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The RNA Viruses in Samples of Endemic Lake Baikal Sponges

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Abstract: Sponges are unusual representatives of the animal kingdom; their viromes, as part of the associated community, began to be studied quite recently, and, accordingly, these studies are gaining momentum. The diversity of viruses in sponges is high, and they most likely play a significant role in the composition of the sponge holobiont, especially under stress conditions. The objects of our metagenomic study were RNA viruses of two common endemic species of Baikal sponges, *Lubomirskia baikalensis* and *Baikalospongia bacillifera*. As a result of viral RNA sequencing, we were able to identify fragments of viral genomes related to those from the RefSeq NCBI complete viral genome database. Most of the similar genomes belonged to viruses isolated from various invertebrates; some of the scaffolds were related to known plant viruses, and one of them was related to a vertebrate virus. The similarity of the putative proteins of viral scaffolds from the Baikal sponges with proteins of known viruses turned out to be low (20.7–67.3%), indicating the detection of novel viruses. The samples of diseased and visually healthy sponges were clustered separately, suggesting a shift in sponge virome composition during the course of the disease. In a comparative analysis, the viromes of the Baikal and marine sponges differed significantly, demonstrating the influence of the host species, habitat, and geographical location on virome composition in the sponge holobiont.

Keywords: RNA viruses; sponges; virome; viral diversity; invertebrate; freshwater ecosystems

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1. Introduction

Sponges are unique representatives of the animal kingdom. These ancient inhabitants (phylum Porifera) are complex symbiotic communities consisting of various microorganisms (bacteria, algae, fungi, protozoa, and others) [1]. Sponges are widespread in both freshwater and marine ecosystems and have important properties and functions in aquatic environments by passing large masses of water through themselves (known as filtering) and purifying them [2–4].

An inherent part of a sponge community (holobiont) is viruses, the diversity of which depends on the species of the sponges themselves, the composition of sponge-associated microorganisms [1], and the macroorganisms that inhabit sponges (gammarus, molluscs, etc.) [5]. Sponge viromes began to be studied relatively recently; the first works were mainly concerned with the study of DNA-containing viruses in marine sponges (most of which are bacteriophages) [6–9]. But some works [10–13] also included investigations of RNA-containing viruses in sponges, which revealed a high level of diversity of RNA viruses in sponge holobionts. Earlier, we carried out the first viromic studies (of DNA viruses) of Baikal sponges [14–16]. To the best of our knowledge, the viral communities of other freshwater sponges have not yet been studied.

As is known, during the past decade, sponges have been affected and died all over the world [17]; to date, the sponge populations of Lake Baikal have declined catastrophically. [18]. All of this happens against the backdrop of noticeable changes in the structure

of coastal phyto- and zooplankton communities, indicating eutrophication of the coastal zone [19,20]. Hydrochemical and sanitary–microbiological analyses of the tributaries of rivers and the shallow zones in area of settlements indicate an increased anthropogenic impact [21–24]; however, mass death of sponges is observed throughout the Baikal water area, regardless of the level of anthropogenic load [25]. Sponge disease may be caused by various pathogenic agents of a bacterial, fungal [26,27], or viral nature. Numerous studies of the microbial communities of healthy and diseased Baikal sponges failed to reveal any specific pathogen [28], and the research of viral communities seems to be especially relevant for understanding the mechanisms of the onset and course of sponge disease (or damage).

At the beginning of this century, large-scale studies of RNA viruses of a wide range of aquatic invertebrates unfolded [10,29,30]. These RNA sequencing (viromic and meta-transcriptomic) studies uncovered a wide variety of new RNA viruses in natural ecosystems. The databases were replenished with numerous genomes of viruses assembled from the samples of various inhabitants of water bodies. Prior to the era of metagenomic research, only a few dozen invertebrate viruses were known, usually of commercial species [31,32]. It has become known that RNA viruses predominate DNA viruses in eukaryotic viromes, in contrast to bacterial and archaeal ones [33]. A huge level of genetic diversity of RNA viruses; shuffling of gene modules among different viral genomes; wide host range for many groups of viruses; and simultaneous monophyleticity of many other viruses with closely related hosts, indicating a long-term virus–host co-divergence, were revealed. Evolutionary concepts of RNA viruses have been revised, and it has been hypothesized that plant and vertebrate RNA viromes originated through multiple horizontal virus transfer (HVT) from marine invertebrate viromes [29,34,35].

The aim of this work was to study the viral RNA isolated from samples of the Baikal sponges *Lubomirskia baikalensis* and *Baikalospongia bacillifera*, members of the endemic family Lubomirskiidae (class Demospongiae, order Spongillida), and to obtain the first data regarding the diversity of RNA viruses circulating in Baikal sponges. In the present study, we used samples of both diseased and visually healthy sponges. This allowed us to trace changes in RNA viral communities over the course of the sponge diseases, as was previously shown for DNA viruses [16].

2. Materials and Methods

2.1. Sampling and Sample Processing

The *Lubomirskia baikalensis* and *Baikalospongia bacillifera* sponges were sampled in sterile tubes by divers using lightweight diving equipment. The *L. baikalensis* sponges (two specimens) were sampled in March 2015 in the Maloye More Strait, near the Malye Olkhonskiye Vorota Strait (the middle basin of Lake Baikal, 53°01′05.1″ N, 106°55′43.5″ E). Two branches of 2–3 cm^3 in volume were collected from the same sponge: one branch looked healthy and another one had lesions (bleaching or brown spots) (Figure 1, Table 1). We also collected samples from *L. baikalensis* sponge individuals without visible damages; however, we failed to isolate viral RNA from these samples in an amount sufficient for the preparation of libraries. The *B. bacillifera* sponges were sampled in the southern basin of Lake Baikal, near the Bolshiye Koty settlement (51°54′07.5″ N, 105°06′12.0″ E), in May 2018. The four specimens of *B. bacillifera*, of 5–7 cm^3 in volume, were collected and used in this study: two specimens looked healthy, and two others had necrosis and brown spot-like lesions (Figure 1, Table 1). Sponge samples were processed, and then concentrates of virus-like particles (VLPs) were obtained as described in [14,15]. Briefly, the sponge samples were washed in sterile Baikal water, thoroughly homogenized by blender, and centrifuged (3000 rpm, 30 min for *L. baikalensis* or 400 g for 15 min followed by 16,000× *g* for 30 min for *B. bacillifera*). The aqueous fraction was passed through a syringe filter with a pore size of 0.2 mm (Sartorius, Göttingen, Germany) and treated with DNase I (50 U/mL) and RNase A (100 mg/mL) enzymes (Thermo Fisher Scientific, Waltham, MA, USA) to remove contaminating nucleic acids. Viral RNA was extracted with TRI Reagent (Molecular Research Center, Cincinnati, OH, USA) [36] according to the manufacturer's

recommendations. Specifically, the samples were diluted 4-fold with TRI Reagent and incubated for 5 min at room temperature (RT); 0.2 mL of chloroform per 1 mL of TRI Reagent was added, and the mixture was shaken and incubated (RT, 15 min). Then, the samples were centrifuged (12,000× *g*, 15 min), and the collected upper phase was precipitated (12,000× *g*, 15 min, 4 °C) with isopropanol (0.5 mL per 1 mL TRI Reagent).

Figure 1. Sampling sites and *Lubomirskia baikalensis* and *Baikalospongia bacillifera* sponges used for the analysis of RNA viruses. Individuals of *L. baikalensis* collected in March 2015 from the Maloye More Strait showed signs of damage: discoloration (#1) and brown spots (#2). Four specimens of *B. bacillifera* were collected in May 2018 from the Bolshiye Koty region; two of them, #3–#4, were visually healthy, and two, #5–#6, showed necrosis and brown spot-like lesions. Numbers of samples for analysis are given in parentheses (with the letter "h" at the end—healthy, with "d"—diseased).

Table 1. Description of sponge datasets used for analysis.

Dataset Name [1,2]	Sample Description	Geographic Location	Latitude and Longitude	Data	Nucleic Acids	Experiments	Reference
L.b.Sv1.1h	*Lubomirskia baikalensis*, healthy branch	Russia: Lake Baikal	53.02 N 106.93 E	March 2015	RNA (V)	SRX19982199	This study
L.b.Sv2.1d	*Lubomirskia baikalensis*, branch with bleaching	Russia: Lake Baikal	53.02 N 106.93 E	March 2015	RNA (V)	SRX19982200	This study
L.b.Sv3.2h	*Lubomirskia baikalensis*, healthy branch	Russia: Lake Baikal	53.02 N 106.93 E	March 2015	RNA (V)	SRX19982201	This study
L.b.Sv3a.2d	*Lubomirskia baikalensis*, branch with brown spots	Russia: Lake Baikal	53.02 N 106.93 E	March 2015	RNA (V)	SRX19982202	This study
B.b.Sv2478.3h	*Baikalospongia bacillifera*, healthy	Russia: Lake Baikal	51.90 N 105.10 E	June 2018	RNA (V)	SRX19982203	This study
B.b.Sv2480.4h	*Baikalospongia bacillifera*, healthy	Russia: Lake Baikal	51.90 N 105.10 E	June 2018	RNA (V)	SRX19982204	This study
B.b.Sv2473.5d	*Baikalospongia bacillifera*, necrosis, brown spots	Russia: Lake Baikal	51.90 N 105.10 E	June 2018	RNA (V)	SRX19982205	This study
B.b.Sv2475.6d	*Baikalospongia bacillifera*, necrosis, brown spots	Russia: Lake Baikal	51.90 N 105.10 E	June 2018	RNA (V)	SRX19982206	This study
Ch.reniformis_1	*Chondrosia reniformis*, m/p	Spain: Mediterranean Sea, RM	42.08 N 3.20 E	July 2016	RNA/DNA (V)	SRX5385349, SRX5385338	[13]
Ch.reniformis_2	*Chondrosia reniformis*, m/p	Spain: Mediterranean Sea, RM	42.08 N 3.20 E	July 2016	RNA/DNA (V)	SRX5385350, SRX5385337	[13]
Ch.reniformis_3	*Chondrosia reniformis*, m/p	Spain: Mediterranean Sea, RM	42.08 N 3.20 E	July 2016	RNA/DNA (V)	SRX5385353, SRX5385336	[13]
Ap.aerophoba_1	*Aplysina aerophoba*, m/p	Spain: Mediterranean Sea, PL	42.30 N 3.29 E	July 2016	RNA/DNA (V)	SRX5385319, SRX5385345	[13]
Ap.aerophoba_2	*Aplysina aerophoba*, m/p	Spain: Mediterranean Sea, PL	42.30 N 3.29 E	July 2016	RNA/DNA (V)	SRX5385320, SRX5385346	[13]
Ap.aerophoba_3	*Aplysina aerophoba*, m/p	Spain: Mediterranean Sea, PL	42.30 N 3.29 E	July 2016	RNA/DNA (V)	SRX5385347, SRX5385351	[13]
P.ficiformis_1	*Petrosia ficiformis*, m/p	Spain: Mediterranean Sea, RM	42.08 N 3.20 E	July 2016	RNA/DNA (V)	SRX5385334, SRX5385330	[13]
P.ficiformis_2	*Petrosia ficiformis*, m/p	Spain: Mediterranean Sea, RM	42.08 N 3.20 E	July 2016	RNA/DNA (V)	SRX5385333, SRX5385329	[13]
P.ficiformis_3	*Petrosia ficiformis*, m/p	Spain: Mediterranean Sea, RM	42.08 N 3.20 E	July 2016	RNA/DNA (V)	SRX5385332, SRX5385339	[13]
Ag.oroides_1	*Agelas oroides*, m/p	Spain: Mediterranean Sea, RM	42.08 N 3.20 E	July 2016	RNA/DNA (V)	SRX5385321, SRX5385325	[13]
Ag.oroides_2	*Agelas oroides*, m/p	Spain: Mediterranean Sea, RM	42.08 N 3.20 E	July 2016	RNA/DNA (V)	SRX5385322, SRX5385326	[13]
Ag.oroides_3	*Agelas oroides*, m/p	Spain: Mediterranean Sea, RM	42.08 N 3.20 E	July 2016	RNA/DNA (V)	SRX5385323, SRX5385327	[13]
Med.sw.PL (2)	Seawater	Spain: Mediterranean Sea, PL	42.30 N 3.29 E	July 2016	RNA/DNA (V)	SRX5385341, SRX5385342	[13]
Med.sw.RM (2)	Seawater	Spain: Mediterranean Sea, RM	42.30 N 3.29 E	July 2016	RNA/DNA (V)	SRX5385343, SRX5385344	[13]
Hym.sp_1	*Hymeniacidon* sp.	Japan: Tokyo bay	35.34 N 139.64 E	April 2014	RNA (T)	DRX171015	[12]
Hym.sp_2	*Hymeniacidon* sp.	Japan: Tokyo bay	35.34 N 139.64 E	April 2015	RNA (T)	DRX171017	[12]
GBR.sw (3)	Seawater	Australia: Great Barrier Reef	18.83 S 147.63 E	October 2014	RNA (V)	SRX2883311, SRX2883316, SRX2883317	[11]
Rh.odorabile_1	*Rhopaloeides odorabile*	Australia: Great Barrier Reef	18.83 S 147.63 E	October 2014	RNA (V)	SRX2883299	[11]
Rh.odorabile_2	*Rhopaloeides odorabile*	Australia: Great Barrier Reef	18.83 S 147.63 E	October 2014	RNA (V)	SRX2883312	[11]
Rh.odorabile_3	*Rhopaloeides odorabile*	Australia: Great Barrier Reef	18.83 S 147.63 E	October 2014	RNA (V)	SRX2883313	[11]
Hal.panicea_A (37)	*Halichondria panacea*	United Kingdom: North Sea, BN	55.99 N–2.45 W	2014	RNA (T)	SRX4378335	[10]
Hal.panicea-B (11)	*Halichondria panacea*	United Kingdom: North Sea, BN	55.99 N–2.45 W	2014	RNA (T)	SRX4378332	[10]

[1] The last two characters in the names of the Baikal samples indicate the number of the sponge and the state of the individual: "h"—visually healthy, "d"—diseased/ damaged. [2] The numbers in brackets indicate the number of samples or data sets (replicates). Abbreviations: PL—Portlligat; RM—Montgrí, Medes Islands and Baix Ter Natural Park; BN—Barns Ness; m/p—mesohyl and pinacoderm; V—viral; T—total.

2.2. Library Preparation and Sequencing

The preparation and sequencing of DNA libraries were performed at the Genomics Core Facility of the Institute of Chemical Biology and Fundamental Medicine, Siberian Branch, of the Russian Academy of Sciences (ICBFM SB RAS, Novosibirsk, Russia), and at the Center of Shared Scientific Equipment "Persistence of microorganisms" of the Institute for Cellular and Intracellular Symbiosis, Ural Branch, of the Russian Academy of Sciences (ICIS UB RAS, Orenburg, Russia). Shotgun libraries were prepared by using and following the NEBNext Ultra II RNA Library Prep Kit (New England Biolabs, Ipswich, MA, USA) protocol. Sequencing of the libraries was conducted on the MiSeq platform (Illumina, San Diego, CA, USA) using a MiSeq Reagent Kit v3 (2 × 300 cycles).

2.3. Initial Shotgun Metagenomic Datasets on RNA Viruses in Marine and Freshwater Samples

For comparative analysis, we also used the available NCBI SRA datasets (Illumina platforms, paired-end reads) (Table 1) from the marine sponge (class Demospongiae) samples (and water samples, if available).

2.4. Primary Processing and Taxonomic Analysis of Initial Virome Reads

The quality visualization of the virome datasets (paired reads) was carried out using the FASTQC program. Trimming of the reads by quality was carried out using the Trimmomatic v.0.39 program (MAXINFO:40:0.05 AVGQUAL:15 MINLEN:100) [37].

Taxonomic classification was carried out with the Kaiju v.1.9.0 software [38] using the NCBI nr (non-redundant) protein database. To increase the sensitivity of the Kaiju software, the parameter length was specified as 6, and the mismatch parameters were specified as 30. To filter out false positive results, the e-value was chosen as $\leq$0.00001 and bit score $\geq$ 50. The results of the Kaiju analysis were grouped in a table (Kaiju taxon count table) where the elements contained information about the representation of taxa in the number of reads per sample. Based on taxonomic identifiers, information on RNA viruses was extracted from the "Kaiju taxon count table" in the form of a table of virotype representation. For further analysis, this table of virotype representation was used in two variants: absolute values (number of reads from a sample per RNA virotype) and relative values (percentage of sample reads per RNA virotype).

2.5. Assembly of Virome Reads

All RNA datasets from the Baikal sponges were aggregated into one array for de novo cross-assembly, as applied in our previous studies of viral communities [16]. For cross-assembly, the meta SPAdes assembler, optimized for metagenomics, was used with default parameters [39]. The Bowtie2 [40] and SAMtools [41] software were used to map paired-end reads on scaffolds and to calculate the total coverage of scaffolds in the assembly and the coverage of scaffolds by reads from each sample. The scaffolds with total coverage of more than five and a length of $\geq$500 bp were used for further analysis.

2.6. Viral Scaffolds Detection

We identified the RNA viral scaffolds and open reading frames (ORFs) within them using the VirSorter2 tool [42] with an integrated RNA viral database. The results of RNA-virus identification were checked by the CheckV program [43], according to the instructions (https://bitbucket.org/berkeleylab/checkv/src/master/, accessed on 23 December 2022).

The Bowtie2 [40] and SAMtools [41] results were used to determine the number of reads mapped on each predicted viral scaffold from each sample. Counts of the predicted viral proteins (ORFs) in samples were defined as the number of reads mapped on a scaffold containing a given protein. Consequently, the count table of viral scaffold representation in the analyzed samples was constructed. TPM (transcripts per million) normalization, recommended for metagenomics, was used to normalize the count table for scaffold length and number of reads per sample [44].

2.7. Taxonomic Assignment of Viral Scaffolds

Taxonomic identification of the viral scaffolds was carried out by comparisons of predicted viral proteins in scaffolds with the NCBI RefSeq complete viral proteome database [45]. The comparison was carried out using the DIAMOND algorithm [46] with a "more-sensitive" option, i.e., e-value $\leq$ 0.00001 and bit score $\geq$ 50. For each protein in the scaffold, the best match in terms of the bit score value was selected. If a single scaffold had multiple proteins that matched different taxa (NCBI RefSeq ID), the one with the largest number of matching proteins was chosen as the most closely related virus taxon (virotype) for this scaffold. If all identified proteins belonged to different taxa, the level of bit-score of the matched proteins was taken into account, and the NCBI RefSeq taxon (ID) with the highest bit-score was selected as the virotype.

Host prediction for the Baikal viral scaffolds was carried out based on the Virus–Host database [47].

2.8. Functional Assignment of Viral Scaffolds

The predicted viral proteins (ORFs) were matched with functional motifs of proteins in the KOfam database [48] using KofamScan software [49], and with functional motifs in the Pfam database using PfamScan software [50] with default options. For ORFs containing RNA-dependent RNA polymerase (RdRp) domains, i.e., most likely non-structural polyproteins, an additional analysis was performed to search for the closest analogues in the nr NCBI protein database using the online BLASTp application. The 50 top BLAST hits were chosen if the number of hits was more than 50, and all hits if their number was less than 50. For each ORF, a correlation analysis of the relationship between the similarities of BLASTp matches with the alignment length was performed (Spearman coefficients correlation with testing using Spearman statistics, with a p-value $\leq$ 0.05 indicating a significant correlation).

2.9. Statistical Analysis of Taxonomic and Functional Diversity

The potential underestimated number of RNA virus Kaiju virotypes (species richness) in the total communities was evaluated using Chao1 [51] and ACE [52] indices. The Shannon and Simpson indices [53] of viral diversity were also calculated.

For further analysis, the Kaiju proportion of reads for RNA virotypes and TPM per samples of the Baikal sponges were standardized into ranges from 0 to 1 by the "decostand" function of the "vegan" package for the R [54]. Standardized values of the Kaiju proportions of reads for RNA virotypes and TPM per sample were visualized using canonical correspondence analysis (CCA). Gradient vectors of the RNA viral family composition and scaffolds' TPM per sample composition were fitted on a CCA scatter plot. The reliability of linear approximation for gradient vectors was assessed by multivariate linear regression analysis. Only vectors that showed reliable directionality were displayed. Biodiversity analysis and CCA ordination were carried out using the "vegan" package for the R [54], according to the instructions.

The Kaiju proportion of reads for the first 30 dominant RNA virotypes and TPM per samples of the Baikal sponges RNA viral scaffolds were visualized with a heat map using the "gplots" package [55] for the R with columns (samples) clustering, and were grouped in order of similarity (Euclidean distance with the complete-link clustering method).

The influence of different factors on scaffolds' TPM per sample composition was assessed with PERMANOVA analysis (2000 *permutations* and Euclidean distance) [56] in the R package "vegan" [54]. The influence of the factor was considered significant at a p-value $\leq$ 0.05.

3. Results

3.1. Analysis of Virome Reads

Eight data sets were obtained from six individuals of the sponges *L. baikalensis* and *B. bacillifera* after separation of the viral fraction (<0.2 μm) from the samples, RNA isolation and sequencing, and primary processing of the reads (Table 1). The resulting metadata were

analyzed using the Kaiju software, and the overall composition of the viromes (percentage of eukaryotes, bacteria, archaea, and viruses) was determined (Supplementary Table S1). Additionally, the available datasets from marine sponges were used for comparison (Table 1). The percentages of unidentified sequences in the Baikal samples were 22–46%; in other samples they reached up to 81–83% (in particular in the sets A and B of *Halichondria panicea* sponges). Large proportions consisted of bacterial and eukaryotic reads (respectively, 15–27% and 22–51% in the Baikal samples, 11–60% and 0.02–26% in the compared data) (Supplementary Table S1). It should be noted that these values were strongly influenced by the method of sample preparation; first of all, it mattered whether the viral fraction was enriched or not, as did what kinds of nucleic acids were isolated and sequenced (viral fraction, V, or total sample, T; RNA or both RNA and DNA, indicated in Table 1). Another important point is the depletion of ribosomal RNA during the preparation of libraries. In the case of the Baikal samples, we sequenced RNA isolated from viral fractions, and depletion was not performed; therefore, these datasets contained a large amount of rRNA, despite the fact that the rRNA during centrifugation at high speed (17,000× *g*, 3 min) in the samples was significantly reduced [57].

The percentage of RNA viruses in the samples was generally low: 0.001–0.12% in the Baikal sponges and 0.0004–2.4% in the marine sponges (Supplementary Table S1). The exception was the sample of *Hymeniacidon* sp. (*Hym*.sp_2, 27.3%), as well as the control seawater sample from the Great Barrier Reef (GBR.sw, 50.2%). A small portion of the sequences in the Baikal sets were affiliated with DNA-containing viruses (0.001–0.3%); in marine datasets, their proportion was generally higher (0.02–15.4%: minimum in *H. panicea* sponges, maximum in Mediterranean water samples, Med.sw.RM). In fact, in the case of the *H. panicea* sponges [10], enriched viral RNA was isolated, but in the study of the Mediterranean samples [13], the total viral RNA and DNA were used for analysis. Viral diversity indices for all datasets are shown in Supplementary Table S2; the number of virotypes in the Baikal samples ranged from 8 to 98, while in marine samples, it ranged from 13 to 466.

The difference between analyzed freshwater and marine sponges was noticeable at the levels of both families and virotypes (Figure 2, Supplementary Table S3). On the CCA plot (Figure 2A), apart from the Baikal ones, the most distant samples were also *Rhopaloeides odorabile* (from GBR) and *Hymeniacidon* sp. (Japan) sponges; the Mediterranean and *Halichondria panicea* (North Sea) sponges occupied an intermediate position (located approximately in the center of the graph). A distinctive feature of the Baikal sponges was the greater representation of the families *Tombusviridae*, *Marnaviridae*, and *Luteoviridae.* In Figure 2B, we were able to see the top virotypes for different sets. For almost all marine (with the exception of *Hym*.sp.), as well as for some Baikal, samples (B.b.Sv2480, L.b. Sv2.1d and Sv1.1h), the most numerous was a certain unclassified *Picobirnavirus* (*Picobirnavirus* sp. strain PBV/roe_deer/SLO/D38-14/2014, [58]). Moreover, *Cylindrotheca closterium RNA virus 03* and *Beihai sobemo-like virus 9* dominated in GBR sponge samples (up to 43% of viral reads); *Sponge holobiont-associated RNA virus* (up to 82.4%) in *Hymeniacidon* sp.; but *Barns Ness breadcrumb sponge hepe-like virus 1* (28.3%) and *Barns Ness breadcrumb sponge aquatic picorna-like virus 1* (11%) in *H. panicea*. Samples of Mediterranean sponges and *H. panicea* were highly represented in retrovirus sequences (*Alpha-*, *Gammaretroviruses*). Datasets from individual studies tended to be grouped separately, and their distribution was consistent with geographical distances (Figure 2A,B).

Read-based analysis was mainly used to compare datasets of the Baikal (freshwater) and other (marine) sponges. Further and more accurate analyses and reliable descriptions of the Baikal samples were carried out based on assembled scaffolds.

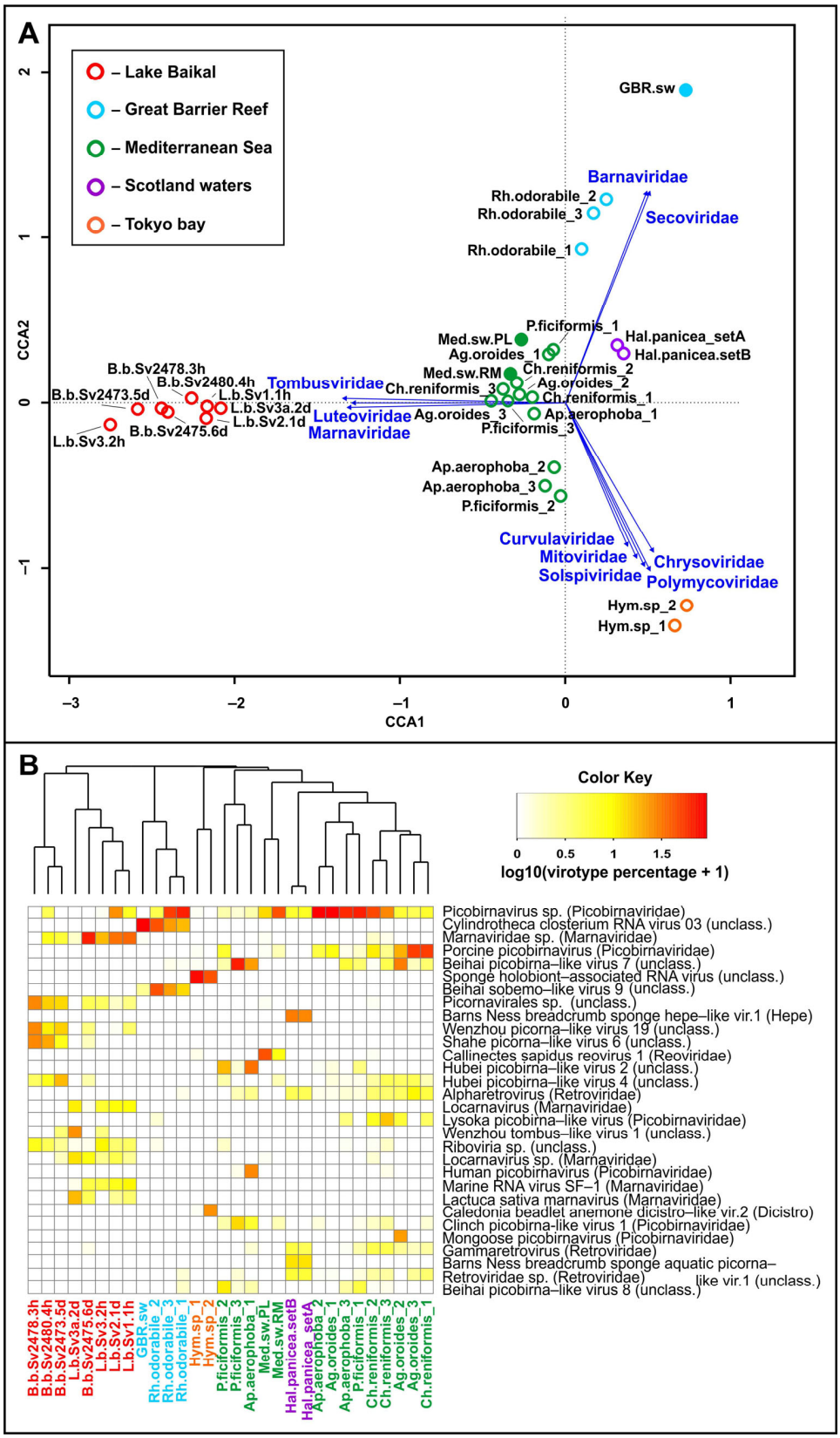

Figure 2. Comparative analysis of the Baikal and marine samples based on the number of reads belonging to different RNA virotypes. (**A**)—CCA scatter plot with gradient vectors of RNA viral families; (**B**)—heatmap of top 30 dominant virotypes with clustering by Euclidean distances. Abbreviations: "Hepe-"—*Hepeviridae*, "Dicistro-"—*Dicistroviridae*, "vir"—virus.

3.2. Assembly and Analysis of Viral Scaffolds

Assembly of reads from the Baikal sponges, further identification of viral sequences, and open reading frames (ORFs) using VirSorter 2 allowed 31 viral scaffolds, over 500 nucleotides in length (maximum length 9067 bp), to be revealed; in terms of the number of reads, this amounted to 0.03–0.5% of the total sequences. Some ORFs from 28 scaffolds were related to proteins from the NCBI RefSeq database; the similarity of hypothetical proteins with known viral proteins ranged from 20.7 to 67.3% (Supplementary Table S4). Taxonomic identification of the assembled scaffolds was carried out according to the previously published algorithm [16]. The identified virotypes (Table 2, Supplementary Table S5) belonged to the families *Tombusviridae*, *Picobirnaviridae*, and *Marnaviridae*, but the most were unclassified RNA-containing viruses (realm *Riboviria*).

Table 2. RNA virotypes revealed in the Baikal sponge datasets.

Species	Family *	Genus *	Host/Isolation Source	No. of Scaffolds
Tobacco necrosis virus D	*Tombusviridae*	*Betanecrovirus*	Plants	45
Changjiang tombus-like virus 20	-	-	/Crustaceans	15, 17, 37
Wenzhou narna-like virus 5	-	-	/Mollusk	24, 9, 532
Beihai picorna-like virus 82	-	-	/Crustaceans	69
Wenzhou picorna-like virus 19	-	-	/Mollusk	3
unclassified	-	-		5, 25, 374
Picobirnavirus green monkey/KNA/2015	*Picobirnaviridae*	*Picobirnavirus*	Vertebrates	48
Wenzhou tombus-like virus 15	-	-	/Mollusk	145, 173
Marine RNA virus SF-1	*Marnaviridae*	*Locarnavirus*	/Wasterwater	6
Ubei picorna-like virus 3	-	-	/Insecta	2
Hubei odonate virus 1	-	-	/Insecta	1
Furcraea necrotic streak virus	*Tombusviridae*	*Macanavirus*	Plants	328
Wenzhou picorna-like virus 41	-	-	/Mollusk	4
Wenzhou weivirus-like virus 1	-	-	/Mollusk	10
Beihai tombus-like virus 15	-	-	/Crustaceans	13
Hubei sobemo-like virus 3	-	-	/Mollusk	167, 286
Beihai noda-like virus 5	-	-	/Crustaceans	11
Beihai tombus-like virus 8	-	-	/Mollusk	49
Hubei unio douglasiae virus 3	-	-	/Mollusk	28
Wenzhou tombus-like virus 16	-	-	/Mollusk	83
Beihai picorna-like virus 56	-	-	/Mollusk	8
Sanxia narna-like virus 1	-	-	/Crustaceans	168

* Unclassified viruses are marked with "-".

In the CCA analysis (Figure 3A), there was a trend of separation of diseased and healthy branches/individuals, and the influence of damage factors was confirmed statistically (p-value in PERMANOVA 0.042). Note that the distances between healthy individuals of *L. baikalensis* and *B. bacillifera* were much smaller than between diseased and healthy individuals and between all diseased specimens. In Figure 3A, one can also see a clear trend in the separation of the sponge species plots. Reliable virotype vectors were divided into two groups, mainly according to "sponge-species" affiliation; that is, one group was directed towards *L. baikalensis* and another towards *B. bacillifera* (Figure 3A). Depending on the quantitative representation of different virotypes, samples of the sponges *L. baikalensis* and *B. bacillifera* also formed two separate clusters (Figure 3B). However, despite the significance of the differences between the *L. baikalensis* and *B. bacillifera* samples (p-value in PERMANOVA 0.025), we cannot state the host–species- specificity of the identified viral communities since the samples of the two species were taken at different stations (at a distance of about 176 km, Figure 1) and during different seasons: in March 2015 (during the freeze-up period; the bottom water temperature at this time was about 0 °C with an average pH of 7.6 [59]) and May 2018 (after ice breaking; temperature 2–3 °C, average pH 8.2 [59]), respectively. The differences between the viromes of *L. baikalensis* and

B. bacillifera most likely formed under the influence of all factors (sponge species, habitat, seasonal or longer-term changes in environmental parameters), but to a different extent.

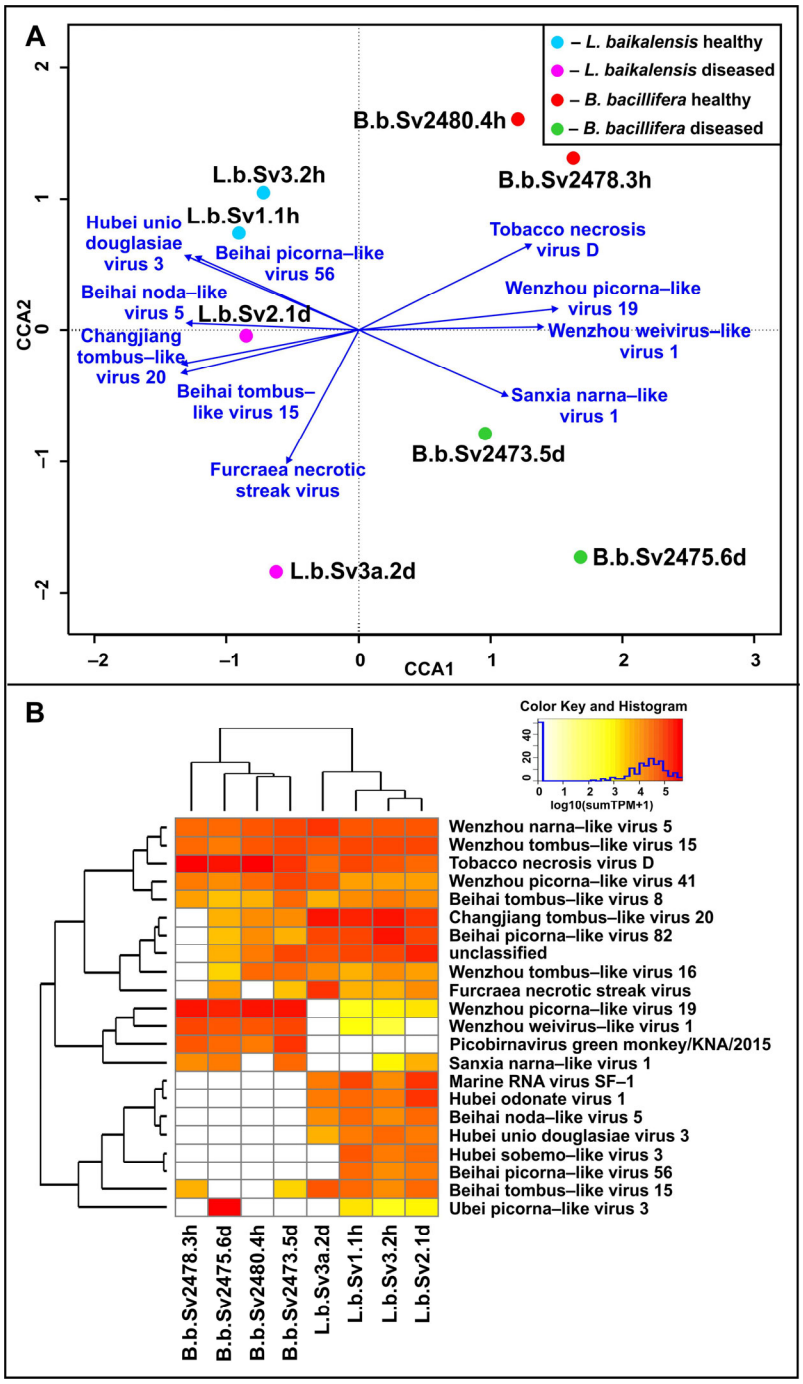

Figure 3. Representation of RNA virotypes in the samples of the Baikal sponges *L. baikalensis* and *B. bacillifera*: (**A**)—CCA analysis based on the revealed virotypes; (**B**)—heatmap and clustering (Euclidean distances) of the virotypes and samples.

Among the samples of *L. baikalensis*, the most numerous RNA-containing virotypes (by number of reads) were *Changjiang tombus-like virus 20* and *Beihai picorna-like virus 82* (Figure 3B, Supplementary Table S5). *Tobacco necrosis virus D* and *Wenzhou picorna-like virus 19* dominated in *B. bacillifera*. *Marine RNA virus SF-1* (*Sanfarnavirus 1*, according to ICTV) dominated in *L. baikalensis* #1 samples (L.b.Sv1.1h and L.b.Sv2.1d); this virotype, as well as *Hubei odonate virus 1*, quantitatively prevailed in the branch with a lesion (L.b.Sv2.1d).

Viruses similar to *Wenzhou narna-like virus 5*, *Furcraea necrotic streak virus*, and *Wenzhou picorna-like virus 41* were markedly more numerous in the affected branch of sponge *L. baikalensis* #2 (L.b.Sv3a.2d). For *B. bacillifera* sponges, some virotypes also predominated in the affected specimens (eg, *Picobirnavirus green monkey/KNA/2015* in B.b.Sv2473.5d, or *Ubei picorna-like virus 3* in B.b.Sv2475.6d), but others, on the contrary, dominated in apparently healthy individuals (eg, *Tobacco necrosis virus D*).

3.3. Potential Hosts for Identified Viruses

According to the Virus–Host database (https://www.genome.jp/virushostdb, accessed on 1 February 2023), only three viruses (*Tobacco necrosis virus D*, *Furcraea necrotic streak virus*, and *Picobirnavirus green monkey/KNA/2015*) from the list of virotypes were well-characterized and had known host ranges. The first two viruses infected various plant species, and the third virus infected vertebrates. The genomes of the remaining viruses were obtained from natural samples by assembling metagenomic (meta-transcriptomic) reads, so-called metagenome-assembled genomes (MAGs). As follows from Table 2, most of them (9 virotypes) were obtained from mollusk samples (two of them were also found in fish), five from crustaceans, and two from insects.

3.4. Diversity of RNA-Viral Proteins

According to the Pfam database, a small portion of the scaffolds (7 scaffolds in total) contained the protein domains of Viral-coat, Rhv, DicistroVP4, and Phospholip_A2_4 as parts of structural polyproteins. Most of scaffolds contained the functional domains of enzymes involved in replication: RNA helicase (4 out of 19) and RNA-dependent RNA polymerase (RdRp and Tombus_P33, 16 out of 19) (Figure 4A, Supplementary Table S6). In addition to these proteins, using the KOfam database, some proteases (Enterovirus proteins 2C, 3C or 3CD), NTPase, kinases, etc. were also identified (Supplementary Table S7).

Our analysis showed a wide range of percent similarity and length alignment between putative functional polyproteins and BLAST hits from the nr NCBI database (Figure 4B,C). For the top 50 BLAST hits, the minimum similarity was 20.7% and the maximum was 99.5%; on average, from 23 to 42%. The largest spread in alignment length was observed for the longest scaffolds (NODE_1, NODE_2, NODE_3, NODE_4 and NODE_8) (Figure 4C). At the same time, the following trend could be traced: the longer the alignment, the lower the percentage of similarity, and vice versa. The significance of this negative correlation was confirmed for RdRps longer than 500 amino acid residues (p-value < 0.05), except for the scaffold NODE_11, where the length of the predicted ORF was 935 aa, but the correlation was absent. The observed results may be associated with a large variability in the sequences, lengths, and orders of different genes in the genomes of RNA viruses, and, accordingly, proteins in the structure of polyproteins. BLAST hits, identified in the nr NCBI database, are often "hypothetical proteins", and can be either polyproteins or particular proteins (RdRp, Rhv, or others).

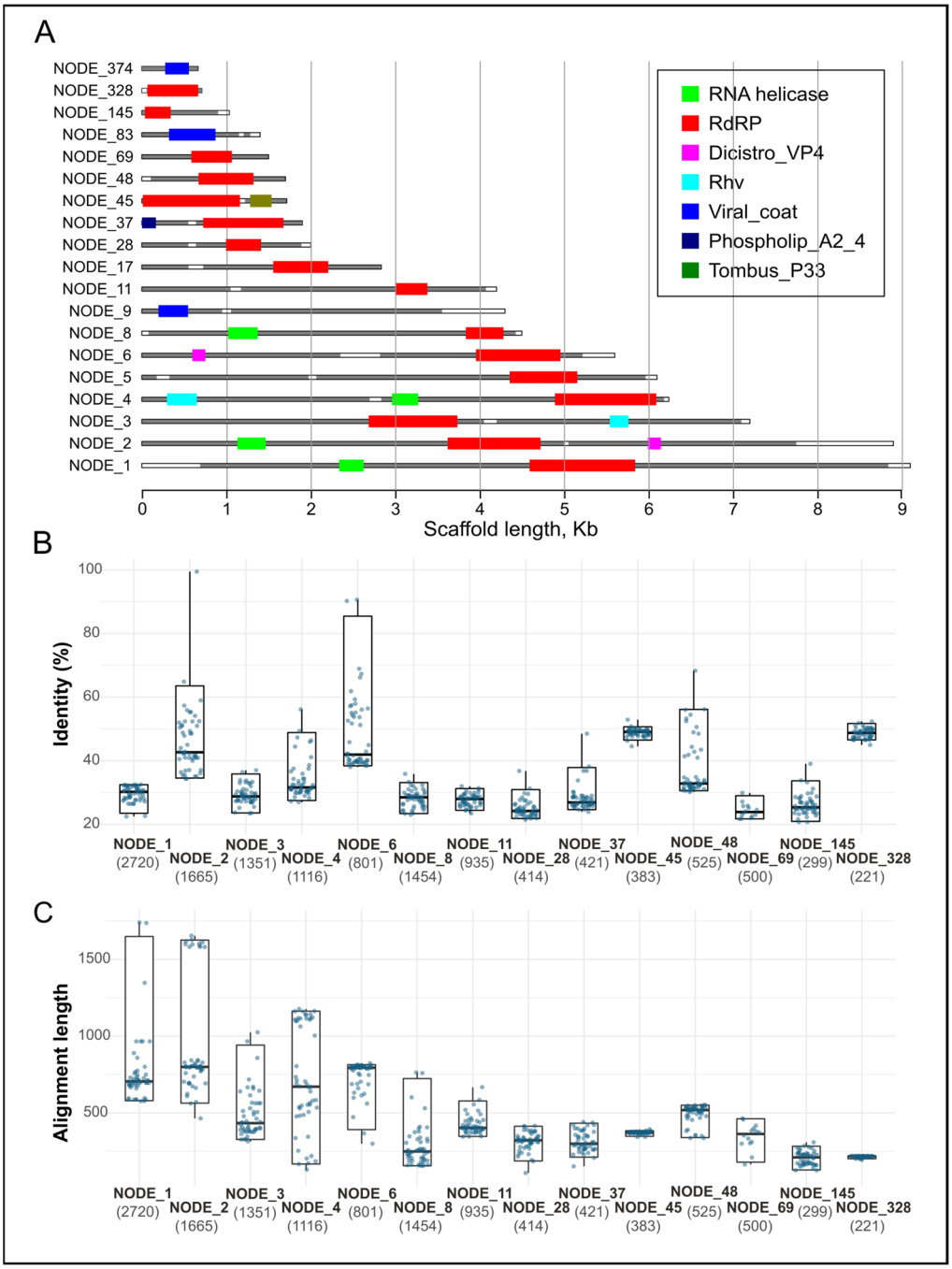

Figure 4. Layout of identified Pfam domains in viral scaffolds from the Baikal sponges ((**A**); the gray lines show the identified ORFs), percent similarity (**B**), and alignment length (**C**) of the ORFs, including non-structural domains, with the top 50 BLAST hits from the nr NCBI database. In brackets after the number of the scaffold (in (**B**,**C**)), the length of the ORF (aa) is indicated.

4. Discussion

In this study, we first evaluated the diversity of RNA-containing viruses in the Baikal sponges *Lubomirskia baikalensis* and *Baikalospongia bacillifera*, representatives of the endemic family Lubomirskiidae, by shotgun sequencing of RNA from an isolated fraction enriched with VLPs. First, we analyzed the composition of the sponge viromes and established the taxonomic affiliation of the reads, along with similar datasets of marine sponges from the SRA NCBI database (Table 1). Next, the reads from the Baikal samples were assembled,

and the identified viral scaffolds and open reading frames of potential viral proteins in their structures were analyzed.

The proportion of RNA viral reads in the Baikal samples was small, but we were able to detect a variety of sequences belonging to RNA viruses. Several families were identified in the analysis of the scaffolds, namely, *Tombusviridae*, *Picobirnaviridae*, and *Marnaviridae*: these three families were also the most numerous in the direct analysis of the reads (Table S3). *Tombusviridae* is a family of ssRNA plant viruses [60]. The hosts for *Picobirnaviruses* (viruses with dsRNA genomes) were, until recently, thought to be mammals, but it is now known that these viruses can infect birds, reptiles, and invertebrates [29,61,62]. *Marnaviridae* (ssRNA viruses) is a family in the order *Picornavirales* that infects various photosynthetic marine protists [63]. In general, the picorna-like viruses are a unique group that infects a remarkable variety of organisms: humans, mammals, insects, plants, and even marine algae [47].

Most of the scaffolds in our study were similar to viruses with undetermined classifications (unclassified *Riboviria*) whose genomes were assembled in the metatranscriptomics studies of various invertebrates from aquatic biotopes [29] (Table 2); among them there were narna-like, picorna-like, noda-like, and sobemo-like viruses. Thus, the identified RNA viruses in sponge holobionts most likely infect nonbacterial hosts, namely, invertebrates and protists, in contrast to DNA viruses revealed previously in *B. bacillifera* sponges [16]. Our results also indicate a wide variety of poorly studied RNA viruses circulating among a wide range of aquatic organisms. Recent genomic and metagenomic studies have actively replenished the database with new genomes [10,29,30]; however, additional studies are required in order to establish the range of hosts and other properties of the identified viruses [64].

The similarity of proteins (structural and non-structural) identified in viral scaffolds from the Baikal sponges and those from the RefSeq NCBI database turned out to be low (20.7–67.3%, Supplementary Table S4). BLAST analysis with the nr NCBI database for ORFs with the RdRp and other function domains revealed a maximum level of similarity (99.5%) only between the NODE_2 scaffold and MAG from river sediment (URG14928.1, [65]), and between the NODE_6 scaffold and the nonstructural proteins of *Flumine marna-like viruses 8* and *9* (UQB76253.1 and UQB76036.1, respectively) ([66]; in those case, the similarities were 90.6 and 90.3%, respectively. However, the alignment length in these cases was no more than 560 aa (<33% of the total length of the putative polyproteins). For the rest of the identified BLAST hits, the similarity did not exceed 68.3% (Figure 4). This indicates that we have identified previously unknown genomes of RNA viruses. It is interesting that the most similar BLAST hits were isolated from freshwater ecosystems (from sediment or shellfish in China and from water samples in New Zealand). Thus, the number of known viruses circulating in freshwater ecosystems is limited.

The samples of two species of Baikal sponges (*L. baikalensis* and *B. bacillifera*) differed in terms of the composition of the identified genotypes (scaffolds) and were clustered separately, which, first of all, would seem to indicate differences in the compositions of viruses of the two sponge species and to confirm the previously obtained host-specific patterns in the viral assemblages [7,8,11]. However, it should be noted once more that sampling of *L. baikalensis* and *B. bacillifera* was carried out at different stations and in different seasons (Table 1), and based on the results of this study, we cannot confirm the host–species-specificity of viral communities of these two sponge species. We are also unable to determine the influence of other factors, including habitat ("site-specificity") [7,8,17], season (as shown for the sponge microbiomes [67]), or more long-term dynamics of environmental parameters.

Nevertheless, we were able to show a significant difference between damaged (or diseased) and visually healthy samples of the *L. baikalensis* and *B. bacillifera* sponges. Furthermore, the proportion of viral reads (Supplementary Tables S1 and S5) and the number of virotypes (α-diversity, Supplementary Table S2) in the affected sponges tended to be higher than in the visually healthy sponges, with the exception of *L. baikalensis* #2, where the number of viral reads and virotypes in the branch with damage (L.b.Sv3a.2d) was much

lower than in the visible healthy branch (L.b.Sv3.2h). The distribution of CCA plots in Figure S3 indicates significant shifts and heterogeneity in the composition of viral communities of diseased samples relative to visually healthy ones. Taking into account the obtained results, and based on the available knowledge and many years of experience in monitoring of the states of the sponges (starting from 2015) [18,28], the following picture emerges. Under stress caused by unfavorable factors (climatic, anthropogenic and/or others), disturbances in metabolism, weakening of immunity, and gradual death of sponge cells and their symbionts (necrosis) occur. Following damage and loosening, the body of the sponge, starting from the surface, is colonized by atypical species of bacteria, algae, protozoa, or other organisms, for which a favorable environment rich in nutrients appears [68]. Various observed patterns or types of lesions (spots, necrosis, discoloration, etc. [28]) are result from colonization of sponges by various groups of invaders. Against the background of these stressful conditions, viral and other infections of the holobiont also appear; the hosts for viruses can be associated microorganisms as well as the sponges themselves. Active reproduction of some viruses that present in "healthy" holobionts may also occur. In general, in the event of damage (or disease), the diversity and quantity of viruses and various microorganisms can be replaced and enriched, or, conversely, can become poorer; thereby, the composition of the sponge holobiont varies greatly. This is recorded in the analyses of not only viral, but also microbial, communities of Baikal sponges [69].

Many researchers of marine sponges and coral reefs conclude that diseases of these aquatic organisms occur due to stress caused by changes in their environments. There is ample evidence that the rise in temperature and the influence of anthropogenic factors [17,70,71] preceded the marine sponge disease outbreak, exacerbating the course of the disease [72,73]. Sponges are unique holobionts, and it is the complex interaction of diverse microbial communities that ensures their stability and plasticity [74]. Stress causes changes in the physiological state of the host and leads to a loss of control over the sponge microbiome [17]. One of the most studied adverse factors for marine sponges is elevated temperatures. Under the influence of heat stress, there is a significant decrease in the expression of genes involved in cytoskeletal processes, signaling, and detoxification; an increase in the expression of heat shock proteins (Hsp70); and, at the same time, a change in the composition of the microbiome, which ultimately leads to cell apoptosis and sponge tissue necrosis [75]. The trend of warming of the atmosphere, as well as the surface of the water, in the Baikal region [76–78] correlates with the global trend [79]. Thus, the increase in the temperature of the surface water layer of Lake Baikal from 1890 to 2000 (from May to October) averaged about 1.0 degree [76], and during the periods of summer stratification (August–September) for 1977–2003, by 2.0 °C [77]. The increase in the atmospheric temperature over the past 65 years was 1.5 °C [78]. In addition, in the early 2000s (2000–2007), there was a change in the oxygen regime and a tendency for a decrease in oxygen concentration to occur in the summer and autumn months compared to the studied period of the previous century (1948–1951) [59]. Anthropogenic impact has also had a serious detrimental effect on aquatic organisms. For example, the influence of various chemical pollutants on the key stages of life and behavior of corals has been demonstrated [80]. Organic substances coming from wastewater settle on the surfaces of sponges, causing a decrease or blocking of filtration activity [81,82], and prolonged exposure can lead to the death of sponges [83]. An increase in the anthropogenic load on the coastal zone of Lake Baikal and the presence of organic layers on the surfaces of sponges (according to our observations) are adverse factors for sponges' health and prerequisites for their disease and death.

The list of virotypes includes three well-studied viruses: *Tobacco necrosis virus D*, *Furcraea necrotic streak virus*, and *Picobirnavirus green monkey/KNA/201*. *Tobacco necrosis virus D* [84], together with *Leek white stripe virus* and *Beet black scorch virus*, form the genus *Betanecrovirus* within the family *Tombusviridae*. *Furcraea necrotic streak virus* [85] also belongs to the family *Tombusviridae*; it is the only member of the *Macanavirus* genus that causes the so-called "macana disease". The symptoms of diseases caused by these and other tombusviruses are similar: mottling, wrinkling, and necrotic spots (rounded or in the form

of stripes) appear on the surfaces of the leaves of plants. Among the BLAST hits for the proteins from viral scaffolds determined using the nr NCBI database, there were other plant viruses that caused necrosis in plants, such as the aforementioned *Leek white stripe virus* and *Pelargonium ringspot virus* (family *Tombusviridae*), as well as the *Southern cowpea mosaic virus* (*Solemoviridae*) (data not shown). Given the common symptomatology of sponge diseases, a reasonable assumption arises regarding the circulation of an unknown RNA virus (or viruses) in sponges which is closely related to a virus affecting plants. This is consistent with the data obtained by [29,34,35] on the close relationship between plant viruses and those of aquatic invertebrates.

The average values of the Shannon and Simpson indices (Supplementary Table S2) in freshwater sponges turned out to be higher than in marine ones (Shannon/Simpson—2.49/0.83 in the Baikal vs. 2.0/0.67 in the marine sponges); in our previous comparative study of DNA viruses [16], these values were almost the same (Shannon/Simpson—3.2/0.89 in *B. bacillifera* vs. 3.2/0.93 in *Ianthella basta* marine sponges [11]). The largest number of virotypes (α-diversity) among all datasets was found in the marine sponges *Hymeniacidon* sp. and *Halichondria panacea* (458–466 and 308–363, respectively). In the first case, a technique to enrich viral dsRNAs in samples was used [12]; in the second, pools of a large number of individuals (37 individuals in set A and 11 in set B) were analyzed [10]. The viral diversity between different individuals of the same species could vary greatly; for example, 26 to 199 virotypes were found in *Aplysina aerophoba* and 20 to 116 in *Petrosia ficiformis* in one study [13].

We were able to show significant differences in the composition of the viromes of the Baikal and marine sponges at the level of families and virotypes. For example, representatives of the families *Tombusviridae* and *Marnaviridae*, highly represented in the Baikal sponges, were absent or present in small numbers in the marine sponges. Dominant virotypes in the Baikal sponge samples were generally absent in marine sponges (such as *Wenzhou picorna-like virus 19*, *Shahe picorna-like virus 6*, *Wenzhou tombus-like virus 1*, *Marine RNA virus SF-1*, and *Lactuca sativa marnavirus*), and vice versa (Figure 2B, Supplementary Table S3). Among the dominant virotypes in distinct marine samples were sponge-associated viruses, whose genomes were identified only in relevant studies (namely, *Sponge holobiont-associated RNA virus* in *Hymeniacidon* sp. sponges [12], or *Barns Ness breadcrumb sponge hepe-like virus 1* and *Barns Ness breadcrumb sponge aquatic picorna-like virus 1* in the *H. panacea* [10]), demonstrating the "sponge-species" specificity and the uniqueness of some viruses in sponge holobionts. It is interesting that, in *Hymeniacidon* sp. sponges, despite having the largest number of virotypes, the Shannon/Simpson diversity indices were among the lowest (average: 1.50/0.53; Supplementary Table S2). This is associated with an uneven distribution of virotypes in normalized (TPM) data and a strong predominance of *Sponge holobiont-associated RNA virus* (up to 82.4%). The clustering (Figure 2A,B) also indicates the difference between the Baikal and other analyzed samples, which is not surprising given the remoteness of water bodies and the differences in their habitats (primarily the influence of the salinity, as well as the unique cold climate of Lake Baikal). Earlier, we obtained similar results when studying the diversity of DNA-containing viruses in samples of the Baikal sponge *Baikalospongia bacillifera* [16].

5. Conclusions

Sponges are unique animals, and an integral and ecologically significant component of many marine and freshwater ecosystems. As shown in a review [86], more than 70 studies have been published in which marine sponges were proposed as bioindicators of pollution of aquatic ecosystems with toxic elements (in particular, heavy metals), and the application of these organisms in bioremediation has been considered. Whether sponges can act as bioindicators of viral contamination of aquatic ecosystems due to anthropogenic pollution is still unknown.

Viruses (of sponges and symbionts) are a diverse and inherent part of the sponge holobiont and most likely play a significant role in the development of pathological processes;

this is supported by a trend towards an increase in the proportion of viral reads and the number of virotypes in the affected samples. However, prerequisites or aggravating factors for the development of a viral disease, as in the case of any other organism, are still unfavorable conditions (i.e., stress). Sponges represent a complex symbiosis (holobiont), where each component plays an important role and the interactions between all components ensure the smooth and uninterrupted operation of the whole organism. Accordingly, dysbacteriosis or other imbalance in this biosystem, which can be a result of a viral infection, may lead to visible lesions and even death of the sponge. However, to confirm this assumption, additional studies are needed; first of all, more detailed studies of the virotypes we have identified, some of which may affect the sponges themselves, should be conducted.

New members of realm *Riboviria* have been identified in large numbers in recent studies of a wide range of aquatic organisms, including sponges [10–13,29,30,87,88]. However, these new data, including those from our research, are far from exhaustive, and an increasing variety of viral RNAs will be revealed in future studies.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/d15070835/s1, Table S1: General taxonomic composition of the datasets (in %) used in the study; Table S2: The viral diversity indices for the datasets used in the study; Table S3: The list of RNA-viruses (virotypes) revealed in datasets on the base of the reads; Table S4: The scaffolds assembled from the Baikal sponge datasets and their closest relatives from the RefSeq NCBI database (the number of identified proteins in scaffolds and their matches with those of virotypes are indicated); Table S5: The counting of reads from the Baikal samples per virotype; Table S6: Pfam domain, identified in the Baikal scaffolds; Table S7: Identification of proteins with the KEGG Orthology database.

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Data Availability Statement: Raw virome reads for the samples of *Lubomirskia baikalensis* and *Baikalospongia bacillifera* were submitted to the National Center for Biotechnology Information (NCBI), Sequence Read Archive (SRA) database (BioProject PRJNA956241, BioSamples SAMN34206360-SAMN34206367). The direct URL to the data is as follows: https://www.ncbi.nlm.nih.gov/sra/PRJNA956241 (accessed on 31 May 2023).

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Conflicts of Interest: The authors declare no conflict of interest.

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