism	18	18
- Lysis	1	1
- Hypothetical protein	98	96
Shared core genes	123	
Average nucleotide identity	98.51%	

ortholog), and Athena, IW18, and Gary (which share both N- and C-terminal orthologs). The *Vibrio* phage CHOED is a phage with podovirus morphology, like those analyzed in the present study [40]. This cluster may represent an evolutionary link within diverse mosaic phage genomes [41]. A total of 24 ORFs were predicted in the third region (nt 32,427–48,611), which are involved in the replication of phage genomic DNA, including DNA helicase (gp94), DNA polymerase (gp96), and DNA primase (gp101). An N4-like single-strand DNA-binding protein (gp104) is located in the right half of the genome for transcription of late genes [42]. The fourth region (nt 48,640–75,022) encompasses 17 genes that are mostly encoded in a leftward orientation. These ORFs were predicted to be involved in packaging and encode a terminase large subunit (gp125), a portal protein (gp120), structural proteins (major capsid protein, gp117; tail proteins, gp122, and gp123), and vRNAP (gp111), which is a signature feature of N4-like viruses [36]. Detailed annotations are provided in Supplementary Table S1.

Phylogenetic analysis of vicoquintaviruses

Several complete genome sequences of *Vibrio* phages belonging to the family *Schitoviridae* are available, including those of phages that infect various *Vibrio* species. Among these are the phages, pVco-5 and pVco-7, which specifically target *V. coralliilyticus*, and phages infecting other *Vibrio* species, including *V. alginolyticus* (BUCT194 and PhilmVa-1), *V. cholerae* (phi 1, 12VC501, VCO139, JSF3, and JA-1), *V. parahaemolyticus* (VBP32, VBP47, and PJN101), *V. owensii* (vB_VspP_SBP1 and phi50-12), and *V. splendidus* (vB_VspP_pVa5). In addition, whole-genome sequences of phages targeting *V. hangzhouensis* (vB_VhaP_PG11) are available. Phylogenetic analysis based on

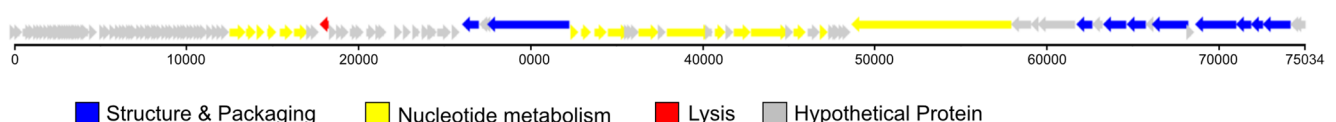


Fig. 3 Genomic map of *Vibrio* phage pVco-7. The open reading frames (ORFs) are grouped according to their predicted functions. Four categories are represented: structure and packaging (blue), nucleotide metabolism (yellow), lysis (red), and hypothetical proteins (gray)

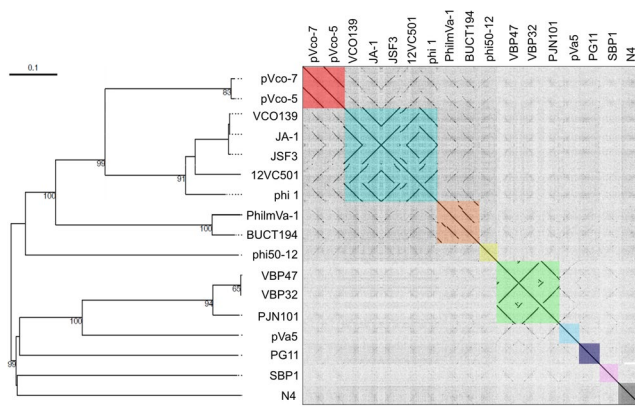


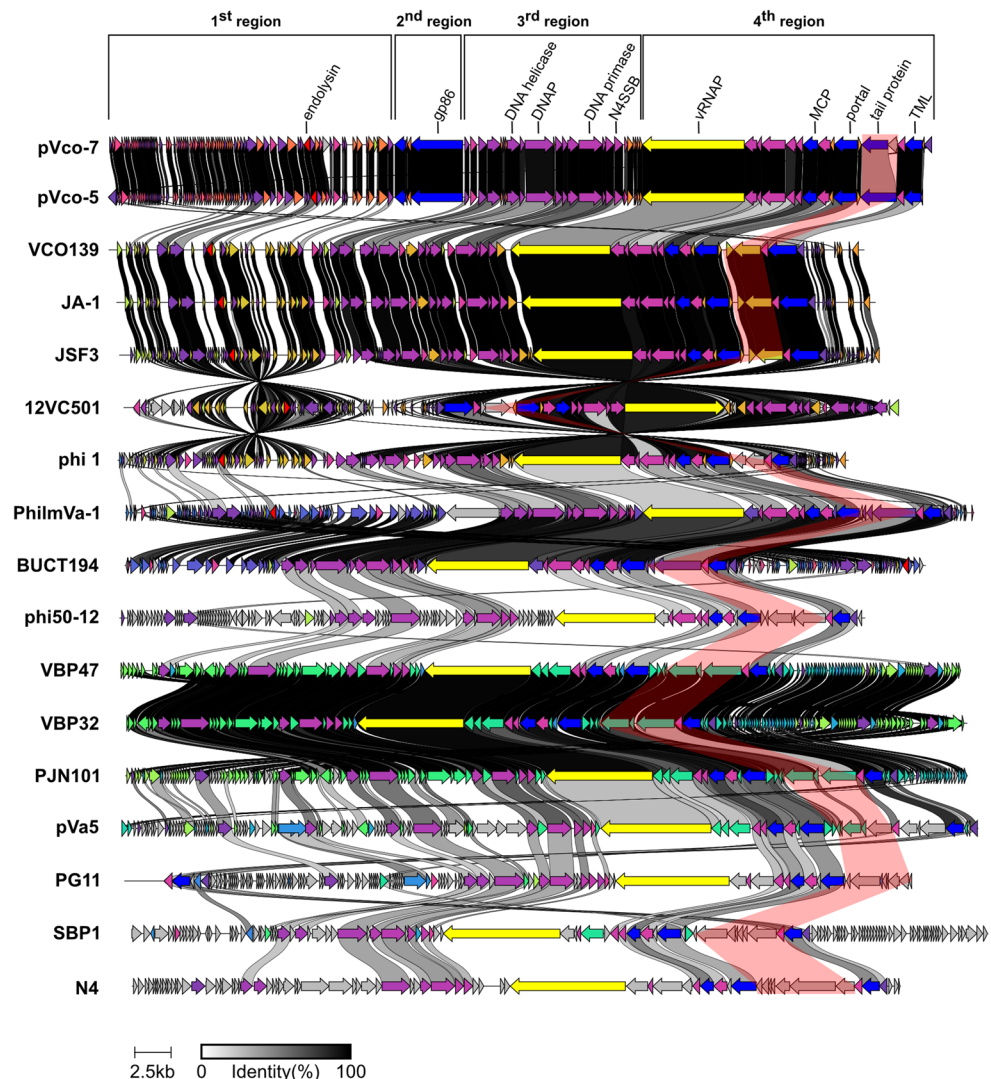
Fig. 4 Phylogenetic and dot plot analysis of whole genomes of pVco-7 and N4-like *Vibrio* phages. pVco-7 is indicated by a black arrowhead (►). The genera are differentiated by color: *Pacinivirus*, cyan; *Vicoquintavirus*, red; unclassified *Schitoviridae* I, orange; *Penintadodekavirus*, yellow; *Stoningtonvirus*, green; *Galateavirus*, blue; unclassified *Schitoviridae* II, navy; *Electraviridae*, purple; *Enquatrovirus* (N4), black

whole-genome sequences and a dot-plot assay were used to classify the phages (Fig. 4). The analysis included phage N4, a distantly related member of family *Schitoviridae*. A comparative genomic analysis is shown in Fig. 5. Notably, virion-associated RNA polymerase, a distinguishing characteristic of members of the family *Schitoviridae*, exhibited low intraspecies but high interspecies similarity. This gene could therefore be used for phylogenetic analysis of *Vibrio* phages in the family *Schitoviridae* [43].

Comparative analysis of tail fiber genes of members of the genus *Vicoquintavirus*

The intrinsic host specificity of bacteriophages originates from their tail fibers [44], and the genomic region encoding tail-fiber-associated genes exhibits the highest variability (Fig. 5). Significant variability was observed in the *V. coralliilyticus* phages pVco-5 and pVco-7. The tail fiber protein

Fig. 5 Comparison of gene clusters of pVco-7, pVco-5, and N4. The analysis was performed using Clinker (threshold, 0.3). Homology is represented by lines, with the shading intensity corresponding to the degree of sequence similarity. DNAP, DNA polymerase; N4SSB, N4-like single-stranded DNA-binding protein; vRNAP, virion-associated RNA polymerase; MCP, major capsid protein; TML, terminase large subunit



of pVco-5 (gp122) is encoded by a single gene, while its counterpart in pVco-7 is divided into two genes (gp122 and 123) by a frameshift due to the insertion of a cytosine 885 nucleotides downstream of gp123 (Fig. 5). Considering the role of the C-terminus of the tail protein in host recognition, this frameshift might affect the binding properties of pVco-7 [45]. This might result in reduced competitiveness of pVco-7 against other resistant strains, as we reported previously [10]. Indeed, it has been observed that no resistant strains emerge after treatment with pVco-5 of *V. coralliilyticus*, while resistant colonies do emerge after pVco-7 treatment (unpublished data). Furthermore, the bactericidal and preventive effects of pVco-5, which is presumed to use the same host receptor on the R7 strain, were partially compromised, although complete resistance was not observed [10]. Further studies incorporating structural analysis or targeted mutagenesis are needed to test the hypothesis that variations in the C-terminus of the tail protein (gp123) and related proteins contribute to host recognition, specificity, and adsorption efficiency. These findings also underscore the evolutionary importance of tail fiber regions as hotspots for adaptive change [46]. The observed frameshift mutation in pVco-7, which results in a split tail fiber gene, might provide a selective advantage for the phage [47, 48]. This supports the view that the modularity of the tail fiber contributes significantly to the rapid evolution of host specificity in N4-like viruses [49].

While our study provides valuable insights into the genomic differences and host interactions of two N4-like vicoquintaviruses, these are based primarily on *in vitro* assays and bioinformatic predictions, and their functional relevance remains to be confirmed in a real-world aquaculture setting. *In vivo* challenge experiments using oyster larvae would be an appropriate next step to validate these findings. Moreover, the cross-sectional nature of our genomic analysis prevents causal inference. Therefore, future longitudinal or experimental approaches, such as mutagenesis or structural studies of tail fiber proteins, are needed to test hypotheses regarding host specificity and resistance.

In conclusion, a lack of information on N4-like *Vibrio* phages of the family *Schitoviridae* poses a challenge for treating vibriosis in marine bivalves, particularly oysters. The variation in the tail fiber gene of pVco-5 and pVco-7 highlights the evolutionary plasticity of receptor-binding proteins in N4-like phages, providing insights into the coevolutionary strategies phages employ to persist within dynamic microbial communities [34]. The findings of this study provide fundamental insights into the host recognition strategies used by vicoquintaviruses, the evolutionary characteristics of *Vibrio* phages, and their potential as alternatives to conventional antibiotic-based treatments [50].

Supplementary Information The online version contains supplementary material available at <https://doi.org/10.1007/s00705-025-06386-3>.

Acknowledgements This research was supported by the Basic Science Research Program through the National Research Foundation of Korea (NRF), funded by the Ministry of Education (RS-2022-NR075245), and a National Research Foundation of Korea (NRF) grant, funded by the Korean government (MSIT) (RS-2021-NR062056).

Author contributions Hyoun Joong Kim: conceptualization, data curation, formal analysis, investigation, methodology, writing—original draft, writing—review and editing. Jun Kwon: data curation, formal analysis, investigation, methodology, writing—review, and editing. Chan Hee Lee: data curation, formal analysis, and validation. Ji Hyung Kim: conceptualization, formal analysis, validation, writing—review and editing. Se Chang Park: conceptualization, data curation, formal analysis, supervision, writing—review and editing. Sang Guen Kim: conceptualization, data curation, formal analysis, investigation, methodology, project administration, software, supervision, validation, visualization, writing—original draft, writing—review and editing, funding acquisition

Data availability All data generated or analyzed during this study are included in this published article and the supplementary information files.

Declarations

Ethical approval Not required

Conflict of interest The authors have no competing interests to declare that are relevant to the content of this article.

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