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Evaluation of the Viral Diversity of Artemia Cysts from Saline Lakes in Kazakhstan Using Viral Metagenomics Analysis

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Abstract: Artemia (brine shrimp) holds significant value as a live feed for larval fish and crustaceans, owing to their distinctive dietary requirements. However, it is vital to acknowledge that Artemia also carries potential risk as a vector of infection. We conducted a metagenomic analysis to explore the virome present in Artemia cysts collected from inland salt lakes across four distinct regions in Kazakhstan. This study identified the presence of dsDNA phages and RNA virus sequences, with a predominant representation from the *Reoviridae*, *Nodaviridae*, *Dicistroviridae*, *Picornaviridae*, *Astroviridae*, *Tombusviridae*, and *Solemoviridae* families. In general, this study has significantly enhanced our understanding of the virome of Artemia cysts in the saline lakes of Kazakhstan; however, the interactions between these putative viruses and brine shrimp and other aquatic animals need further research.

Keywords: Artemia (brine shrimp); Artemia cysts; metagenomics analysis; viral diversity; saline lakes

Key Contribution: This study assesses the viral diversity of Artemia cysts from saline lakes in Kazakhstan to address a knowledge gap in this area. Thus, it provides valuable insights for further research aimed at investigating the evolutionary dynamics of viruses in their natural reservoirs.

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1. Introduction

Viruses are extensively dispersed and abundant entities on Earth, particularly in both marine and freshwater environments [1–3]. The utilization of a conventional culture-based technique in the investigation of environmental viruses frequently leads to an underestimation of their prevalence and ecological importance [4]. However, the emergence of metagenomic analysis provided a reliable and efficient method for identifying genetic material from individual or pooled samples within a significantly shortened period [5,6]. Also, this methodology enables the discernment of known and novel viral species, thereby enabling the exploration of ecological associations between microorganisms and their respective natural environments [7,8]. Using metagenomics, we obtain deeper insights into the variety, dynamics, and ecological functions of viruses, enriching our knowledge of their influence on ecosystems.

In recent years, due to the significant impact of viruses on microbial communities and ecosystem functions, researchers have become increasingly interested in investigating the diversity of viruses in salt lakes [9–11]. Salt lakes are fascinating ecosystems characterized by high salt concentrations and unique physical and chemical properties [12]. These saline systems are distributed around the world, with prominent examples including the Great Salt Lake in Utah [13], the Dead Sea in the Middle East [14], and the Aral and Caspian Seas in

Central Asia [15,16]. Hypersaline viral communities across different salinity environments exhibit a remarkable degree of genetic consistency, indicating the formation of a cohesive community [10]. Moreover, viral populations exhibit similarities to the viruses found in hypersaline ponds and lakes that possess diverse salinity levels [9,17]. The awareness of the variety of viruses present in saline water environments contributes to our understanding of the intricate associations between viruses and their hosts, thereby unveiling the ecological dynamics that influence these exceptional habitats.

Artemia, also known as brine shrimp, is a small crustacean that lives in global saline water environments, and this species contributes to upholding ecosystem equilibrium and productivity [18] while also providing sustenance for a wide array of aquatic organisms that rely on these habitats for survival [19–21]. Kazakhstan is an important player in the production of Artemia cysts. Its bitter-salty water basins contain substantial Artemia cyst reserves, accounting for 15 to 20% of the world's total production (1.0–2.0 thousand tons per year) and making it one of the leading producers [22]. The Pavlodar region is the main provider of Artemia cysts, accounting for approximately 73.2–76.3 percent of the country's total. It is estimated that Kazakhstan's vast saltwater bodies could generate approximately 11,000 tones of Artemia cysts annually. This highlights Kazakhstan's crucial involvement in the global supply of Artemia cysts to support aquaculture.

Artemia, despite its widespread use as a live feed for fishes, shrimps, and mollusks, presents a potential concern as both a reservoir and vector for some viruses [23,24]. Several cases have proven that Artemia serves as a mechanical vector of fish pathogens, such as lymphocystis disease virus (LCDV) [24], natural nerve necrosis virus (NNV) [25], and *Vibrio anguillarum* [26]. These diseases have the ability to impact a wide range of fish species inhabiting freshwater, brackish, and marine environments [27,28]. Recent research has revealed the ability of Artemia to serve as a vector for some types of crustacean viruses, such as infectious myonecrosis virus (IMNV), white spot syndrome virus (WSSV), and hepatopancreatic parvo-like virus (HPV). These viral infections impact a range of shrimp species, including *Litopenaeus vannamei*, and *Penaeus monodon* post-larvae [23,29,30]. Notably, research indicates Artemia's role in the transmission of Macrobrachium rosenbergii nodavirus (MrNV) and extra small virus (XSV), which causes significant mortality in the early life stages of the giant river prawn (*Macrobrachium rosenbergii*) [31]. All these facts highlight the importance of studying the role of Artemia in the transmission of several diseases in aquatic ecosystems.

The primary objective of this study is to evaluate the viral diversity present in Artemia populations inhabiting inland saline lakes in four distinct locations in Kazakhstan. This will be achieved with the utilization of metagenomic analysis. The methodology facilitates a thorough investigation of viral populations present in salt lakes located in both the northern and southern areas. The findings of this study contribute to the advancement of knowledge regarding viral diversity in saltwater lake ecosystems.

2. Materials and Methods

2.1. Sampling Sites and Collection

The Artemia cysts analyzed in this study were sourced from the Fisheries Research and Production Centre (Almaty, Kazakhstan) and were collected from several saline lake ecosystems in four regions of Kazakhstan, namely, North Kazakhstan, Pavlodar, Almaty, and Kyzylorda. Figure 1 and Supplementary Table S1 depict the sampling sites and their respective position coordinates, water temperatures, and salinities. Sampling was carried out in the Pavlodar and Kyzylorda regions in August 2021, while in the North Kazakhstan and Almaty areas, sampling was undertaken in August 2022. Late summer (August) is regarded as the optimal time for sampling because it coincides with the period when Artemia exhibits high cyst productivity and thus maximizes the chances of collecting mature cysts and obtaining a sample that accurately represents the entire population. The water salinity level in the reservoirs was determined using PAL-SALT (model: 4250) (Atago, Tokyo, Japan).

Figure 1. Locations of Artemia cysts collection spots in four regions of Kazakhstan. The different colored shapes on the map represent the sampling locations allocated to each region, with each color corresponding to specific geographical region lakes: green indicates the North Kazakhstan region, orange denotes the Pavlodar region, purple signifies the Almaty region, and blue represents the Kyzylorda region. Source: Map from Wikipedia.

Artemia cysts, collected from a shored mass of cysts in salty lakes with the utilization of a plankton net (150 µm mesh size), were washed with the same lake water in order to remove any debris. To ensure proper preservation of the samples, they were promptly placed in a sealed icebox after collection and thereafter maintained in a biomedical freezer set at a temperature of −80 °C upon their arrival at the laboratory.

2.2. Viral Nucleic Acid Extraction and Sequencing

For viral nucleic acid extraction, approximately 10 mg of Artemia cysts were collected from each of the 12 saline lakes. These samples were then grouped based on their individual regions, resulting in the creation of four composite pools based on the sampling region. The pooled samples were homogenized using a TissueLyser (Qiagen, Hilden, Germany) in conjunction with lysis buffer (Buffer RLT Plus, Qiagen, Hilden, Germany) and 5 mm stainless steel beads, achieving comprehensive mixing using vigorous vortexing. Following this, the homogenized samples were centrifuged at a force of 10,000 times the acceleration due to gravity using a benchtop microcentrifuge for a duration of 5 min at a temperature of 4 °C in order to remove any remnants of cyst debris. The centrifugation stage was performed twice to provide optimal separation. In addition, a 0.45 µm filter (Merck Millipore, Burlington, MA, USA) was used to exclude particles of eukaryotic and bacterial cell sizes from every pooled sample. DNase kits (Turbo DNase from Thermo Fisher Scientific in Vilnius, Lithuania, Baseline-ZERO from Epicentre, Charlotte, NC, USA, and Benzonase from Novagen, Darmstadt, Germany) and RNase kits (Promega, Madison, WI, USA) were utilized to enzymatically degrade unprotected nucleic acid present in the filtrates that were enriched with viral particles [32,33]. The residual viral RNA and DNA were subsequently isolated using the QIAamp viral RNA Minikit (Qiagen, Hilden, Germany) in accordance with the protocol provided by the manufacturer.

Four pooled samples were produced, each containing virus sequences of both DNA and RNA, subjected to reverse transcription (RT) and double-stranded DNA construction utilizing a QIASeq Stranded RNA Library Kit (Qiagen, Hilden, Germany) along with a random hexamer primer at a concentration of 100 pmol. Subsequently, four libraries were generated using the NEBNext Ultra DNA Library Prep Kit for Illumina (New England Biolabs, Ipswich, MA, USA) and sequenced on the MiSeq Illumina platform using MiSeq Reagent v.3 kit (2 × 300) (Illumina, San Diego, CA, USA). The obtained raw data were submitted to the NCBI SRA database with the accession number PRJNA1000065.

2.3. Metagenome Data Analysis

Data processing was performed on a Linux machine located locally, utilizing the DIAMOND + MEGAN analytic combination as described in reference [34]. Initially, the raw metagenomic data obtained from each pool underwent preprocessing using Fastp software (version 0.20.0) with the following parameters: "-n 0 -l 30 -5 -r -W 5 -cut_mean_quality 20" [35]. This preprocessing step aimed to exclude low-quality sequencing tails and adapters. This stage involved determining the overall quantity of clean reads acquired from the four pools. To ensure contamination control, the sequencing reads were aligned to the scaffold-level genome of *Artemia* sp. *Kazakhstan ARC1039* (Assembly accession: ASM2916890v1) using BWA-mem v. 0.7 tools [36] and afterward removed from the dataset.

Subsequently, the quality-controlled reads were de novo assembled applying MEGAHIT v1.2.9, with "-k-min 21 -k-max 141 -k-step 20" [37]. In order to taxonomically annotate the assembled contigs, a comparison was made against the NCBI's nr database (ftp://ftp.ncbi.nih.gov/blast/db/FASTA/nr.gz) (accessed on 10 May 2023) using DIAMOND Blastx v. 0.9.24. The e-value cutoff used was $<10^{-5}$ [38]. Subsequently, the contigs were taxonomically classified, and the results were visualized using the default setting in MEGAN (MEtaGenome Analyzer, version 6.24.23, Tübingen, Germany) [34,39]. The MEGAN outputs, which were received in the form of summarized reads, were utilized to present a comprehensive overview of the taxonomic classification of the sequencing reads (Table S2). Exclusion criteria were applied to the contigs originating from cellular organisms, including bacteria, archaea, and eukaryotes. The primary objective of this study was to specifically target and acquire contigs resembling those of viruses.

To eliminate false positive virus hits, further analysis of virus-like reads was performed using DIAMOND Blastx v. 0.9.24 (E-value $< 10^{-10}$) and compared with the NCBI virus reference sequence (RefSeq) database (https://ftp.ncbi.nih.gov/refseq/release/viral/) (accessed on 20 May 2023) to identify sequences with higher similarity to viral sequences compared to non-viral sequences. The obtained results were further analyzed using MEGAN (v. 6.24.23) software with the lowest common ancestor (LCA) parameter. A minimum score cutoff of 100 and an e-value threshold of $1 \times 10-10$ were applied during the analysis.

In order to attain a full classification of virus-like sequences within our dataset, an extensive collection of detailed information on various virus groupings was undertaken. These categories included double-strand DNA (dsDNA), single-strand positive-sense RNA (ssRNA(+)), and double-strand RNA (dsRNA). In addition, we gathered data from two key sources regarding well-documented virus hosts, including bacteria, invertebrates, vertebrates, and plants. The first material cited in this study was obtained from the ICTV website, which may be accessed at http://talk.ictvonline.org (Davison, 2017) (accessed on 20 June 2023) [40]. The second source utilized in this study was the ViralZone database, a comprehensive repository of genetic and taxonomic data pertaining to viral diseases. The database may be accessed at the following URL: http://viralzone.expasy.org (accessed on 22 June 2023) [41].

2.4. Phylogenetic Analysis

The phylogenetic analyses in our work were carried out with predicted amino acid sequences, as well as the nearest viral relatives identified using the top BLASTx matches and representative individuals from the corresponding viral species. The sequence alignment was performed using Clustal W in MEGA-X (v. 10.2.6) [42], utilizing the default values. The aligned sequences underwent trimming to correspond to specific nucleotide orders of the viral sequences that were retrieved. The maximum likelihood method was used to construct a phylogenetic tree in MEGA-X (v. 10.2.6) [43]. The analysis incorporated 500 bootstrap resamples to provide statistical support. Geneious Prime software (version 2023.2.1) was utilized to anticipate potential open reading frames (ORFs) [44].

3. Results

3.1. Overview of Sequencing Outcomes

The present investigation involved the gathering of twelve batches of Artemia cysts derived from saline lakes situated in the northern and southern regions of Kazakhstan (Figure 1). The Illumina MiSeq platform was utilized to generate a substantial number of raw paired-end reads (8,108,513 in total) from the four pooled cyst samples. After starting the Fastp filtering method, a significant fraction of the raw reads, ranging from 91.8% to 93.3% (7,486,652 reads), were successfully retained. After aligning the reads with the genome of *Artemia parthenogenetica*, the average number of reads in each pool was found to be 1.85 million (95% CI: 1.22–2.48 million reads), as shown in Table 1. All raw sequences obtained during this experiment are stored in the NCBI Sequence Read Archive (www.ncbi.nlm.nih.gov/sra accessed on 22 June 2023) under the accession codes SRR25455638, SRR25455639, SRR25455640, and SRR25455641.

Table 1. Summary of read and contig counting of pooled cysts of Artemia from four regions of Kazakhstan in each data processing step.

Sampling Region	Raw Paired End Reads	Filtered Paired End Reads	Host-Genome Removed Paired End Reads	GC%	Contigs Post-Assembly	Average Contig Length (bp)	Min Contig Length (bp)	Max Contig Length (bp)	No. of Viral Contigs	Percent of Virus Contigs	SRA Accession No.
North_KZ	1,968,713	1,836,619 (93.3 %)	1,788,961 (90.9%)	44.3%	18,182	487	206	9836	44	0.24 %	SRR25455641
Pavlodar	2,121,769	1,948,430 (91.8%)	1,941,957 (91.5%)	53.0%	5526	468	200	9067	24	0.43 %	SRR25455640
Almaty	2,533,969	2,330,154 (91.9%)	2,313,732 (91.3%)	48.1%	5205	507	200	9717	18	0.34 %	SRR25455639
Kyzylorda	1,484,062	1,371,449 (92.4%)	1,357,628 (91.5%)	42.1%	10,950	473	200	11,780	19	0.17%	SRR25455638
Mean/ (95% CI)	2,027,128 (1.33–2.72 M)	1,871,663	1,850,570 (1.22–2.48 M)	46.88 %	9966	484	201	10,100	26	0.26 %	-
Total	8,108,513	7,486,652	7,402,278	-	39,863	-	-	-	105	-	-

3.2. Contig Analysis

The utilization of MEGAHIT resulted in the generation of a total of 39,863 contigs with variable lengths. The contig selection process for each individual region is summarized in Table 1. The analysis of pooled cysts in four geographic areas demonstrated notable variations in the number of contig lengths. The North Kazakhstan region demonstrated the largest count of contigs, totaling 18,182, while the samples obtained from the Almaty region revealed the lowest count of 5205 contigs. Moreover, the average contig lengths observed in the samples from each location had a range of 468 bp to 507 bp, whereas the maximum contig length displayed variation, extending from 9067 bp to 11,780 bp.

Following the assembly of contigs, the homology-based identification step was conducted by running BLASTx tools in Diamond and applying an e-value threshold of less than 10^{-5}. Contigs originating from cellular organisms (eukaryotes, bacteria, and archaea) were omitted from the obtained results. Furthermore, contigs lacking significant similarity to any known amino acid sequences were also excluded. Subsequently, a total of 168 contigs as possible viral contigs (containing phages) were collected for further research, which were identified among the remaining contigs. These putative viral contigs accounted for a proportion that varied from 0.88% to 1.77% of the total contigs found in each pooled sample, as shown in Supplementary Figure S1.

To enhance the accuracy of the dataset, two measures were implemented. Initially, we eliminated virus contigs that were identified as false positives and those exhibiting limited levels of amino acid similarity to established viral sequences. Moreover, to confirm the existence of viral contigs, we performed an analysis of contigs that were identified as potential viral sequences and classified as unknown RNA or DNA viruses. Subsequently, a sum of 105 contigs was identified as the best match with viral reads. The percentage of contigs associated with viruses in each pooled sample ranged from 0.17% to 0.43% (Table 1).

3.3. Composition of the Artemia Viral Community

The identification of viral sequences in our study was mostly based on homology, which resulted in a restricted range of DNA and RNA virus groups. This was determined using an examination of sequence contigs. The contigs demonstrating a significant level of similarity to the viral genome were methodically categorized into seven viral families, comprising a total of nine distinct species. In addition, our research effectively identified a collective of seven phage families, comprising a set of 25 unique species (Tables 2, S3 and S4). In addition, there was a cluster of viruses that remained unclassified.

Table 2. Contig count for viral species, families, and orders with the range of alignment length, alignment identity percentage, and contig length.

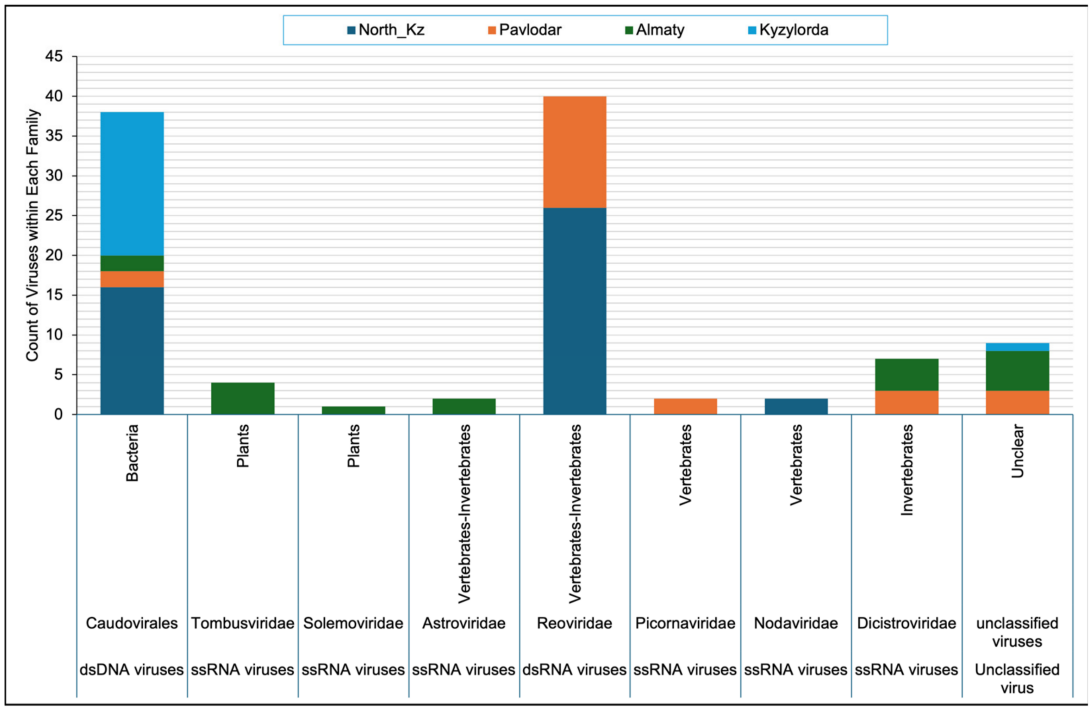
Region	Order	Family	Species	Alignment Length [a], (nt)	Contig Identity [b], %	Contig Length, (nt)	Number of Contigs	Protein Product
North_KZ	Reovirales	unclassified Reovirales	Cimodo virus	109–493	71.5–100	337–1500	26	hypothetical protein
	Nodamuvirales	Nodaviridae	Covert mortality nodavirus	112–204	87.5–90.2	337–612	2	RdRp
Pavlodar	Reovirales	unclassified Reovirales	Cimodo virus	106–153	69–100	302–459	14	hypothetical protein
	Picornavirales	Dicistroviridae	*Cripavirus* sp.	100–308	65.6–67.5	304–922	3	nonstructural polyprotein
		Picornaviridae	*Picornaviridae* sp.	107–200	72.5–75.7	322–605	2	RdRp
Almaty	Picornavirales	Dicistroviridae	*Dicistroviridae* sp.	114–239	32.2–55.1	364–1626	4	putative replicase
	Stellavirales	Astroviridae	Microbat bastrovirus	376	37.8	1107	1	non-structural polyprotein
			Nelson Astrovirus-like 1	289	47.8	879	1	hypothetical protein
	Sobelivirales	Solemoviridae	*Solemoviridae* sp.	392	44.1	4992	1	hypothetical protein
	Tolivirales	Tombusviridae	*Tombusviridae* sp.	232–679	37–41	788–6137	4	putative replicase

[a] Contig sequences over 100 nt in length were characterized. [b] Determination of identity levels using BLASTx with virus reference strains from NCBI RefSeq database (e-value < 1×10^{-10}).

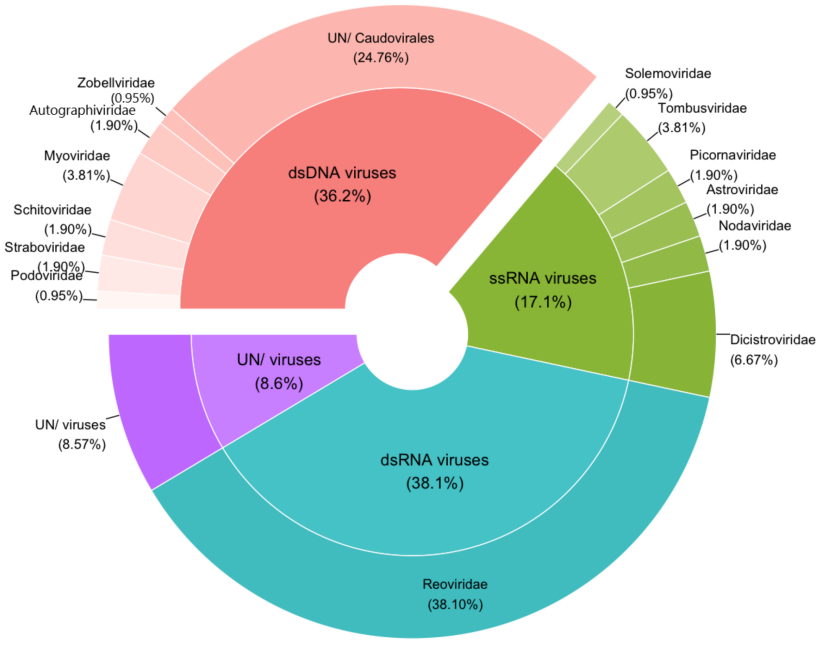
The classification of these viral families was established using the analysis of their unique patterns of host infection. There are seven families of double-stranded (ds) DNA viruses that were identified, most of which host bacteria, and one of these group remains unclassified *Caudovirales* (Table S3). In addition, a total of six families were formed, including single-stranded (ss) RNA viruses, with a particular focus on positive-strand viruses. It is important to state that two of these viral families were shown to have associations with vertebrate hosts, whereas another two were recognized for their ability to infect plants. Furthermore, a specific family demonstrated a unique capacity to infect invertebrates, while the other family consisted of a virus capable of infecting both vertebrates and invertebrates. A *Reoviridae* family is identified within the taxonomy of viruses, which is defined by its association with a non-enveloped double-stranded (ds) RNA virus. This virus family exhibits a distinct capacity to infect a diverse range of hosts, including both vertebrates and invertebrates. Additionally, the presence of unclassified RNA and DNA viruses was identified (Figure 2a).

A significant proportion of the viral sequences were linked to the dsRNA viruses within the *Reoviridae* family, making up 38.1% of the overall reads of virus-like sequences. The second largest virus group was dsDNA viruses, especially phages classified as *Caudoviricetes*, which consist of six virus families and unclassified *Caudovirales*. These accounted for 36.2% of the overall viral reads. Furthermore, an analysis of the viral sequence reads revealed that 17.1% of them were associated with ssRNA viruses. These ssRNA viruses belonged to several families, including *Dicistroviridae* (6.67%), *Tombusviridae* (3.81%), *Astroviridae* (1.9%), *Picornaviridae* (1.9%),

Nodaviridae (1.9%), and *Solemoviridae* (0.95%). The viral reads that accounted for the remaining 8.6% were classified as unclassified viruses, as depicted in Figure 2b.



(**a**)



(**b**)

Figure 2. Distribution of virus sequence reads within a specific virus family. (**a**) The distribution of several virus families among taxonomic classes. The *y*-axis of the graph depicts the number of individual viral contigs belonging to each viral family, while the *x*-axis displays the taxonomic categorization and corresponding viral family. (**b**) Proportion of virus sequences in different virus families within the classification. The abbreviation "UN" signifies the unclassified virus family.

3.4. Distribution of Viral Families in Lakes of Different Regions

Using careful analysis of the data obtained, we found that some patterns in the viral diversity of Artemia cysts among different regions emerge in Table 2 and Figure 2a. The Artemia cysts from the Almaty region are notable for their exceptional viral variety, which encompasses four distinct viral species from different families: *Dicistroviridae, Tombusviridae, Astroviridae*, and *Solemoviridae*. In materials from the Pavlodar region, which is known for its hypersaline ecosystems, there are observations of four distinct viral species belonging to three viral families: *Reoviridae, Picornaviridae*, and *Dicistroviridae*. The subject of our inquiry now turns to the saline lakes found in the North Kazakhstan region, where two viral species can be identified belonging to the viral families *Reoviridae* and *Nodaviridae*. On the other hand, the Kyzylorda region is notable for its high occurrence of contigs that are classified within bacteriophage families. It is noteworthy that, other than an unidentified DNA virus, no other viral contigs were identified in this study. After completing a thorough comparative examination of viral diversity in different places, a significant observation becomes apparent. Although viral species and families exhibit diverse compositions, the overall amount of diversity is very low.

3.5. Characteristics of Selected Viruses

After characterizing the host properties, the dataset underwent a process of excluding plant viral reads (*Tombusviridae, Solemoviridae*), bacteriophages, previously unidentified virus reads, and those that provided too few sequences reads (*Astroviridae*). This facilitated a shift in our attention toward a comprehensive examination of specific viruses associated with both vertebrate and invertebrate hosts, which are classified under the viral families *Reoviridae, Nodaviridae, Dicistroviridae,* and *Picornaviridae*. As part of this study, we performed an analysis and comparison of the partial genome sequences of these viruses with their closest relatives using phylogenetic analysis.

- Reoviridae

The viral family *Reoviridae* is categorized within the taxonomic order *Reovirales*. The viruses under consideration exhibit a non-enveloped, segmented double-stranded RNA genome and have the ability to infect a diverse array of hosts, encompassing mammals, birds, reptiles, fish, insects, and plants [45]. It is worth noting that the *Reoviridae* family was found to be highly abundant in the pooled Artemia cysts collected from the North Kazakhstan and Pavlodar regions. Close relatives of unclassified *Reoviridae*, specifically the Cimodo virus (CMDV), were discovered during our investigation. CMDV was isolated from mosquitoes and consists of a total of 12 genomic segments [46]. The virus that was recognized is classified as an Artemia reo-like virus. A total of 40 contigs belonging to the Artemia reo-like virus were identified from the pooled samples originating from two distinct regions. It was observed that a notable similarity exists between the Artemia reo-like virus and the CMDV. The amino acid identities between them displayed a range from 71.5% to 100% in the North Kazakhstan region and 65.6% to 100% in the Pavlodar region, highlighting the genetic similarity between them.

To further investigate the genetic links between the Artemia reo-like virus and closely related reference sequences, comprehensive phylogenetic research was undertaken. This study involved creating a phylogenetic tree based on the sequence of a particular segment of the Artemia reo-like virus genome. The partial contig sequence (length 1500 nt) was selected because of its high amino acid identity and coverage of over 80% of the NTP-binding domain of segment 6 in CMDV. The result showed that there is a closer relatedness between the Artemia reo-like virus and CMDV (Accession No. KF880765.1) (Figure 3). Our study suggests that the Artemia reo-like virus belongs to the unclassified *Reoviridae* based on the result of a homology similarity and phylogenetic analysis.

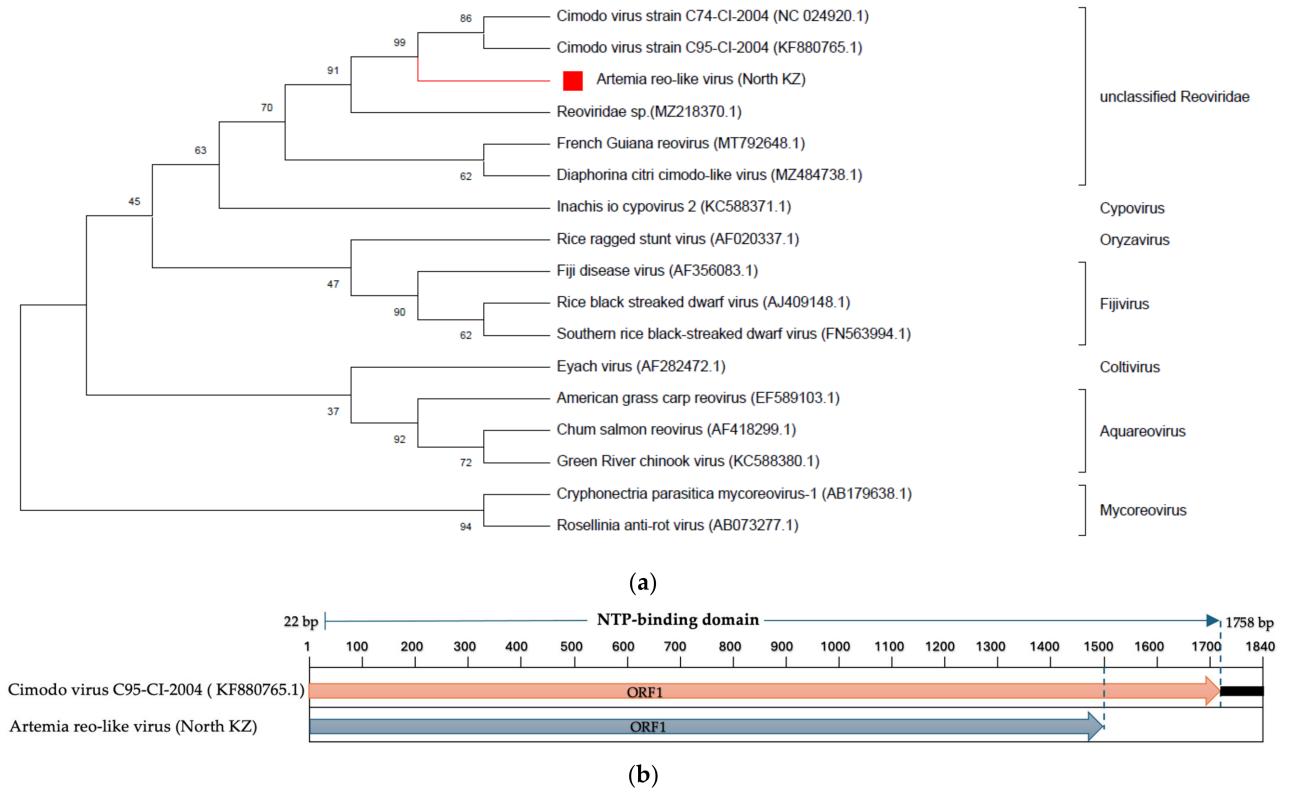


Figure 3. Phylogenetic relationships of the Artemia reo-like virus. (**a**) The phylogenetic tree was constructed using a maximum likelihood method, utilizing the alignment of the conserved portion of the partial hypothetical protein of Artemia reo-like virus (segment 6) and a set of representative sequences from phylogenetically related viruses. The numerical values assigned to the branches in the tree diagram represent bootstrap support levels. The virus found in this specific investigation is prominently indicated using a red square. (**b**) The predicted genome organization of the Artemia reo-like virus (North. KZ), with positions and lengths of the ORF indicated, along with closely related virus genes highlighted for reference.

- Nodaviridae

The family *Nodaviridae* is comprised of small, non-enveloped RNA viruses with positive-sense, single-stranded genomes. This family can be further divided into two separate genera known as *alphanodavirus* and *betanodavirus.* These viral species demonstrate a remarkable ability to infect a wide range of hosts, including insects, fish, and crustaceans [47].

In the course of our inquiry, we encountered a couple of viral sequences belonging to the *Nodaviridae* family, which we designated as an Artemia noda-like virus, in Artemia cysts from the North Kazakhstan region. During the BLASTx analysis, an identity level of amino acid similarity, specifically 90%, was observed between the contig in query and the sequence of the covert mortality nodavirus (CMNV). The analyzed sequences had similarities to the RNA-dependent RNA polymerase gene (RdRp) of CMNV isolated from *Larimichthys* (marine ray-finned fish), with the contigs covering approximately 11–20% of the open reading frame 1 (3152 nt) of CMNV (accession no. MW625911.1).

Following the previous evaluation of similarities, a thorough examination of the phylogenetic relationships was conducted (Figure 4). This investigation used the contig sequence of the Artemia noda-like virus, focusing on the longest length, along with other viruses, particularly those resembling the CMNV. Based on phylogenetic research utilizing the partial genome sequence of Artemia noda-like virus, it was observed that this virus's partial genome exhibited clustering with the CMNV belonging to the unclassified *Nodaviridae.*

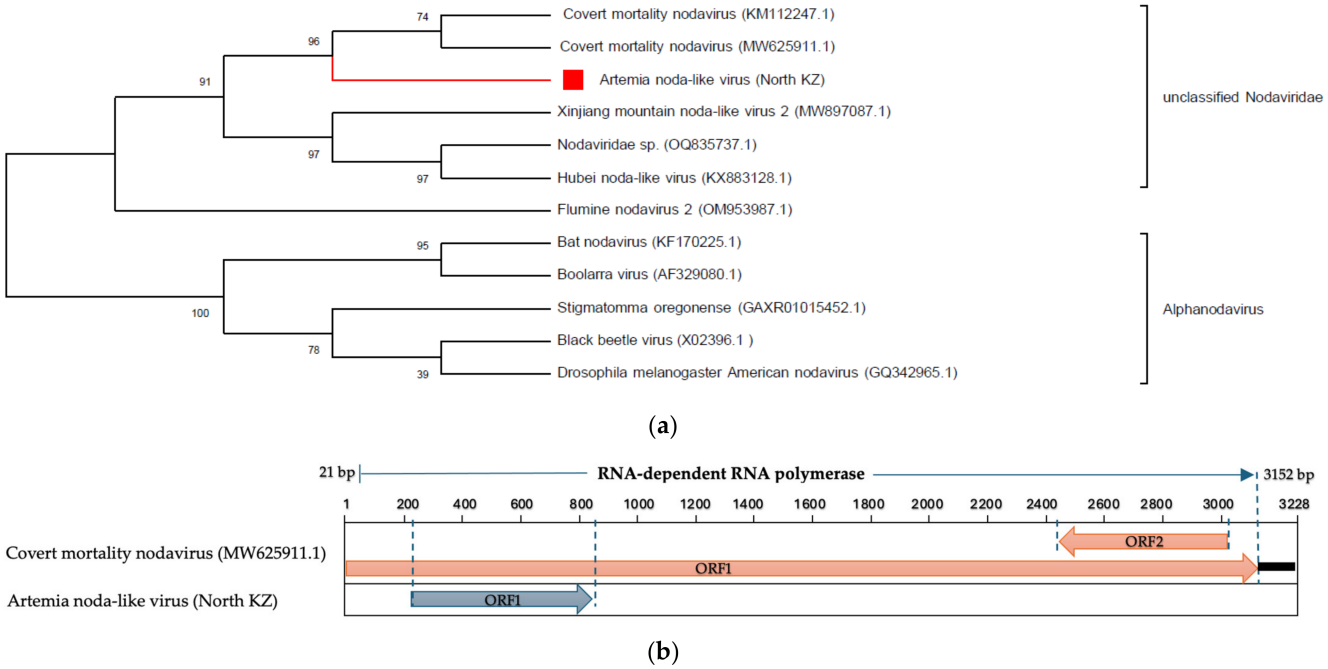


Figure 4. Phylogenetic relationships of Artemia noda-like virus. (**a**) The phylogenetic tree was constructed using a maximum likelihood methodology, utilizing the alignment of the RdRp partial genome of the Artemia noda-like virus alongside a carefully chosen set of representative sequences from phylogenetically similar viruses. The numerical values assigned to the branches in the tree diagram represent bootstrap support levels. The virus found in this specific investigation is prominently indicated using a red square. (**b**) The predicted genome organization of the Artemia noda-like virus (North. KZ), with positions and lengths of the ORF indicated, along with closely related virus genes highlighted for reference.

- Dicistroviridae

The *Dicistroviridae* family comprises a group of small, non-enveloped RNA viruses with single-stranded genetic material that demonstrates a host range including insects and crustaceans. The family can be classified into two genera, namely, *Cripavirus* and *Aparavirus*. Viruses belonging to the *Dicistroviridae* family exhibit tiny icosahedral geometries and feature positive-sense RNA genomes that are typically 8–10 kilobases in size [48,49].

The current study detected sequences of Artemia dicistro-like viruses in both the Pavlodar and Almaty regions. Within the pooled sample of Artemia cysts originating from the Pavlodar region, a limited number of sequences (Artemia dicistro-like virus 1) displayed the highest resemblance to the *Cripavirus* genus. These sequences demonstrated an amino acid identity ranging from 65% to 68% for hypothetical polyprotein genes of *Cripavirus* sp. The Artemia dicistro-like virus 1, which has close phylogenetic clustering with unclassified *Cripavirus* (Figure 5a), was discovered to occupy approximately 18% of the ORF1 (5141 bp) of the *Cripavirus* sp. (Figure 5b), with a genome length of 9426 bp (accession no. MZ679108.1). On the other hand, the genomic sequence of Artemia dicistro-like virus 2 was detected in the Almaty region, exhibiting a resemblance to *Dicistroviridae* sp. The amino acid sequence similarity between them varied from 32% to 55%. In the phylogenetic analysis, the queried sequence was subjected to clustering with the *Dicistroviridae* sp. (accession no. MZ679056.1) (Figure 5a).

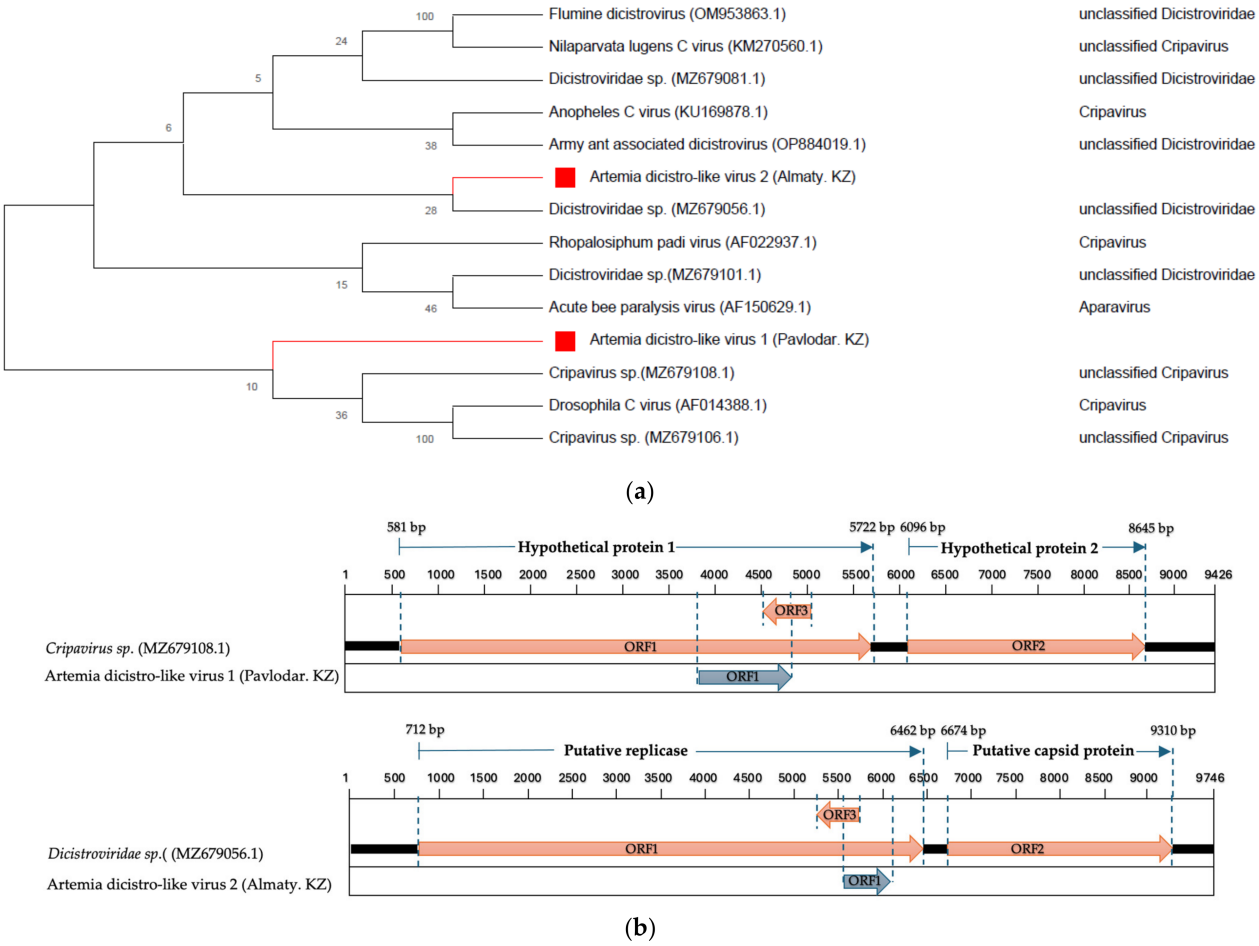


Figure 5. (**a**) Phylogenetic relationships of Artemia dicistro-like virus 1 (Pavlodar. KZ) and Artemia dicistro-like virus 2 (Almaty. KZ). The nonstructural polyprotein genes of those viral contigs were aligned with representative selected sequences from phylogenetically related viruses using a maximum likelihood approach to create the phylogenetic tree. The numbers indicated on the branches are bootstrap support values. The red square clearly highlights the virus that was found in this investigation. (**b**) The predicted genome organization of Artemia dicistro-like virus 1 (Pavlodar. KZ) and Artemia dicistro-like virus 2 (Almaty. KZ), with positions and lengths of the ORF indicated, along with closely related virus genes highlighted for reference.

- Picornaviridae

The family *Picornaviridae* comprises viruses that exhibit small, non-enveloped particles that possess a linear single-stranded RNA (+) genome with a size ranging from 7.1 to 8.9 kilobases (kb). The present genome exhibits polyadenylation and is comprised of a single ORF that encodes a polyprotein. These viruses exhibit significant diversity and possess a broad spectrum of hosts, infecting not just human beings but also several vertebrate and invertebrate species [50,51].

Artemia picorna-like viral sequences, closely resembling an unidentified virus from the *Picornaviridae* family, were identified in the Pavlodar region. Two contigs were generated by assembling. The initial contig exhibited a 76% amino acid identity of the partial polyprotein gene of *Picornaviridae* sp. (accession no. ON162097.1). The second contig, spanning a length of 605 bp, presented a 73% amino acid identity in the incomplete RdRp gene of *Picornaviridae* sp. (accession no. ON161882.1). In order to further understand the evolutionary relationship, we performed a phylogenetic analysis utilizing the most extensive contig sequence (Figure 6). In this analysis, the Artemia picorna-like virus showed

clustering alongside the unclassified *Picornaviridae*, specifically with the Clinch picorna-like virus (accession no. MT341476.1).

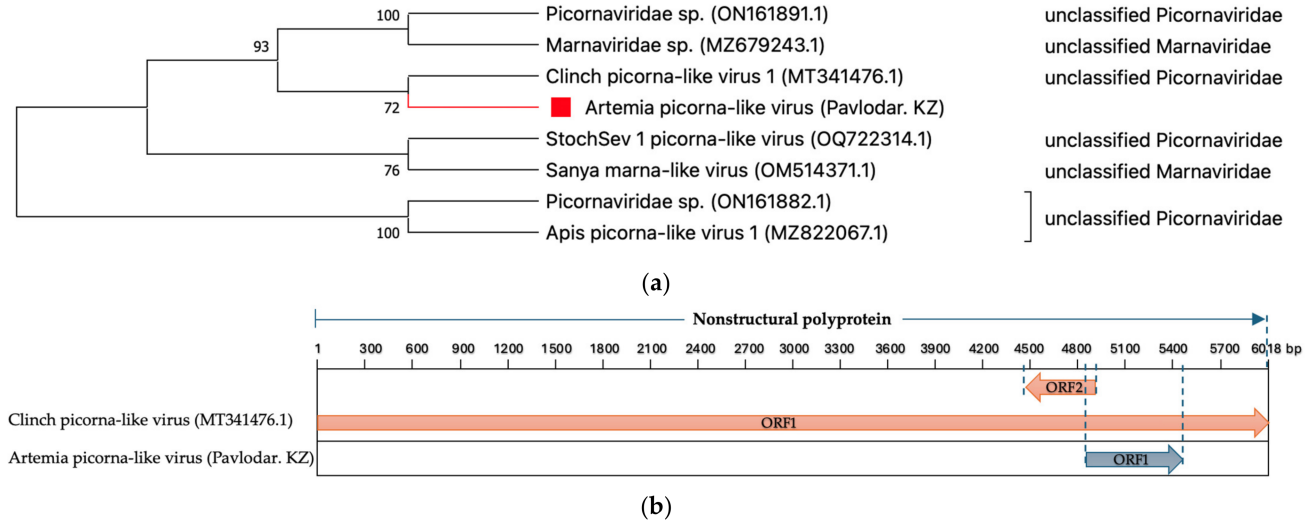


Figure 6. Phylogenetic relationships of Artemia picorna-like virus. (**a**) The phylogenetic tree was constructed using a maximum likelihood method. This method utilized the alignment of the incomplete gene of the Artemia picorna-like virus with a set of carefully chosen representative sequences from viruses that are phylogenetically related. The numerical numbers depicted on the branches signify bootstrap support values. The virus found in this specific study is prominently emphasized within a red square. (**b**) The predicted genome organization of the Artemia picorna-like virus (Pavlodar. KZ), with positions and lengths of the ORF indicated, along with closely related virus genes highlighted for reference.

4. Discussion

Metagenomic research, coupled with homology-based data processing, provided evidence for all three categories of viruses, encompassing both single-stranded and double-stranded RNA/DNA viruses. The viral groups under consideration consist of a combined total of 15 viral families and 35 viral species. These groups can infect a wide range of hosts, including vertebrates, invertebrates, bacteria, and plants. The present study highlights the existence of dsDNA viruses, with a specific emphasis on the abundance of bacteriophages. The seven viral families (*Autographiviridae, Myoviridae, Podoviridae, Straboviridae, Schitoviridae, Zobellviridae,* and unclassified *Caudovirales*) in this specific category accounted for 36.2% of the detected viral families. Among them, the viruses within unclassified *Caudovirales*, tailed bacteriophages, were the most abundant viral family in pooled Artemia cysts, especially in the North Kazakhstan and Kyzylorda regions (Figure 2b). The prevalence of phages was not a surprise since earlier studies have demonstrated the predominance of phage families in comparison with other viruses in the saltwater ecosystem [9,10,17,51]. However, it is crucial to acknowledge that bacteriophages did not constitute the primary focus of our investigation.

The findings of our analysis shed light on the dominant viral group, the unclassified *Reoviridae*, which accounts for 38% of the overall viral readings. As previously mentioned, this particular family is classified under a category of dsRNA viruses renowned for their capacity to infect a diverse array of hosts [45]. Significantly, our research observed a plausible occurrence of cross-species transmission involving the Artemia reo-like virus detected in pooled Artemia cysts originating from the northern region of Kazakhstan. Significantly, the present virus has a considerable degree of genetic resemblance to the hypothetical protein of the Cimodo virus, which belongs to the *Reoviridae* family and was originally discovered in mosquitoes inhabiting an African rainforest. CMDV is purportedly responsible for the classification of a new genus in the *Spinareovirinae* subfamily [46].

The veracity of this association was additionally corroborated by the use of phylogenetic research, as depicted in Figure 3. Multiple study articles have provided evidence for the presence of diverse reo-like viruses, such as the Hubei reo-like virus, the French Guiana reovirus, and the Shenzhen reo-like virus [52–54]. A recent study documented the discovery of the *Diaphorina citri* cimodo-like virus within the Asian citrus psyllid insect [55]. Moreover, the cimodo-like virus in brine shrimp has been detected in brine shrimp cyst products from some countries, including Russia, China, Iran, and the United States [56].

Moreover, the earlier result implies a plausible ecological interdependence between brine shrimp and mosquitoes, maybe facilitated by an unknown intermediary species. The complex interaction among viruses linked to aquatic animals highlights their ability to move across various species within aquatic ecosystems. According to these observations, additional extensive investigation is imperative to ascertain the potential transmission of these viruses to fish and other animals reliant on brine shrimp or inhabiting similar environments. It is imperative to comprehend the potential effects of these interactions and transmission pathways in order to obtain insight into the broader implications for aquatic ecosystems and their inhabited species.

The present study not only emphasizes the widespread occurrence of the *Reoviridae* family but also reveals the intriguing existence of the *Nodaviridae* family in brine shrimp cysts. In the context of our research, we identified a viral strain classified under the *Nodaviridae* family, specifically referred to as the Artemia noda-like virus, present within the Artemia cysts in North Kazakhstan lakes. By using phylogenetic analysis and homology-based identification techniques, we identified sequences present in the Artemia noda-like virus that exhibit similarities with the CMNV, an unclassified virus belonging to the *Nodaviridae* family. Previous studies have demonstrated that CMNV possesses the ability to cause several types of pathology to the tissues and organs of prominent farmed crustaceans [57,58]. Moreover, CMNV has exhibited its capacity to surpass species barriers and infect a wide array of cultivated and wild fish species, obtained from various habitats including prawn ponds or drainage channels that have been impacted by CMNV [59,60]. This statement emphasizes the inherent capacity of CMNV to surpass taxonomic divisions, thus highlighting its ability to infect both vertebrate and invertebrate taxa.

The identification of the Artemia noda-like virus in the northern region of Kazakhstan prompts inquiries regarding species interactions and the potential involvement of Artemia in the transmission of the virus. The discernible correlation between brine shrimp and other aquatic organisms underscores the necessity for comprehensive investigation in order to reveal the implications for aquatic ecosystems and various taxa in the North Kazakhstan region. Furthermore, an examination of CMNV-like sequences within the Artemia cysts has the potential to provide valuable knowledge regarding the virus's ability to adapt and its effects on both invertebrates and vertebrates.

The identification of *Dicistroviridae* genome sequences among Artemia cysts originating from the Pavlodar and Almaty regions serves as a noteworthy illustration of their capacity to thrive in various ecological habitats. It is postulated that the occurrence of an unclassified *Cripavirus* within Artemia cysts found in the salt lakes of the Pavlodar region could potentially contribute to the partial impact of these viruses on the indigenous invertebrate fauna in the indicated region. In contrast, the Artemia dicistro-like virus 2 found in the Almaty region exhibits lower levels of amino acid similarity when compared with the unclassified *Dicistroviridae*. Based on published studies, the presence of the *Dicistroviridae* family has been observed in aquatic invertebrates dwelling in saltwater habitats. Two examples of viruses include the Crustacea picorna-like virus N14, which was found in the South China Sea, and the Bivalvia picorna-like virus D23, which was discovered in the East China Sea [61]. Nevertheless, the coverage levels of the genomes belonging to the *Dicistroviridae* family exhibited comparatively low values in both regional samples. Further investigation is necessary to ascertain the classification of these viruses within the *Dicistroviridae* family.

The identification of members belonging to the *Picornaviridae* family within the Pavlodar region contributes to the advancement of our knowledge regarding the diversity of viruses. The family *Picornaviridae* is considered one of the most prominent and substantial collections of ssRNA viruses [50,51]. Significantly, there has been substantial growth in the *Picornavirales* order in recent years. This expansion can be attributed to the identification of previously unidentified picornaviruses using advanced sequencing techniques, such as next-generation sequencing, across a wide range of animals, including vertebrates, arthropods, algae, insects, plants, and humans [61,62]. The investigation conducted in our study examined the detection of an unidentified virus belonging to the *Picornaviridae* family in the samples collected from the Pavlodar region. Although, the virus has a restricted number of contigs, posing a significant challenge in accurately identifying the true viral species.

Moreover, the prevalence of plant viruses, vertebrate viruses, and unidentified viruses was seen in almost all pooled samples. This statement highlights the critical necessity for additional investigative studies aimed at understanding the complex viral diversity present among populations of Artemia.

The saline lakes in our study display considerable variability in terms of salinity, ranging from 80 to 240 g/L (Table S1), and are characterized as hypersaline (a salinity exceeding 35 g/L) [63]. Although there is a lack of information regarding the organisms inhabiting the saline lakes of Kazakhstan, the collection of Artemia cysts provided an opportunity to detect zooplankton (Table S1), suggesting the presence of aquatic life in this saline lake environment. As a result, the hypersaline lakes located in Kazakhstan exhibit a low level of water species diversity. The higher salinity in these aquatic environments creates favorable conditions for the Artemia population, although, at the same time, it reduces the presence of other species [63]. Therefore, the sole dominance of Artemia species in this extremal ecosystem enables us to predict an association between the identified viral contigs and this brine shrimp species.

It is important to acknowledge that the most concerning fish and crustacean viral diseases listed by the World Organization for Animal Health (WOAH, OIE) [64] were not identified in the samples examined throughout the period of this investigation. The reduced richness of species diversity in hypersaline ecosystems, the absence of fish in this community, and the lack of *Penaeid* shrimp-raising farms in the vicinity of Kazakhstan's saline lakes may serve as possible reasons for the low risk of exposure to WOAH-listed infections from Artemia cysts to aquatic animals. Additionally, flamingos, as the only bird species that exclusively feed on Artemia, are considered possible avian vectors of viruses in brine shrimp-rich hypersaline shallow lakes. However, their nesting spots in Kazakhstan and migrating routes do not overlap with the artemia cyst-rich sampling sites in our study.

This raises the possibility that pathogens may not exist within this saline lake ecosystem, possibly due to the less likely occurrence of cross-species viral transmission through direct contact or water. Also, populations of brine shrimp often do not possess the capability for active migration between distinct saline habitats unless they receive assistance from external factors such as wind, birds, or human-mediated dispersal [65,66]. In their native habitat, these brine shrimp mostly depend on passive dispersal processes, such as wind currents, bird migrations, or occasional human activity, to facilitate their transit among various saline habitats [56]. This observation suggests that the viruses identified in Artemia cysts may not have originated from adjacent aquaculture operations. This information is crucial for comprehending the potential sources of viral infections and their pathways in lakes.

In addition, it is vital to point out the viral diversity that was discovered within the examined populations of Artemia cysts. The limited diversity observed in this study may be influenced by various factors, including specific sampling locations, salinity degrees, timing of sampling, and temperature conditions [9,10,17,67]. It is important to note that the temperatures and seasonal circumstances in these regions exhibit variations based on their respective geographical locations. The two regions in the northern part of Kazakhstan present an average summer temperature that normally spans from 20 to 30 °C. On the

other hand, the two regions located in the south of the country have raised temperatures, often ranging from 25 to 35 °C. It is notable that the presence of the Artemia reo-like virus associated with Artemia cysts was found only in the northern regions. Nevertheless, no viral contigs were identified in the Kyzylorda region. The Artemia dicistro-like virus was detected in cyst samples collected from the Almaty region. Despite the fact that these patterns may suggest a possible temperature effect, it should be highlighted that there is presently no evidence to support this hypothesis. To investigate the relationship between temperature and viral presence, more research is required. Also, changes in salinity and temperature affect the development of the infectious myonecrosis virus in *L. vannamei*. Low-salinity brackish water is known to trigger outbreaks of IMNV [68]. Although IMNV was not specifically investigated in this study, we predict that the high salinity environment of Artemia cysts might affect virus survival and transmission.

It is essential to determine the role of the identified viruses in the infection pathology of aquatic animals. To evaluate the effects of these viruses on aquaculture, it is necessary to conduct exhaustive experiments with targeted species.

5. Conclusions

Overall, the present research constitutes the pioneering assessment of the virome within Artemia cysts from the saline lakes in Kazakhstan. Our research uncovered a restricted diversity of viral sequences that frequently resembled unclassified RNA viruses. Notably, we identified putative RNA viruses, such as the nearly completed genome segment sequence of Artemia cysts-associated Artemia reo-like virus, as well as partial genomes of Artemia cysts-associated Artemia noda-like virus, Artemia dicistro-like viruses, and Artemia picorna-like viruses. This study establishes a crucial foundation for future research into the virome of Artemia cysts, which aims to confirm the association between these detected putative viruses and their host. In addition, our study sheds light on the intricate interaction between salinity levels, temperature fluctuations, and low species diversity, which collectively shape the unique virome of Artemia in saline lakes in Kazakhstan. It emphasizes the need for continued investigation into these complexities and their implications for the viral communities harboring Artemia cysts in hypersaline environments. Further research is needed to elucidate the effects of these viruses on different aspects of Artemia's life cycles and productivity.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/fishes8100487/s1, Table S1: Information about the sampling locations in the four regions (sampling site, coordination, temperature, salinity, sampling dates, and aquatic organisms); Table S2: Overview of sequencing reads classification at the domain level (DIAMOND Blastx and MEGAN-LCA); Figure S1: Proportion of the classification level of sequencing reads for different regions; Table S3: Summary of the total count of viral readings obtained from 15 distinct viral families and unclassified viruses; Table S4: Overview of contigs of Bacteriophage species identified using BLASTx in Artemia cysts collected from four regions.

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Institutional Review Board Statement: All components of this study did not involve human participants and did not use laboratory animals, and the sampling would have minimal impact on the natural habitat and behavior of Artemia. Thus, our study received ethical approval from the local ethics committee of the Research and Production Center for Microbiology and Virology. (No. 07-01-02/61-2023/04/28).

Informed Consent Statement: Not applicable.

Data Availability Statement: The obtained raw data were submitted to the NCBI Sequence Read Archive (SRA) database with the BioProject accession number PRJNA1000065.

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