



RESEARCH ARTICLE

Viral metagenomics analysis of feces from coronary heart disease patients reveals the genetic diversity of the *Microviridae*

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Recent studies have declared that members of the ssDNA virus family *Microviridae* play an important role in multiple environments, as they have been found taking a dominant position in the human gut. The aim of this study was to analyze the overall composition of the gut virome in coronary heart disease (CHD) patients, and try to discover the potential link between the human gut virome and CHD. Viral metagenomics methods were performed to detect the viral sequences in fecal samples collected from CHD inpatients and healthy persons as controls. We present the analysis of the virome composition in these CHD patients and controls. Our data shows that the virome composition may be linked to daily living habits and the medical therapy of CHD. *Virgaviridae* and *Microviridae* were the two dominant types of viruses found in the enteric virome of CHD patients. Fourteen divergent viruses belonging to the family *Microviridae* were found, twelve of which were grouped into the subfamily *Gokushovirinae*, while the remaining two strains might represent two new subfamilies within *Microviridae*, according to the phylogenetic analysis. In addition, the genomic organization of these viruses has been characterized.

KEYWORDS gut virome; coronary heart disease (CHD); viral metagenomics; *Microviridae*; *Gokushovirinae*

INTRODUCTION

Coronary heart disease (CHD) is the second highest risk factor for cardiovascular death in China, where the burden of CHD has been increasing (Feng et al., 2016). Recent studies revealed that there may be a link between the gut microbiota and CHD (Wong et al., 2012). Modulation of the gut microbiota is suggested to have the potential to reduce the risk factors associated with CHD (Emoto et al., 2016). Being the main member of the gut microbial ecosystem, gut viruses may also have a potential impact on the microbial ecosystem of the hosts.

Viruses, the most abundant biological entities in the biosphere, have been classified and determined in multiple normal species and ecosystems, such as marine ecosystems, freshwater lakes and the human gut (Wommack and Colwell, 2000; Zhang et al., 2006; Breitbart et al., 2008). However, because of the high diversity of their genomes and morphology, it is hard to cultivate novel viruses or deeply explore their populations. So far, many viruses have not been discovered, and it is difficult to discuss their distribution and composition.

The metagenomic approach, which is a new technology within the last two decades, has provided an in-depth look at the molecular diversity of viruses in a range of environments, including the human gut system (Breitbart et al., 2002; Minot et al., 2011). *Microviridae* is one of the main families of bacteriophages with ssDNA. They are widely spread in marine environments, freshwater habitats, human gut or feces, stromatolites (Angly et al.,

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2006; Desnues et al., 2008; Lopez-Bueno et al., 2009; Tucker et al., 2011; Roux et al., 2012a; Roux et al., 2012b) and so on. Based on the nucleotide sequences and the phylogeny of the major capsid protein (VP1), *Microviridae* are further divided into seven subgroups, including microviruses (genus *Microvirus*), gokushoviruses (subfamily *Gokushovirinae*), alphaviruses (subfamily *Alphavirinae*), the recently described pichoviruses (subfamily *Pichovirinae*), the new clade *Stokavirinae* (stoka: small in Sanskrit), Group D and *Aravirinae* (ara: little in Sanskrit) (Roux et al., 2012b; Quaiser et al., 2015).

In this study, using the viral metagenomics method, we have investigated 43 stool samples from 37 CHD inpatients and six healthy people, and analyzed their virome composition. Twelve divergent *Microviridae* genomes were determined from the samples, and phylogenetic analysis and genome comparisons were performed. The data from the present study provide new insight of the diversity, distribution and abundance of the *Microviridae* subfamilies in humans, especially in CHD patients.

MATERIALS AND METHODS

Samples and viral metagenomic analysis

Thirty-seven fecal samples were collected from CHD inpatients from the Central Hospital in the Minhang District of Shanghai, China, while six fecal samples were collected from healthy residents, without a history of cardiovascular disease, living in communities in the Minhang District of Shanghai, China, as controls. All of the CHD patients had coronary angiographies (CAGs) and percutaneous coronary interventions (PCIs) performed in the cardiovascular department of the hospital, and CHD was defined when the narrowness diameter of the left main coronary artery, the left anterior descending branch, the circumflex artery, the right coronary artery and other main factors were up to or exceeded 50%; while the control members had no history of cardiovascular disease. All the subjects met the following conditions: (1) no application of any antacids, probiotics, antibiotic or antimicrobial agent for the past one or more months; (2) no other digestive system diseases; (3) no surgical operations on their digestive systems; (4) no drunkenness, smoking or diabetes or diseases that might affect the gut microbiota; (5) resident in southern China; (6) aged between 50 and 85 years old.

All samples were preserved at -80°C . Fecal samples were re-suspended in ten volumes of phosphate-buffered saline and vigorously vortexed for 5 min. Four hundred microliters of supernatant was collected after centrifugation (10 min, 15,000 g) and filtered through a 0.45 μm filter (Millipore) to remove eukaryotic and bacterial cell-sized particles. The filtrates enriched in viral particles were treated with a mixture of DNases (Turbo DNase

from Ambion, Baseline-ZERO from Epicentre and benzonase from Novagen) and RNase (Fermentas) to digest unprotected nucleic acid at 37°C for 90 min. The remaining total nucleic acid was then isolated using a QIAamp Mini Viral RNA kit (Qiagen) according to the manufacturer's protocol. Eight separate pools were randomly generated, five of which contained nucleic acids from five specimens, while three included nucleic acids from six specimens. Eight libraries were then constructed using a Nextera XT DNA Sample Preparation Kit (Illumina) and sequenced using the MiSeq (Illumina) platform with 250 bp paired-end reads with dual barcoding for each pool.

Bioinformatics analysis was performed according to a previous study (Deng et al., 2015). Briefly, paired-end reads of 250 bp generated by MiSeq were debarcoded using vendor software from Illumina. An in-house analysis pipeline running on a 32-node Linux cluster was used to process the data. Clonal reads were removed and low sequencing quality tails were trimmed using Phred quality score ten as the threshold. Adaptors were trimmed using the default parameters of VecScreen, which is NCBI BLASTn (Altschul et al., 1997) with specialized parameters designed for adaptor removal. Human host reads and bacterial reads were subtracted by mapping the reads to human reference genome hg19 and bacterial RefSeq genomes release 66 using bowtie2 (Langmead and Salzberg, 2012). The cleaned reads were de novo assembled by SOAPdenovo2 version r240, using Kmer size 63 with default settings (Luo et al., 2012). The assembled contigs, along with singlets, were aligned to an in-house viral proteome database using BLASTx with an *E*-value cutoff of $< 10^{-5}$. The significant hits to virus sequences were then aligned to an in-house non-virus-non-redundant (NVNR) universal proteome database using BLASTx. Hits with a more significant adjusted *E*-value to NVNR sequences than to virus were removed.

Phylogenetic analysis

Phylogenetic analyses were performed based on predicted amino acid sequences, their best BLASTp matches in GenBank and representative members of related viruses. Sequence alignment was performed using CLUSTAL W (version 2.1) with the default settings. A phylogenetic tree with 1000 bootstrap resamples of the alignment data sets was generated using the maximum-likelihood method based on the Jones-Taylor-Thornton (JTT) model in MEGA7.0. Bootstrap values (based on 1000 replicates) for each node are given. Putative ORFs (open reading frames) in the genome were predicted by the NCBI ORF finder.

Nucleotide sequence accession numbers

The genome sequences of SH-CHD 1-SH-CHD 14, respectively, were deposited in GenBank under the ac-

cession numbers KX513864, KX513865, KX513866, KX513867, KX513868, KX513869, KX513870, KX513871, KX513872, KX513873, KX513874, KX513875, KX513876 and KX513877.

RESULTS

The overall composition of the gut virome in CHD patients

Deep sequencing generated eight raw data sets from eight DNA libraries, including seven experimental groups of CHD patients and one control group. We got 11,273,562 reads from the eight libraries, and 162,319 of them showed significant sequence similarity with known viruses. After genetic optimization, BLASTx searching and classification according to viral taxonomy, the mean size of the assembled contigs was about 1919 bp, and 69±29.6 kinds of viruses were found in each library (Table 1). Using the bioinformatics method, we analyzed the viral community compositions of the eight groups.

Primarily, the enteric virome of CHD patients consisted of two dominant families, *Virgaviridae* (69.48%) and *Microviridae* (21.05%), while *Phycodnaviridae* (0.22%), *Mimiviridae* (0.15%), *Closteroviridae* (0.18%) and unknown species (8.57%) accounted for the remaining approximately 9%. *Betaflexiviridae* and *Dicistroviridae* occupied a portion of about 0.05%~0.1%; a small number of viral reads from *Picobirnaviridae*, *Poxviridae*, *Anelloviridae*, *Secoviridae*, *Potyviridae*, *Polyomaviridae*, *Baculoviridae*, *Asfarviridae*, *Iridoviridae*, *Lipothrixviridae*, *Adenoviridae* and *Reoviridae* were also detected. The numbers of the primarily dominant viral species were different among the different libraries. Three of the seven libraries of CHD patients were dominated by *Virgaviridae*, while the other four were dominated by *Microviridae* (Table 1). We calculated the percentage of viral reads of different viral species in each group. Mean percentages were calculated by taking one virus's total sequences in eight libraries divided by the sum of the eight libraries' total sequences after BLAST. We listed the mean percentages of viral reads of different viral species (> 0.1%) from the CHD groups and the percentage of viral reads of different viral species (> 0.1%) in the control group to compare the differences in viral composition between them (Figure 1A, 1B). In comparison to the control group, the percentage of enteric viruses in the CHD patient groups was lower; while the percentage of reads of plant viruses, e.g. viruses of *Virgaviridae*, in the CHD patient group was significantly higher than that in the control group. *Virgaviridae* is a family of rod-shaped plant viruses, and the CHD patients had eaten more plant-based foods rather than animal products to decrease the fat content of their bodies and keep healthy (Adams et al., 2009; Martinez, 2016). The plant viruses in the CHD patient

group were mainly *Virgaviridae*, which exist in a wide range of herbaceous and monocotyledonous and dicotyledonous plant species (Marais et al., 2015; Quaiser et al., 2015; Schroder et al., 2016).

Assembly and compared sequence analyses of the complete genomes in *Microviridae*

In total, 14 complete circular genomes of *Microviridae* were assembled from the eight libraries, the genome sizes ranged from 4500 bp to 6400 bp. Genome analysis indicated that all of these complete genomes contained two major ORFs: VP1, encoding the major capsid protein; and VP4, encoding the replication initiation protein. Another *Microviridae* core gene (encoding minor spike or pilot protein VP2) was detected in all assembled *Microviridae* genomes except SH-CHD 8.

An internal separation divided the newly assembled *Gokushovirinae* viruses into two subgroups (group 1 and group 2) (Figure 2). The separation is consistent with the phylogenetic information, except for SH-CHD 2, which had the same gene order conservation with group 1 but was in group 2. Genomes of *Gokushovirinae* in group 1 shared the same gene content and gene order (genes encoding VP4, VP5 - DNA binding protein, VP3 - internal scaffolding protein, VP1 and other proteins). All assembled *Gokushovirinae* genomes in group 2 displayed a reduced content of group 1 conserved genes, with only three genes present (those for proteins VP1, VP2 and VP4), except SH-CHD 2 and SH-CHD 10. SH-CHD 5, SH-CHD 14, SH-CHD 13 and SH-CHD 1 contained an ORF encoding peptidase M15_3.

Two new clades, SH-CHD 8 and SH-CHD 12, did not have VP3 and VP5 in their genomes. SH-CHD 8 lacked VP2, but possessed a similarly-sized ORF at a position equivalent to that occupied by VP2 in all other members of the *Gokushovirinae*. The mean genome sizes were bigger than for the other assembled viruses in the present study. The sizes of both VP4 (2018 bp on average) and VP5 (1406 bp on average) of SH-CHD 8 and SH-CHD 12 were longer than for the other assembled *Gokushovirinae* genomes (1648 bp and 944 bp on average, respectively).

Phylogenetic analysis of novel *Gukoshorivus*

In order to explore the diversity and the putative evolutionary origin of the microviruses identified in the present study, a phylogenetic tree was established based on the major capsid protein sequence (VP1). The phylogenetic tree included representative *Microviridae* subfamilies as references and our 14 newly assembled viruses (Figure 3). Eight well-supported clades were formed: the *Microvirus* out-group, Group D, *Gokushovirinae*, *Pichovirinae*, *Aravirinae*, *Stokavirinae*, *Microvirus*, *Alpavirinae* and the two strains identified in this

Table 1. The virus composition of the eight libraries. The plant viruses are marked with bold letters

Characteristic	CHD group							Mean percentage	Control group SHu8
	SHu1	SHu2	SHu3	SHu4	SHu5	SHu6	SHu7		
Total numbers of reads	595670	238206	369360	1555948	2562824	2120470	1560126	–	2270958
Reads of viral origin	3387	474	4553	40624	41025	53041	10362	–	8853
Mean size of assembled contigs (bp)	1715	1816	1623	1811	2569	1537	1421	–	2000
Virus taxa/%	—	—	—	—	—	—	—	—	—
<i>Virgaviridae</i>	6.97	24.89	48.74	82.8	31.5	93.9	73.7	69.48	1.78
<i>Microviridae</i>	85.36	67.93	44.85	13.37	41.2	4.97	19.8	21.05	77.18
None	4.19	6.12	5.97	2.82	26.8	0.77	1.23	8.57	15.88
<i>Phycodnaviridae</i>	0.35	0.84	0.09	0.15	0.05	0.05	2.0	0.22	2.78
<i>Mimiviridae</i>	0.26	0.22	0	0.07	0.04	0.08	1.37	0.15	0.42
<i>Betaflexiviridae</i>	1.33	0	0	0.01	0.03	0.002	0.08	0.05	0.002
<i>Closteroviridae</i>	0	0	0.02	0.66	0	0	0.06	0.18	0
<i>Poxviridae</i>	0.37	0	0	0.002	0.01	0.002	0.21	0.03	0.08
<i>Anelloviridae</i>	0.41	0	0	0	0.01	0.002	0.03	0.01	0.10
<i>Picobirnaviridae</i>	0.26	0	0.13	0.01	0.04	0	0.02	0.02	0
<i>Secoviridae</i>	0.09	0	0	0.01	0.002	0.002	0.22	0.02	0.10
<i>Potyviridae</i>	0.06	0	0	0	0.002	0	0.25	0.02	0
<i>Polyomaviridae</i>	0	0	0	0	0	0	0.22	0.01	0
<i>Baculoviridae</i>	0.08	0	0.02	0.01	0.03	0.02	0.03	0.02	0.16
<i>Marseilleviridae</i>	0	0	0.04	0	0.02	0.002	0.13	0.02	0.32
<i>Lipothrixviridae</i>	0.03	0	0	0.002	0.01	0	0.12	0.01	0.05
<i>Astroviridae</i>	0	0	0.02	0	0.01	0.002	0.13	0.00	0
<i>Dicistroviridae</i>	0	0	0	0	0	0.15	0	0.05	0
<i>Asfarviridae</i>	0	0	0	0	0	0.002	0.13	0.02	0
<i>Iridoviridae</i>	0	0	0.02	0.01	0.02	0	0.08	0.01	0.03
<i>Adenoviridae</i>	0.06	0	0	0.002	0.01	0.01	0.04	0.01	0.03
<i>Tymoviridae</i>	0	0	0	0	0	0	0.10	0.01	0
<i>Reoviridae</i>	0.06	0	0	0	0.01	0	0.01	0.01	0
<i>Nudiviridae</i>	0.06	0	0	0	0	0	0.02	0.00	0
<i>Retroviridae</i>	0.03	0	0	0.002	0	0.002	0.03	0.00	0.04
<i>Caulimoviridae</i>	0	0	0.02	0	0	0	0.03	0.00	0
<i>Picornaviridae</i>	0	0	0.04	0	0	0.002	0	0.00	0.20
<i>Alloherpesviridae</i>	0	0	0.04	0	0	0	0	0.00	0.01
<i>Flaviviridae</i>	0.03	0	0	0	0	0.002	0	0.00	0
<i>Togaviridae</i>	0	0	0.02	0	0	0	0	0.00	0
<i>Coronaviridae</i>	0	0	0	0.01	0.01	0	0	0.00	0.40
<i>Arteriviridae</i>	0	0	0	0.01	0.002	0.002	0	0.00	0
<i>Circoviridae</i>	0	0	0	0	0	0	0	0.00	0.04

Note: The dominant species (*Microviridae*) is marked with italic letters. “None” represents viruses that cannot be identified by BLAST.

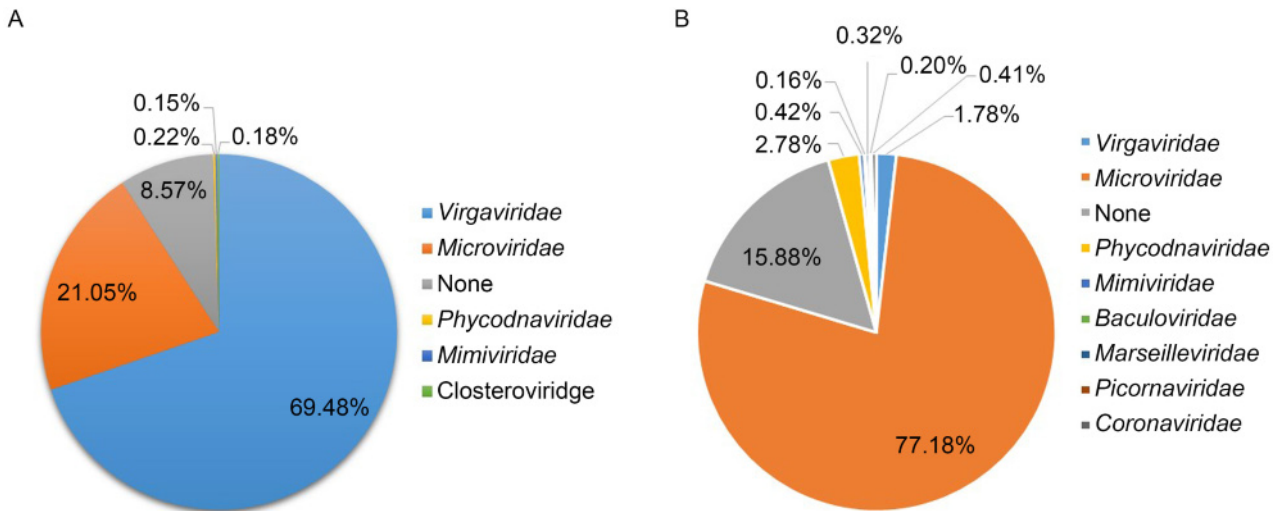


Figure 1. The composition of the gut virome. (A) The mean percentage of viral reads of different viral species in the seven CHD libraries. (B) The mean percentage of viral reads of different viral species in the control group.

study (SH-CHD 12 and SH-CHD 8) (Desnues et al., 2008; Deng et al., 2015).

Twelve of the newly assembled *Microviridae* genomes in the present study were grouped into the *Gokushovirinae* clade, which included another 22 already known representative *Gokushovirinae* genomes. Ten of these twelve new gokushoviruses were from samples from the CHD groups, and the other two viruses were assembled from the control group. The 12 gokushoviruses identified in this study could be further divided into two sub-groups: group 1, including SH-CHD 6, SH-CHD 9, SH-CHD 11 and SH-CHD 4; group 2, including SH-CHD 7, SH-CHD 3, SH-CHD 2, SH-CHD 5, SH-CHD 14, SH-CHD 10, SH-CHD 13 and SH-CHD 1. Gokushoviruses in group 1 were most closely related to *Bdellovibrio phage phiMH2K* (NP_073538.1), while the other group were genetically close to *Microviridae phi-CA82* (ADP89807.1) and *Spiroplasma phage 4* (AAA72621.1) (Figure 3).

The two new clades (indicated by red spots in Figure 3), represented by SH-CHD 12 and SH-CHD 8, are further separated from other assembled *Gokushovirinae*. SH-CHD 12 is predicted to be most close to the members of genus *Stokavirinae*, while SH-CHD 8 falls between genus *Microvirus* and *Alpavirinae*. These two genomes of viruses of the family *Microviridae* may represent two totally new subfamilies or a genus within *Microviridae*.

DISCUSSION

In the present study, we investigated the enteric virome of CHD patients and healthy controls, which showed significant differences in composition, with slight individual variety among the different CHD groups. In total, the percentage of reads of plant viruses in CHD patients

is much higher than that in the healthy control group, while the percentage of reads of enteric viruses in CHD patients is lower than that in the control group. Some research had reported the relationship between the diet and plant viruses. Phan *et al.* noted the presence of plant viruses, from families such as *Virgaviridae*, in the virome of the feces of rodents, concluding that these viruses reflected the diet of the rodents; usually plant viruses are considered incapable of infecting humans (Phan et al., 2011). The CHD patients had eaten more plant-based foods, so as to form better eating habits and keep healthy (Martinez, 2016). The viruses in the CHD patients may have suffered effects from various treatments, such as pharmacological treatment applied after CHD (non-invasive cardiac procedures (PCI, CAG and revascularization) and medicinal inhibition during rehabilitation (Schroder et al., 2016).

In the virome of CHD patients and healthy controls, divergent viral sequences of family *Microviridae* were found. Fourteen complete genomes of *Microviridae* were then assembled and analyzed, among which twelve genomes belonged to *Gokushovirinae*, while the other two genomes may represent two totally new subfamilies or a genus within *Microviridae*. Our data confirmed and complemented the division of the family *Microviridae*, as they had the same genome organizational order (*VP1* genes, *VP2* genes, *VP4* genes, *VP5* genes (partial) and *VP3* genes (partial)) with viruses assembled from human gut (fecal) samples and different from *Gokushovirinae* viruses assembled from marine (fresh) water samples and chlamydia phages with a gene order of *VP1*, *VP2*, *VP3*, *VP4* and *VP5* (Roux et al., 2012b; Brentlinger et al., 2002). All the newly assembled *Gokushovirinae* virus sequences shared the same origin.

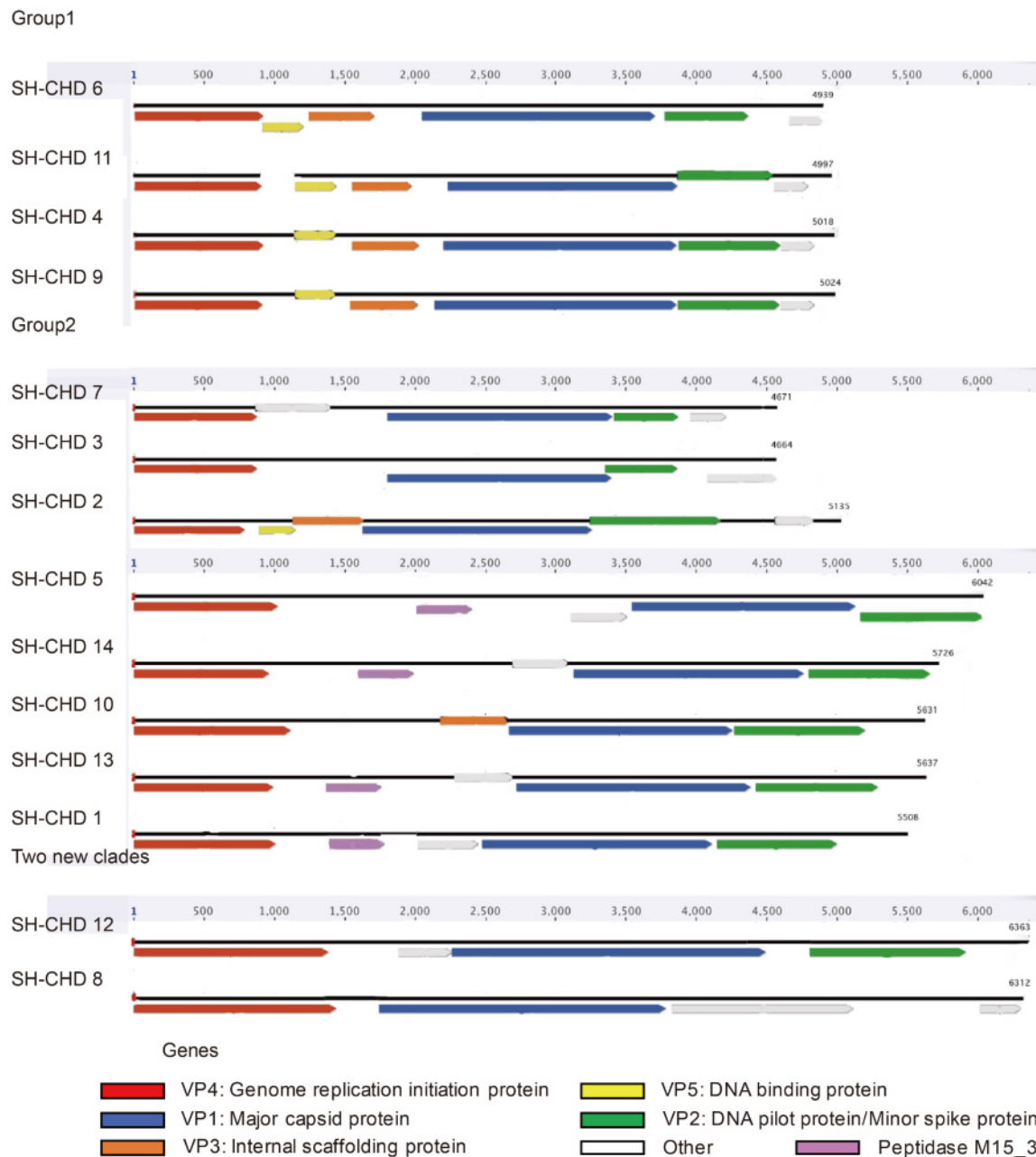


Figure 2. Genome organization of the 14 newly assembled viruses. Linearized genomes are represented for each virus. The ORFs in each genome are colored (VP1, major capsid protein, blue; VP2, DNA pilot protein, green; VP3, internal scaffolding protein, orange; VP4, genome replication initiation protein, red; VP5, DNA binding protein, yellow). Undefined ORFs are colored grey. Peptidase M15_3 is colored purple. The division is consistent with phylogenetic analysis.

Research has found peptidase *M15_3* genes in human gut or feces samples have been horizontally acquired by several members of the *Microviridae* on multiple occasions, and genes transferred between *Microviridae* are rare (Roux *et al.*, 2012b). SH-CHD 13 and SH-CHD 14 with the peptidase *M15_3* genes are viruses assembled from the feces samples of healthy people. It is strange that

among 12 newly assembled viruses from CHD groups, only SH-CHD 5 and SH-CHD 1 involved the peptidase *M15* genes. Given this, those viruses (SH-CHD 13 and SH-CHD 14) should be assigned to the same clade and separated from the same origin, which is exactly consistent with the phylogenetic analysis result.

The discovery of the two new clades also confirmed

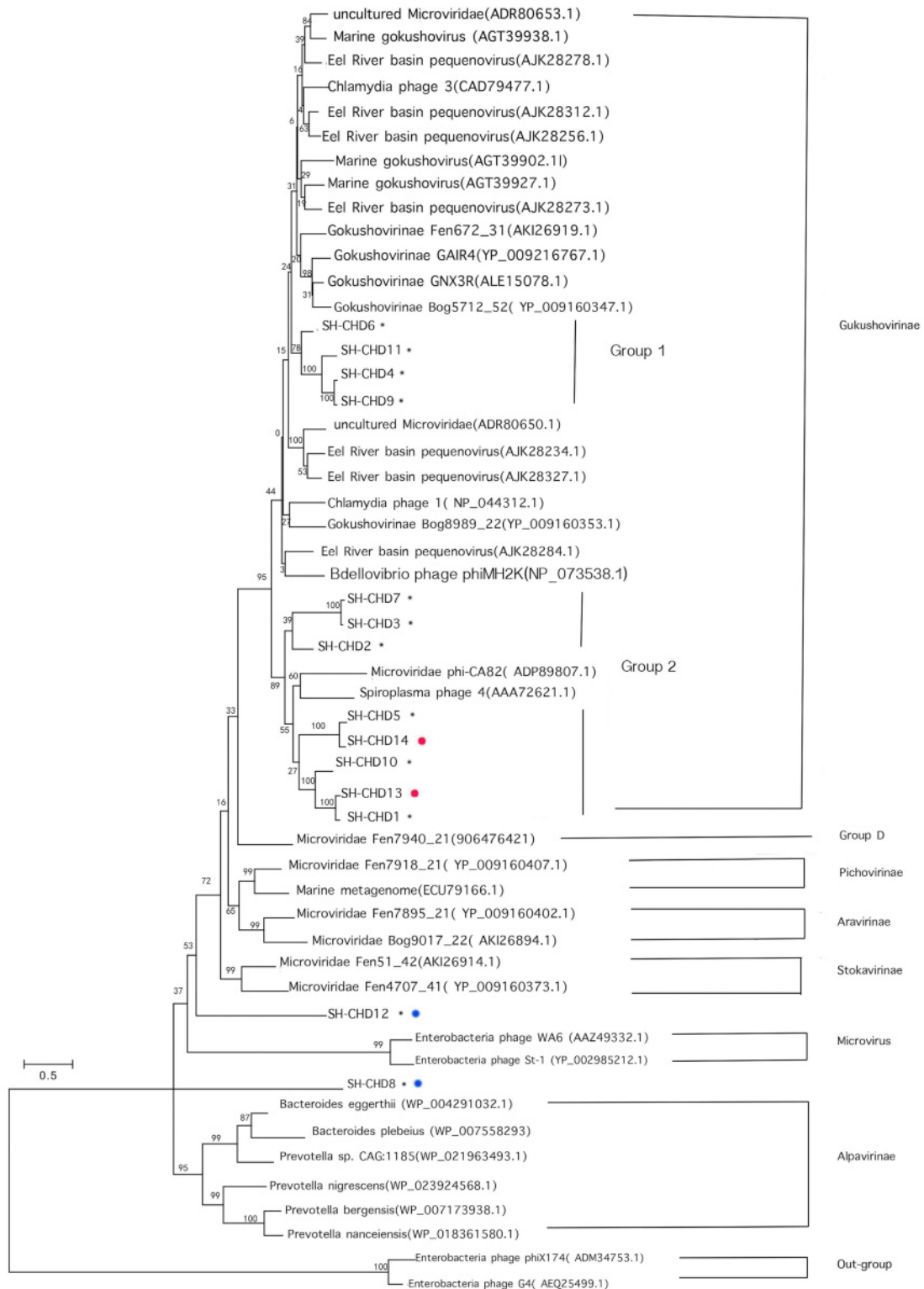


Figure 3. Maximum-likelihood phylogenetic analysis of the major capsid protein (VP1) of the 14 assembled viruses and other environmental microviridae genomes for reference. Black spots indicate viruses assembled from CHD groups. Red spots indicate viruses assembled from feces samples of the healthy control group. Blue spots indicate viruses formed new clades.

the high abundance of *Microviridae*. The consistent type of difference in their genomes (*VP1* genes and *VP4* genes) showed that they were probably diverged from one common *Microviridae* ancestor a long time ago. SH-CHD 8, the only virus without *VP2* genes, may be a new clade of *Microviridae* that separated long ago.

Studies on the gut virome using methods for purifying virus-like particles declared that the normal human gut virome composition was mainly phages of families *Siphoviridae*, *Podoviridae* and *Myoviridae*, followed by members of the family *Microviridae* (Breitbart et al., 2003; Scarpellini et al., 2015). However, in our CHD groups, the families *Microviridae* and *Virgaviridae* were the two dominant ones, while *Siphoviridae*, *Podoviridae* and *Myoviridae* only account for a small percentage of the total (Table 2). *Microviridae* were previously considered to be exclusively lytic phages, but which can, in fact, integrate into bacterial hosts e.g. *Bacteroides* and *Prevotella* spp. in an environment that encourages a temperate (lysogenic) virus-host lifestyle, suggesting that *Microviridae* could be an important viral family in the human gut (Kim et al., 2011; Reyes et al., 2012). However, the deeper interaction between the human gut virome and CHD still needs to be further explored. Research on both the RNA virome and the DNA viral community revealed that among plant viruses, a pepper-associated virus (pepper mild mottle virus (PMMV)) constituted more than 80% of the identifiable gut viruses (Zhang et al., 2006; Minot et al., 2011; Reyes et al., 2012). However, we did not detect PMMV in the CHD patients in the present study. As plant viruses have a close relationship with food intake and the intestinal bacterial qualitative/quantitative composition of human hosts (Scarpellini et al., 2015), the difference of the plant viruses could be explained by the hosts' gut microbiota.

In this study, we have analyzed the composition of the gut virome in CHD patients and healthy controls. Compared with the viral composition of healthy people, viruses of the family *Virgaviridae* were significantly higher

in number in the CHD patient group. The reduction in the number of viruses of the family *Microviridae* in the CHD groups may be because of the illness of the CHD patients, as *Microviridae* could be an important viral family in the healthy human gut (Kim et al., 2011). However, we could not find a clear or direct relationship between the gut virome and CHD. We have presented 14 new assembled *Microviridae* viruses, 12 of them belonging to the subfamily *Gokushovirinae*, and the other two new viruses presenting two novel *Microviridae* subfamilies with special gene orders. These new virus sequences expand the currently known genome information, adding to our knowledge of the diversity and distribution of the *Microviridae* subfamilies.

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COMPLIANCE WITH ETHICS GUIDELINES

The authors declare that they have no conflict of interest. Ethical Approval was given by Ethics Committee of Shanghai Jiaotong University and the reference number is No. SJTU2015085. An informed consent was obtained from all participants. Consent was obtained from all patients for which identifying information is included in this article.

AUTHOR CONTRIBUTIONS

HX CL and ZW designed the experiments. GL, LZ, SQ, YS and LJ carried out the experiments. GL, CL and ZW wrote the paper. All authors read and approved the final manuscript.

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Table 2. Mean viral composition of the seven CHD groups including major phages

Virus taxa	Mean composition(%)
<i>Virgaviridae</i>	68.46
<i>Microviridae</i>	20.74
Other viruses	8.45
None	2.31
<i>Podoviridae</i>	0.01
<i>Siphoviridae</i>	0.04
<i>Myoviridae</i>	0.00

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