



A metagenomic analysis of urban river samples reveals high numbers of sequences related to mycoviruses

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Abstract

Mycoviruses have been classified into 35 virus families so far. In addition to numerous mycoviruses with proven fungal or oomycetes hosts, many newly discovered viruses from environmental water and soil samples and various animal or plant specimens exhibit significant similarity to classified mycoviruses, thereby expanding the known sequence space of fungal and related viruses. In this study, we searched for mycoviruses in two environmental water samples that had been collected from the Teltow Canal and the Havel River in Berlin, Germany. Viral sequences with similarity to members of 16 virus families were identified. The most common viruses in our samples were botourmia-like viruses with moderate similarity to members of the genus *Ourmiavirus*. Notably, 58 of the ourmia-like sequences from the Teltow Canal and Havel River and 10 ourmia-like sequences from other sources exhibited a dicistronic genome layout. The second open reading frame (ORF) of these viruses encoded a putative capsid protein with an S domain that showed little similarity to the structural proteins of the classified ourmiaviruses. The second-largest virus group (59 sequences) was assigned to the order *Ghabrivirales*, and 13 of these sequences exhibited similarity to members of the suborder *Alphatotivirineae* (families *Orthototiviridae*, *Pseudototiviridae*, *Botybirnaviridae*, and *Chrysoviridae*). Thirty-three sequences clustered with members of the suborder *Betatotivirineae* – three of them with members of the family *Artiviridae*. Fifteen highly divergent toti-like sequences suggest the need to establish a new higher-order taxon within the order *Ghabrivirales*. Other virus sequences were assigned to the families *Mitoviridae* (three unuamitoviruses and 10 highly divergent mitovirus-like sequences), *Narnaviridae* (five "alphanarnavirus"-like sequences), *Amalgaviridae* (two zybavirus-like sequences), *Hypoviridae* (one partial RdRP sequence), and *Mymonaviridae* (one partial RdRP sequence), and one was not classified (Sclerophthora macrospora B-like virus). Notable results include a clade of highly divergent mitovirus-like sequences with a standard translation code, three narnavirus-like sequences with a reverse-frame ORF, and a clade of four *Ghabrivirales*-like replicase sequences that were found to have numerous termination codons regardless of which translation table was used.

Introduction

Mycoviruses have been detected in a wide range of host species comprising all of the major taxa of fungi and oomycetes. They exhibit great diversity in their genome structure and replication mechanisms. Moreover, mycoviruses have mostly nonlytic life cycles, as they forgo extracellular routes of infection and make no use of vectors but instead establish persistent asymptomatic infections of their fungal hosts. Mycoviruses propagate by efficient vertical and horizontal transmission routes. Typical vertical transmission routes are cell division and sporogenesis; horizontal transmission includes cell fusion mechanisms such as hyphal anastomosis and heterokaryosis [1]. Although viral

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persistence is common, mycoviruses with icosahedral particles have been described. The complex interactions of most mycovirus–fungus–plant and mycovirus–fungus–animal host systems are still underexplored, as is the ecological role of viral hyperparasitism [2]. Viral infection of some fungi may cause hyper- or hypovirulence of their plant-pathogenic hosts, with the latter feature making them potential viral agents for controlling virulent pathogenic fungi. Furthermore, some viruses can shuttle between fungi and plants or insects [3–5], and fungi may serve as vectors of plant viruses [6, 7].

Aquatic systems are often overlooked as fungal habitats although fungi play important roles in cycling of organic matter and food web dynamics [8]. Moreover, mycoviruses are also important players in these interactions and in ecological control [9]. In addition to their complex ecological role, the great genetic diversity of mycoviruses has attracted the attention of researchers in recent years. Hundreds of mycoviruses belonging to at least 35 virus families have been classified (see Virus Metadata Resource MSL40.v1.20250307, available for download from <https://ictv.global/>), but thousands of mycovirus-like sequences still await classification. Mycoviral genomes may consist of single-stranded DNA (*Genomoviridae*), segmented or unsegmented double-stranded RNA or single-stranded RNA with positive- or negative-strand polarity. Furthermore, positive-stranded RNA viruses with reverse transcriptase activity have also been described (*Metaviridae*, *Pseudoviridae*). Other oddities in the mycovirus world include single-stranded RNA viruses with circular genomes and two open reading frames in ambisense orientation (viruses of the phylum *Ambiviricota*) and multisegmented positive-stranded RNA viruses with a composite RNA-dependent RNA polymerase (RdRP) complex assembled from two separate domains that are encoded by different RNA segments (viruses of the family *Spilipalmiviridae*) [10]. One mycovirus with a double-stranded DNA genome, rhizidiovirus, has been reported, but no sequence data are available [11] and its significance is unclear.

The number of published mycovirus sequences has increased exponentially with the availability of high-throughput sequencing techniques, and virus taxonomists have to cope with the continuously growing amount of sequence data and its classification. Each new published fungal metagenome and each new sequenced virome of environmental samples has revealed novel mycoviruses or mycovirus-like sequences [10, 12, 13]. Often, fungi may contain many different viruses. For example, the facultative plant-pathogenic fungus *Rhizoctonia solani* – which is in fact a complex of many related species, so-called anastomosis groups – hosts as many as 100 different viruses belonging to 18 virus families [14]. Similarly, the oomycete

Bremia lactucae hosts more than 15 viruses of various families [15]. The situation is complicated by cross-kingdom infections, e.g., if fungi serve as vectors for plant viruses, or if viruses shuttle between fungi and plants or between fungi and insects [3–7, 16].

The Teltow Canal and Havel River are two waterbodies traversing the metropolitan area of Berlin, Germany. In our previous investigations of their fluvial viromes, we identified complete or partial genomes of more than 1500 novel RNA viruses, which were reported in a series of papers [17–21]. Here, we add viral sequences detected in samples from these waterbodies that are related to known mycoviruses. The novel viruses exhibit similarity to members of the suborders *Alpha-* and *Betatotivirineae* and the families *Botourmiaviridae*, *Mitoviridae*, *Narnaviridae*, and *Amalgaviridae* (genus *Zybavirus*), as well as to the unclassified *Sclerophthora macrospora* B virus.

Materials and methods

Sample collection and methods of virus enrichment, RNA extraction, Illumina sequencing, sequence data processing, and sequence analysis have been described previously [21]. They are briefly summarized here:

The 50-L Teltow Canal water sample (ID MR233-17) was collected in Berlin, Bäkebrücke (site coordinates: 52°26'03" N, 13°18'57" E), on July 18, 2017. The 50-L Havel River water sample (ID MR644-17) was collected in Berlin, Heerstrasse (site coordinates: 52°30'46" N 13°12'14" E), on June 28, 2018. Both 50-L samples were divided into five 10-L aliquots and further treated as described by Wyn-Jones et al. [22]. For this, the aliquots were stirred for 20 minutes to suspend floating detritus, acidified to pH 3.5 by addition of hydrochloric acid, and loaded on a glass-wool-packed column for adsorption of virus particles. Adsorbed virus particles were consecutively washed with hydrochloric acid (1 M), sodium hydroxide (1 M), and tap water and finally eluted with 3% beef extract/0.05 M glycine buffer, pH 9.5. Eluates were neutralized by addition of sodium hydroxide and passed through a 0.45-µm filter to remove residual detritus and bacteria. Virus particles were sedimented by ultracentrifugation (100,000 × g, 2.5 h at 4 °C). Finally, the sediment was redissolved in 0.5 ml of phosphate-buffered saline by agitation in a ball mill and stored at –80 °C for later RNA extraction.

RNA was extracted using a QIAamp Viral RNA Mini Kit (QIAGEN, Hilden, Germany) according to the manufacturer's instruction and analyzed by Illumina sequencing as described by Zell et al. [21]. Briefly, 100 ng of RNA from the Havel River sample was used for library preparation employing an Illumina TruSeq Stranded Total RNA Library

Preparation Kit, whereas the library from the Teltow Canal sample was prepared from 450 ng of RNA using an Illumina TruSeq Stranded Total RNA Library Preparation Kit combined with a Ribo-Zero Gold rRNA Removal Kit according to the manufacturer's instructions. The protocol was adapted to include all RNA molecules rather than only polyadenylated RNA by adding an RNA precipitation step with isopropanol and redissolving the pellet in Fragment, Prime, Finish Mix (FPF). Thereafter, library quantitation and quality checking were done using a DNA 7500 kit and a 2100 Bioanalyzer instrument (Agilent Technologies, Waldbronn, Germany). Paired-end Illumina sequencing (2×150 bp) was done on a HiSeq 2500 platform using the rapid run mode.

Sequence data were extracted in FastQ format using *bc12FastQ* v2.19.1.403 (Illumina). Adapter sequences were removed using *Cutadapt* v1.8.3 [23]. After duplication extraction, the remaining 70,018,635 read pairs of the Teltow Canal sample and 51,902,006 read pairs of the Havel River sample were used for *de novo* assembly by two methods: (i) Employing *clc_assembler* v5.2.1 (QIAGEN) with the parameters *-p fb ss 50 500*, a total of 537,529 contigs longer than 200 nucleotides were obtained from the Teltow Canal sample, and 162,082 contigs were obtained from the Havel River sample. (ii) Employing the *metaSPAdes* assembler v3.15.3 [24] with standard parameters (*-k auto*), 1,314,849 scaffolds were obtained from with the Teltow Canal sample, and 388,367 were obtained from the Havel River sample. The final sequences were obtained by manual curation, i.e., linking of overlapping contigs and scaffolds.

We used a 2-step procedure for analysis of sequence data. First, virus-specific scaffolds and contigs were identified by comparing the assembled sequences with our in-house virus protein database compiled from all NCBI GenBank entries with the Taxonomy ID 10239, using *DIAMOND* v2.0.10 [25]. *DIAMOND* identified 66,371 virus-specific scaffolds and 41,408 contigs from the Teltow Canal sample and 16,922 scaffolds and 8810 contigs from the Havel River sample. In the second step, selected *DIAMOND* hits were confirmed using *BLAST+* v2.13.0 (<https://ftp.ncbi.nlm.nih.gov/blast/executables/blast+/2.13.0/>) with the *BLASTp* and *tBLASTx* tools. If appropriate, specific *BLASTn* searches were performed with reference sequences downloaded from GenBank. For identification of conserved protein domains, the NCBI web search tools *BLASTp* suite and *Pfam* conserved domain database (CDD) were used (<https://blast.ncbi.nlm.nih.gov/Blast.cgi>, <https://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi>). *ClustalW* and *MUSCLE* implemented in *MEGA X* [26] were employed for protein sequence alignments. Alignments were adjusted manually if necessary. For construction of phylogenetic trees, we used *IQ-TREE* 2.1.3 for Windows [27]. The best-fit substitution models were identified using the automatic model selection option

(*ModelFinder*) implemented in *IQ-TREE*. Branch support was assessed using the ultrafast bootstrap approximation *UFBoot2* with 10,000 replications [28].

All viruses were provisionally assigned to established taxa using the current virus taxonomy (2024 release) based on the most recent Master Species List #40 (<https://ictv.global/msl>; accessed on March 7, 2025) and the most recent Virus Metadata Resource spreadsheet MSL40.v1.20250307 (<https://ictv.global/vmr/current>; released on March 7, 2025).

Results

Analysis of the Teltow Canal and the Havel River viromes revealed the presence of numerous RNA viruses with similarity to fungal viruses. Genomic sequences related to members of 15 virus families known to contain mycoviruses were detected (Table 1). These included – in alphabetical order *Alphaflexiviridae*, *Amalgaviridae*, *Artiviridae*, *Barnaviridae*, *Botybirnaviridae*, *Botourmiaviridae*, *Chrysoviridae*, *Endornaviridae*, *Hypoviridae*, *Mitoviridae*, *Mymonaviridae*, *Narnaviridae*, *Orthototiviridae*, *Partitiviridae*, and *Pseudototiviridae*. In addition, sequences related to the unclassified *Sclerophthora* macrospora viruses A and B and *Plasmopara halstedii* virus A, as well as many unclassified viruses of the orders *Ghabrivirales* and *Cryppavirales* were detected. Furthermore, viruses with similarity to fungus-associated aspivirus-like viruses were identified (Table 1). Notably, the majority of the novel virus sequences exhibited similarity to members of the family *Botourmiaviridae* (positive-strand RNA viruses) or the order *Ghabrivirales* (double-strand RNA viruses), but most of the new sequences were highly divergent compared to those of the established members. As Teltow Canal and Havel River viruses with similarity to alphaflexiviruses, barnaviruses, endornaviruses, and partitiviruses have been described previously [19], they were excluded here. No significant sequences corresponding to mycoviruses of the families *Polymycoviridae* (dsRNA), *Deltaflexiviridae* (+RNA), *Fusariviridae* (+RNA), *Gammaflexiviridae* (+RNA), *Hadakaviridae* (+RNA), *Yadokariviridae* (+RNA), *Discoviridae* (–RNA), *Phenuviridae* (–RNA), *Tulasviridae* (–RNA), *Metaviridae* (RT viruses), or *Pseudoviridae* (RT viruses) were found.

The most characteristic features of the detected mycovirus-like sequences and the high variability in their genomic layouts (Fig. 1) are outlined in the following paragraphs:

Botourmiaviridae

The majority of the sequences obtained in this study were related to botourmiaviruses. Viruses of the family *Botourmiaviridae* (order *Ourlivirales*) infect plants, fungi, and

Table 1 Viral sequences from the Teltow Canal and Havel River with similarity to sequences of mycoviruses

Phylum	Order	Family	No. of sequences	Genome	Reference
<i>Pisuviricota</i>	<i>Durnavirales</i>	<i>Amalgaviridae</i>	2	dsRNA	This study
		<i>Partitiviridae</i>	12	dsRNA	[19]
		<i>Hypoviridae</i>	1	+RNA	This study
	<i>Sobelivirales</i>	<i>Barnaviridae</i>	1	+RNA	[19]
<i>Duplornaviricota</i>	<i>Ghabrivirales</i>	<i>Artiviridae</i>	4	dsRNA	This study
		<i>Botybirnaviridae</i>	4	dsRNA	This study
		<i>Chrysoviridae</i>	3	dsRNA	This study
		<i>Pseudototiviridae</i>	4	dsRNA	This study
		<i>Orthototiviridae</i>	2	dsRNA	This study
		[<i>Ghabrivirales</i> sp.]	42	dsRNA	This study
<i>Kitrinoviricota</i>	<i>Martellivirales</i>	<i>Endornaviridae</i>	20	+RNA	[19]
	<i>Tymovirales</i>	<i>Alphaflexiviridae</i>	28	+RNA	[19]
<i>Lenarviricota</i>	<i>Ourlivirales</i>	<i>Botourmiaviridae</i>	139	+RNA	This study
		<i>Cryppavirales</i>	3	+RNA	This study
		[<i>Mitoviridae</i> sp.]	10	+RNA	This study
	<i>Wolframvirales</i>	<i>Narnaviridae</i>	5	+RNA	This study
	<i>Mononegavirales</i>	<i>Mymonaviridae</i>	1	-RNA	This study
<i>Negarnaviricota</i>	<i>Naedrevirales</i>	<i>Aspiviridae</i>	4*	-RNA	[19]
Unclassified	<i>Plasmopara halstedii</i> virus A-like		1	+RNA	This study
	<i>Sclerophthora macrospora</i> virus A-like		45	+RNA	This study, [19]
	<i>Sclerophthora macrospora</i> virus B-like		1	+RNA	This study

* similarity to fungus-associated aspi-like viruses

possibly oomycetes. The family comprises 12 genera, one of which is the genus *Ourmiavirus*, whose members have a positive-stranded RNA genome with three monocistronic segments, produce non-enveloped bacilliform virions, and infect plants [29]. The viruses of the remaining genera have

Fig. 1 Genome layout of selected novel viruses from the Teltow Canal and Havel River. ORFs are indicated by boxes. Locations of conserved protein domains identified using the Conserved Domain Database (CDD) search tool are indicated. Abbreviations: CP, capsid protein (highlighted in ochre); ORF, open reading frame; Prot, V8-like Glu-specific endopeptidase (COG3591) (green); RdRP, RNA-dependent RNA polymerase (red). An asterisk (*) indicates the presence of a ribosomal frameshift site (UUUCUUA) at nt position 750–756 of TC-zyba-like virus 1, whereas hashtags (#) indicate stop-restart sites (AUGA) in TC-totivirus 1 and TC-pseudototivirus 1, respectively. Nucleotide and amino acid positions are indicated

non-encapsidated monopartite, monocistronic positive-stranded RNA genomes and infect fungi. The sizes of the monopartite genomes of the classified botourmiaviruses range from 2 to 5 kb. The gene segments of the tripartite ourmiaviruses are 2.8 kb, 1.1 kb, and 1 kb long. The RNA of fungus-infecting botourmiaviruses and RNA segment 1 of ourmiaviruses encode a replicase (cd23183), whereas RNA segment 2 of ourmiaviruses encodes a movement protein, and segment 3 encodes a coat protein with an S domain (pfam00729). The RdRP sequences of all botourmiaviruses cluster with those of leviviruses (now assigned to the family *Fiersviridae*), mitoviruses, and narnaviruses on branch 1 of the so-called 'comprehensive RdRP tree' [30]. The viruses of this branch have recently been assigned to the phylum *Lenarviricota* [31].

DIAMOND identified 121 sequences from the Teltow Canal sample and 33 from the Havel River sample encoding a botourmia-like RdRP (cd23183). These sequences were named TC-botourmia-LVs and Havel-botourmia-LVs. Notably, five clades contained dicistronic virus sequences. Altogether, 68 viruses exhibited a dicistronic genome layout with ORF 1 encoding the RdRP (cd23183) and ORF 2 encoding a putative capsid protein with an S domain (pfam00729). The genome of TC-botourmia-LV-17 has a putative third ORF with unknown significance. Another sequence of 2589 nt was identified by the CDD tool as a botourmia-like virus based on a short stretch of 120 amino acids representing a partial RdRP sequence (cd23183), although with low statistical significance (1.31e-12). This RdRP sequence was located on the C-terminal side of a helix domain, which is unusual for botourmiaviruses.

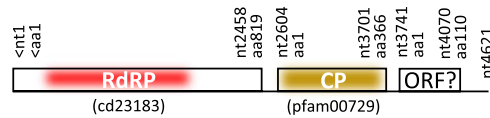
The RdRP sequences of the botourmia-like viruses were aligned with the respective sequences of classified reference viruses, including a number of mitoviruses and narnaviruses as an outgroup. The phylogenetic tree reproduced three branches corresponding to the families *Botourmiaviridae*, *Mitoviridae*, and *Narnaviridae* (Supplementary Fig. S1). None of the Teltow Canal or Havel River botourmia-like viruses clustered with members of any of the fungus-infecting viruses but were related to plant-infecting ourmiaviruses.

To examine the sequences of the structural proteins, we aligned these sequences with the most similar CP sequences

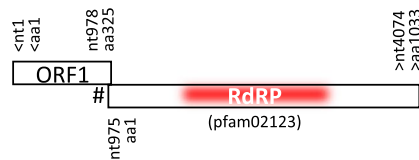
PX098518, Havel-botourmia-like virus 1
(dicistronic botourmia-like virus)



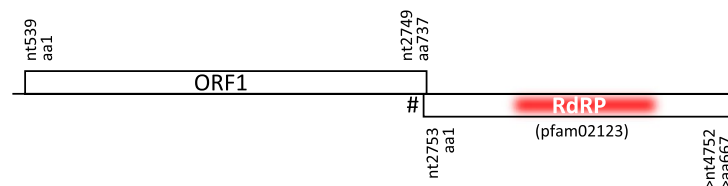
PX098567, TC-botourmia-like virus 17
(with hypothetical third ORF)



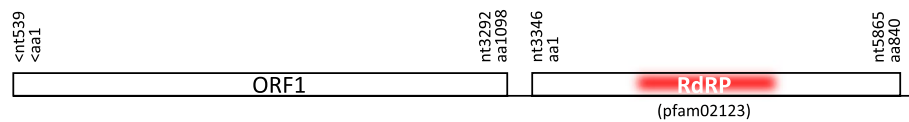
PX098708, TC-Totivirus 1
(*Orthototiviridae*)



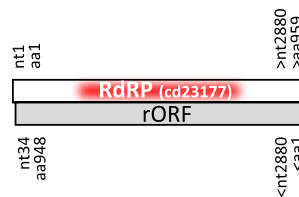
PX098694, TC-Pseudototivirus 1
(*Pseudototiviridae*)



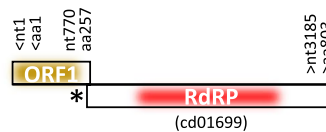
PX098691, TC-Artivirus 1
(*Artiviridae*)



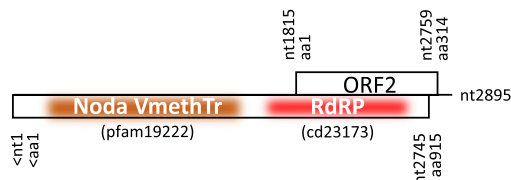
PX098689, Havel-narna-like virus 4



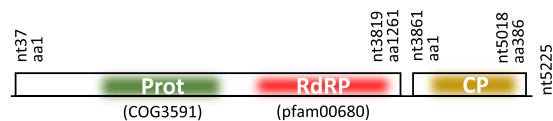
PX098516, TC-zyba-like virus 1
(*Amalgaviridae*)



PX098759, TC-noda-LV-173
(*Sclerophthora macrospora* A-like)



PX098753, TC-Sclerophthora macrospora
B-like virus



identified using BLAST, but this failed to yield a significant alignment with the ourmiavirus CP sequences. A CDD search of the polymerase sequences linked to the CP sequences shown in the phylogenetic tree in Fig. 2 revealed six protein families, i.e. cd23183 (Botourmiaviridae-RdRP, ▲), cd23173 (Nodaviridae-RdRP, ◆), cd23179 (Tolivirales-RdRP, ■), cd23180 (Solemoviridae-RdRP, ○), cd23206 (Tombusviridae-RdRP, □) and cd23242 (Gammacarmovirus-RdRP, □). This finding might suggest that recombination events occurred frequently in the evolution of these RNA viruses.

Ghabrivirales

The taxonomy of the order *Ghabrivirales* is based on the polymerase phylogeny, resulting in three suborders, *Alpha*-, *Beta*- and *Gammatotivirineae*, which together include 19 virus families [31]. Monopartite and multipartite double-stranded RNA genomes with variable gene layouts have been described in this order. The polymerase belongs to the protein family pfam02123 (RdRP4). Where structural data are available, the members of the *Ghabrivirales* have been shown to possess an icosahedral capsid with a size of 35–50 nm. The capsid protein gag of L-A virus (species *Totivirus ichi*, family *Orthototiviridae*) which is the best-studied member of the order *Ghabrivirales*, belongs to protein family pfam09220 and exhibits similarity to the reovirus capsid protein regarding protein folding and arrangement in a T=1/pseudo T=2 capsid symmetry [32].

We investigated 59 sequences from the Teltow Canal and Havel River samples with similarity to members of the order *Ghabrivirales*. Our alignment of RdRP sequences includes reference sequences of all 19 *Ghabrivirales* families plus several unclassified viruses. The resulting tree supports the present taxonomy (Supplementary Fig. S2). Three branches correspond to the three suborders. All 19 families exhibit long branch lengths. Within the suborder *Alphatotivirineae*, two new orthototiviruses, four new pseudototiviruses, and four new botybirna-like viruses were identified. Notably, our orthototi- and pseudototiviruses possess overlapping stop/start codons (UAAUG) at the ORF1-ORF2 junction, which would enable a coupled termination-reinitiation mechanism for the expression of the capsid protein and RdRP rather than a programmed ribosomal shift. Furthermore, 33 toti-like viruses, including three artiviruses, grouped with members of the suborder *Betatotivirineae*. Fifteen toti-like viruses, which clustered distinctly from the three known suborders, were also observed. This clade of divergent viruses suggests the need for another higher-order taxon within the order *Ghabrivirales*. Among these divergent viruses, one subclade has four RdRP-like sequences that contain many internal stop codons regardless of which

Fig. 2 Phylogenetic analysis of 80 capsid protein sequences of members of dicistronic unclassified botourmia-like viruses. The CP sequences of members of five clades of botourmia-like viruses from the Teltow Canal and Havel River (listed in Supplementary Figure S1) were aligned with CP sequences from various hosts and used for construction of a maximum-likelihood tree, using IQ-TREE 2 (optimal substitution model: Q.pfam+F+R5). Presented are GenBank accession numbers, vernacular virus names, and strain designations, if available (in parentheses). Numbers at nodes indicate bootstrap support greater 65% obtained after 10,000 ultrafast replications. The bar indicates amino acid substitutions per site. Color code: blue, unclassified viruses; red, viruses from the Teltow Canal and Havel River. Symbols (◆, ■, ○, ▲, □, ●, ◇) indicate the CDD family of the corresponding RdRP

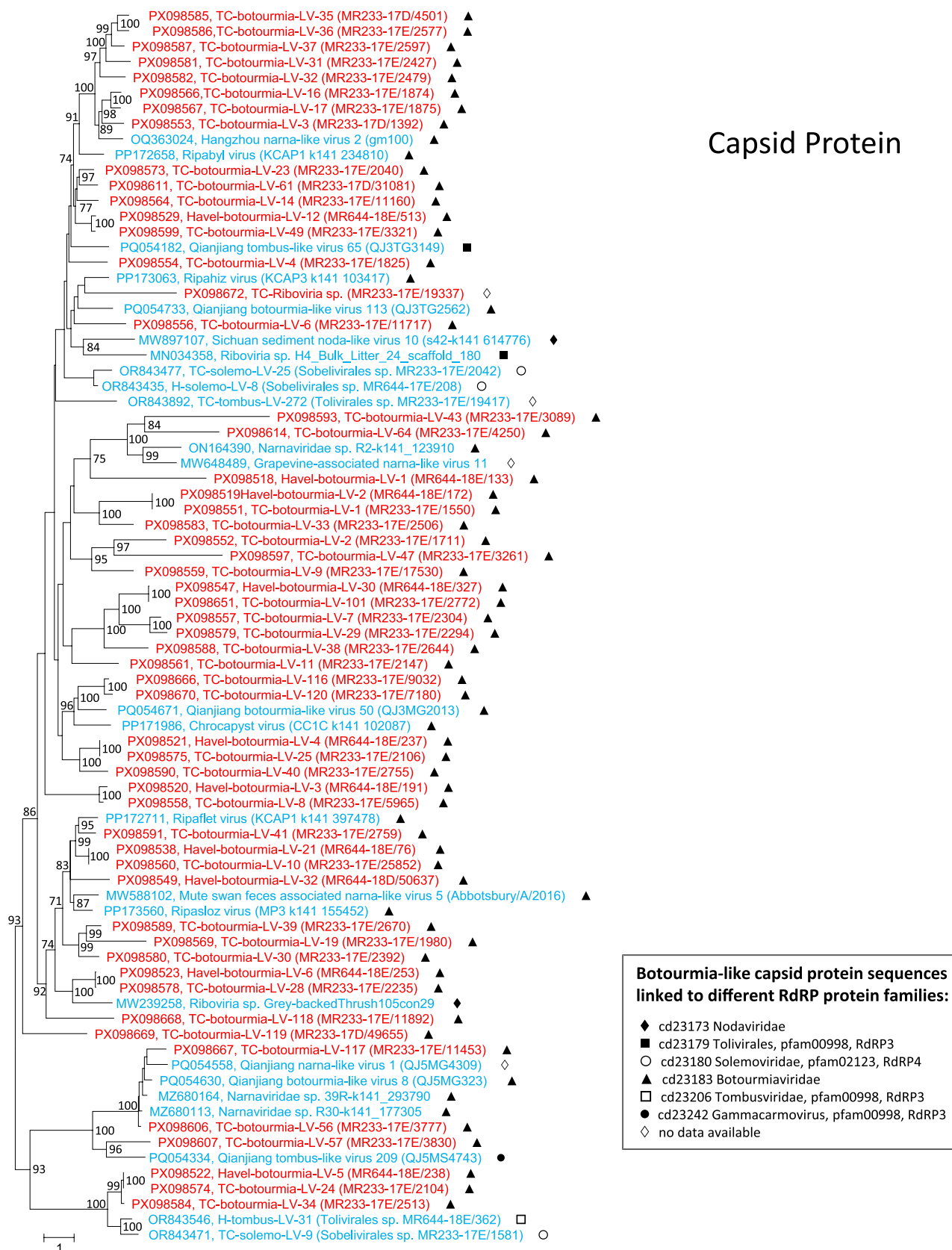
translation table is used. The significance of this finding remains to be elucidated.

In addition, DIAMOND identified three scaffolds representing alphachrysoviruses. One 278-nt scaffold corresponded to segment 1 (RdRP) and exhibited 52% aa sequence identity to *Brassica campestris* chrysovirus 1 (accession no. KP782031). However, this fragment was too short for a proper phylogenetic analysis. Two other scaffolds with lengths of 671 and 546 nt, respectively, showed about 30% aa sequence identity to the major capsid proteins of two different alphachrysoviruses (data not shown). No *Ghabrivirales* sequences with significant similarity to those of members of the *Alternaviridae*, *Fusagraviridae*, *Giardiaviridae*, *Inseviridae*, *Lebotiviridae*, *Megabirnaviridae*, *Megatotiviridae*, *Monocitviridae*, *Ootiviridae*, *Phlegiviridae*, *Pistolviridae*, *Quadriviridae*, *Spiciviridae*, or *Yadonushiviridae* were found.

Narnaviridae and Mitoviridae

Narnaviruses and mitoviruses possess a non-encapsidated positive-stranded RNA genome ranging from 2.1 to 5 kb in length. Its single ORF encodes an RdRP. A recent taxonomic revision removed mitoviruses from the family *Narnaviridae* and placed them into the newly created family *Mitoviridae* based on the low degree of similarity of their replicase sequences. The family *Narnaviridae* (order *Wolframvirales*) is comprised of a single genus with two species. In contrast, the family *Mitoviridae* (order *Cryppavirales*) includes four genera with 105 species [33]. Numerous related viruses from a wide spectrum of hosts await classification. Whereas the two acknowledged narnaviruses infect yeast cells, most mitoviruses use filamentous fungi as hosts and utilize translation table 4, in which UGA encodes tryptophan, indicating persistence in fungal mitochondria [34]. Only a few mitoviruses have been associated with plant hosts [35, 36], and those viruses use the standard genetic code.

By comparing RdRP sequences, we identified five sequences with similarity to those of narnaviruses (cd23177) and 13 with similarity to those of mitoviruses (pfam05919).



Phylogenetic analysis of these RdRP sequences revealed that all of the narna-like viruses were novel and clustered with viruses that were recently proposed to be classified as "alphanarnaviruses" [37] (Supplementary Fig. S3). Notably, three viruses of the "alphanarnavirus" clade (TC-narna-LV-1 and Havel-narna-LV-3 and -4) contained a reverse-frame ORF (rORF, also known as an ambigrammatic sequence), as has been described previously for some narna-like viruses [37–40].

The *Mitoviridae* branch contains monophyletic clades of the four mitovirus genera, albeit with moderate bootstrap support (Supplementary Fig. S3). Three of our mito-like sequences clustered with unamitoviruses (Havel-mito-LV-4, Havel-mito-LV-5, and TC-mito-LV-8). The remaining mito-like sequences from the Havel River and Teltow Canal were too divergent to assign them to an existing mitovirus genus. Two clades of the tree contain viruses which utilize the standard translation table rather than translation table 4. One of these clades comprises nine plant-infecting mitoviruses of the genus *Duamitovirus*. The other clade consists of a group of eight novel virus sequences from the Teltow Canal and Havel River plus six still unclassified "narna-like" viruses that have been proposed recently to be members of the order *Cryppavirales* [33]. These six "narna-like" viruses were associated with hosts as diverse as plants, insects, tunicates, penguins, and rabbits. The use of the standard translation table is compatible with the reported non-fungal hosts and also suggests non-fungal hosts for the eight Teltow Canal and Havel River viruses of this clade. Another novel, untypeable virus is Havel-mito-like virus 3, which also lacks internal UGA codons, suggesting the use of the standard genetic code.

Amalgaviridae

The family *Amalgaviridae* (order *Durnavirales*) consists of three genera: *Amalgavirus*, with plant-infecting viruses; *Unirnavirus*, with viruses of filamentous ascomycetes; and *Zybovirus*, with viruses that infect budding yeast. Members of the *Amalgaviridae* have double-stranded RNA genomes with two open reading frames. ORF 1 encodes a protein of unknown function and is not conserved among the genera. The second ORF is partly overlapping with ORF 1 and is expressed as a fusion protein, either by a -1 or a $+1$ ribosomal frameshift [41]. The fusion protein is a polyprotein with an RdRP1 domain (pfam00680, cd01699). Members of the *Amalgaviridae* cause persistent infections in their hosts without a visible cytopathic effect.

DIAMOND identified two scaffolds with similarity to the zyboviruses. TC-zyba-like virus 1 has a length of 3185 nt and represents the nearly complete ORFs 1 and 2 (Fig. 1). In comparison to Antonospora locustae virus 1, an unclassified

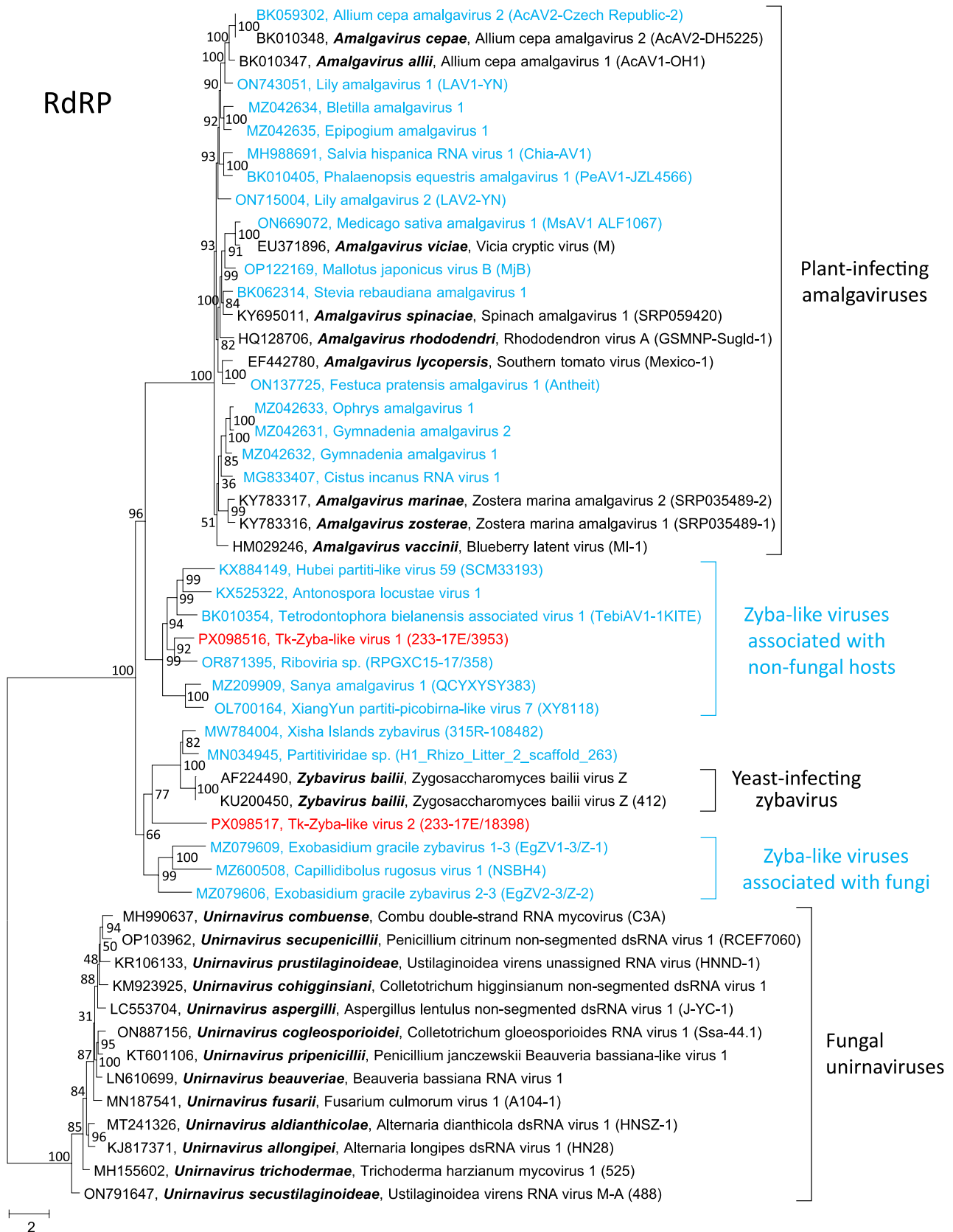
Fig. 3 Phylogenetic analysis of 52 RdRP sequences of members of the family *Amalgaviridae* and related viruses. The replicase sequences of members of all species of the *Amalgaviridae*, unclassified related viruses from the Teltow Canal, Havel River, and various hosts were aligned using MEGA, and a maximum-likelihood tree was constructed using IQ-TREE 2 with the optimal substitution model Q.pfam+F+R6. Presented are GenBank accession numbers, vernacular virus names, and strain designations, if available (in parentheses). Square brackets indicate hosts. Numbers at nodes indicate bootstrap support obtained after 10,000 ultrafast replications. The tree was arbitrarily rooted using unirnavirus sequences. The bar indicates amino acid substitutions per site. Color code: black, classified viruses; blue, unclassified viruses; red, viruses from the Teltow Canal and Havel River

zyba-like virus (accession no. KX525322), the fusion protein lacks fewer than five amino acids at both the N- and C-terminal ends. The sequence of TC-zyba-like virus 2 corresponds to a partial RdRP. A phylogenetic analysis based on the RdRP sequences of all members of the *Amalgaviridae* plus several related but unclassified virus sequences, including both TC-zyba-like sequences, revealed three major clades corresponding to the three amalgavirus genera plus two clades with zyba-like virus sequences. One of these clades includes fungal viruses, while the other is comprised of viruses that are associated with non-fungal hosts (Fig. 3).

Sclerophthora macrospora viruses A and B, Plasmopara halstedii virus A

The oomycete *Sclerophthora macrospora* is an important pathogen that causes downy mildew disease of cereal crop plants of the family Poaceae. Two viruses infecting *S. macrospora* have been isolated so far: *Sclerophthora macrospora* viruses A and B (SmVA and SmVB). A third oomycete-infecting virus is *Plasmopara halstedii* virus A (PhVA). These three viruses have icosahedral capsids with sizes of 32 nm (SmVA), 35 nm (SmVB), and 37 nm (PhVA), respectively [42–44]. SmVA and PhVA have segmented single-stranded RNA genomes with RNA segment 1 encoding noda-like methyltransferase (pfam19222) and RdRP (cd23173) domains, whereas RNA segment 2 codes for a capsid protein with similarity to tombusviruses (pfam00729) [45, 46]. In addition, SmVA possesses a third satellite-like RNA. In contrast, the genome of SmVB consists of a monopartite, dicistronic single-stranded RNA (Fig. 1). The polyprotein of SmVB that is encoded by ORF 1 includes a V8-like Glu-specific endopeptidase, several VPg oligopeptides, and a polymerase that is related to the RdRPs of solemoviruses [47]. The second ORF codes for a capsid protein.

Our phylogenetic analysis of the RdRP sequences of 85 viruses, including SmVA, SmVB, PhVA, solemoviruses, tombusviruses, nodaviruses, and a number of unclassified viruses, revealed six clades of unclassified viruses that clustered on the branch with the *Solemoviridae* members



(Fig. 4, upper branch). Among these are (i) the previously described solemo-like clade C viruses with a barna-like RdRP (cd23184 [19]), (ii) carascovirus and similar viruses from the Teltow Canal, (iii) SmVB and related viruses, and (iv) three clades of Chinese solemo-like viruses from invertebrate samples. The viruses of this branch were characterized by the presence of an S domain (pfam00729) in their capsid protein. Phylogenetic analysis of the CP sequences of these six clades revealed the relatedness of their structural proteins (Fig. 5). However, our attempts to align the CP sequence of the detected TC-SmVB-like virus and *Solemoviridae* members failed. Too little similarity was observed (data not shown) although a CDD search suggested that both capsid proteins belong to protein family pfam00729. Notably, most of the viruses of the upper branch have a serine proteinase domain, either a trypsin-like proteinase (solemoviruses, viruses of the solemo-like clade C, carasco-like viruses, sobemo-like viruses from China) or a V8-like glutamyl endopeptidase, which is a characteristic of SmVB, the SmVB-like virus from the Teltow Canal, and Qianjiang sobemo-like virus 4.

The other oomycetes viruses, SmVA and PhVA, as well as 20 related viruses from the Teltow Canal clustered with nodaviruses, but in a distinct clade (Fig. 4 lower branch, see also reference 19).

Hypoviridae

A single 264-bp scaffold related to a hypovirus was identified. It showed 47% aa sequence identity to *Botrytis cinerea* hypovirus 2, accession no. MN617169 (data not shown).

Mymonaviridae

A 216-nt scaffold was assigned by DIAMOND to *Lentimonavirus lentinulae*, a species of negative-strand RNA viruses of the family *Mymonaviridae*. According to BLASTp, this sequence showed 57% aa sequence identity to the RdRP of *Golovinomyces magnicellulatus* associated virus and approximately 53% identity to the RdRPs of *Erysiphe necator* associated negative-stranded RNA virus 23 and *Plasmopara viticola* lesion associated mononegaambi virus 8 (data not shown).

Discussion

In order to better understand the virome composition of riverine freshwaters, we examined the virus content of two 50-L water samples from the Teltow Canal and Havel River, which traverse the metropolitan area of Berlin, Germany. Both freshwater bodies are affected by human

Fig. 4 Phylogenetic analysis of 85 RdRP sequences of oomycetes viruses, related viruses, and reference viruses. The replicase sequences of *Sclerophthora macrospora* viruses A and B, *Plasmopara halstedii* virus A, unclassified related viruses from the Teltow Canal, Havel River, and various hosts as well as reference viruses (nodaviruses, luteoviruses, and solemoviruses) were aligned using MEGA, and a maximum-likelihood tree was constructed using IQ-TREE 2 with the optimal substitution model VT+R5. Presented are GenBank accession numbers, vernacular virus names, and strain designations, if available (in parentheses). Square brackets indicate family names (solid lines) and protein families of polymerase and coat proteins according to the CDD and pfam databases (broken lines). Numbers at nodes indicate bootstrap support obtained after 10,000 ultrafast replications. The tree was arbitrarily rooted with sequences with a *Nodaviridae* RdRP (cd23173). The bar indicates amino acid substitutions per site. Color code: black, classified viruses; blue, unclassified viruses; red, viruses from the Teltow Canal and Havel River

activity and contain microbes associated with municipal surface water drains, discharged effluents from a wastewater treatment plant, and recreational activities. Our previous work revealed the presence of thousands of novel, highly divergent virus sequences. In recent papers, we focused on picorna-like viruses, hepeliviruses, plant viruses, and invertebrate viruses [17–21], and these included descriptions of mycovirus-like sequences resembling sequences of members of the families *Alphaflexiviridae*, *Barnaviridae*, *Endornaviridae*, *Partitiviridae*, and *Spinareoviridae* and of the order *Elliovirales* [19, 21]. These virus families are known to include fungal viruses. Although environmental samples do not allow us to associate sequences with specific hosts, phylogenetic analysis suggested that the alphaflexiviruses, bunyaviruses, and spinareoviruses from the Teltow Canal and Havel River were not mycoviruses. In contrast, the SmVA- and PhVA-like viruses, endorna-like viruses, gammapartitiviruses, and aspi-like viruses were related to mycoviruses or still-unclassified fungus-associated viruses [19, 21]. Unfortunately, the possible hosts of the barna-like viruses and many of the mycovirus-related sequences in our present study remain uncertain because the related reference virus sequences are associated with both fungal and non-fungal hosts. This illustrates once more the limitations of using environmental samples. Among the detected and analyzed viruses whose hosts are still uncertain are the following viruses:

TC- and Havel-botourmia-like viruses

Although we characterized more than 150 botourmia-like viruses, none of them belonged to the known genera of fungus-infecting viruses with monopartite, monocistronic genomes. Even their similarity to the plant-infecting ourmia viruses was only moderate. Five clades of botourmia-like viruses exhibited a dicistronic genome layout, suggesting that there are a number of new taxa to be created. Chen et al. and Sadiq et al. have also described botourmia-like viruses



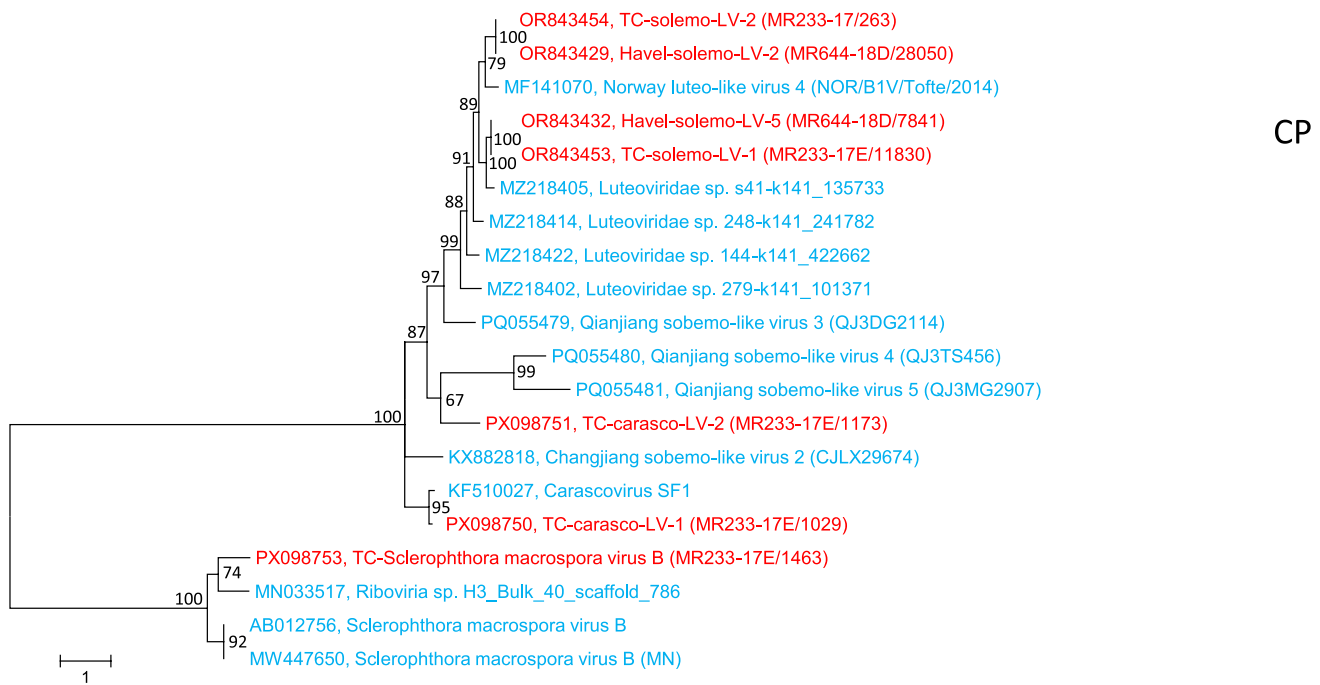


Fig. 5 Phylogenetic analysis of 20 capsid protein sequences of *Sclerophthora macrospora* virus B and related viruses. The CP sequences of *Sclerophthora macrospora* virus B and unclassified related viruses from the Teltow Canal, Havel River, and various hosts were aligned using MEGA, and a maximum-likelihood tree was constructed using IQ-TREE 2 with the optimal substitution model: Q.pfam+F+G4. Pre-

sented are GenBank accession numbers, vernacular virus names, and strain designations, if available (in parentheses). Numbers at nodes indicate bootstrap support greater than 65% obtained after 10,000 ultrafast replications. The bar indicates amino acid substitutions per site. Color code: blue, unclassified viruses; red, viruses from the Teltow Canal and Havel River

with dicistronic genomes [48, 49]. Moreover, GenBank has released a number of similar dicistronic botourmia-like virus sequences from other unpublished metagenomic studies. The presence of a second ORF encoding a putative CP with an S domain suggests that these viruses form virions with icosahedral or hemi-icosahedral capsids. At present, *Ourmiavirus* is the only genus of the family *Botourmiaviridae* whose members are known to have a capsid. Ourmiaviruses have a tripartite genome, with the genes for the RdRP, CP, and movement protein located on separate segments. Our data do not indicate whether the dicistronic botourmia-like viruses have a segmented genome or encode a movement protein, which would suggest a plant host. Qianjiang botourmia-like virus 13 (PQ054635) and TC-botourmia-like virus 17 both have a third ORF, but because no information is available on the expression or function of these hypothetical proteins, the significance of the additional ORF is unclear.

Ghabrivirales-like viruses from the teltow canal and havel river

The association of 59 viruses with similarity to members of the order *Ghabrivirales* with their possible hosts remains inconclusive. Some artiviruses, orthototiviruses, and pseudototiviruses are known to infect both fungi and plants. The

known botybirnaviruses exhibit specificity for fungi, but our TC-botybirna-like viruses are only distantly related to them and may utilize other hosts. Our remaining viruses of the order *Ghabrivirales* either belong to the suborder *Betatotivirineae* ($n = 30$), which includes only a few mycoviruses, or are too divergent to be assigned to any of the existing *Ghabrivirales* suborders, suggesting the need to create a new higher order taxon ($n = 15$). Related viruses of this clade for which host data are available do not appear to be associated with fungi. Nevertheless, this clade contains a branch with four interesting sequences with numerous in-frame termination codons, regardless of which translation table is used. It remains to be determined whether these sequences belong to endogenous, inactive virus-like RNA or indicate the existence of viruses that use a genetic code for protein translation that has not yet been described. Another possible explanation for this phenomenon could be that their genomes contain unusual RNA base modifications that lead to misincorporations during the reverse transcription step of library preparation.

TC- and Havel-mito-like viruses

Hundreds of novel mitovirus-like sequences have been published in recent years (e.g., reference 50), and most of these

viruses are fungal viruses that replicate in mitochondria. Here, we add three new virus sequences from candidate members of the genus *Unuamitovirus* (Supplementary Fig. S3). Another virus using translation code 4, Havel mito-like virus 1, clusters at the root of the *Mitoviridae* branch (Supplementary Fig. S3). For this virus, we failed to identify a related viral sequence with significant similarity (data not shown). However, because few mitoviruses lack internal UGA codons, we suggest that this virus has a non-fungal host. While some mito-like viruses that use the standard translation code are proven plant viruses [35, 36], a few have been associated with various hosts, e.g., Beihai narna-like virus 26 (source: tunicate [50]), Hubei narna-like virus 24 (source: diptera [50]), Melbourne fly narnavirus 1 (source: drosophila), Halley virus (source: penguin), Dwyer narna-like virus (source: rabbit), Gergich narna-like virus (source: rabbit), and eight mito-like viruses from the Teltow Canal and Havel River. All of these viruses cluster in a single clade that is independent of the four established *Mitoviridae* genera (Supplementary Fig. S3). Except for sequence data and the source of the sample, no biological information is available. Hence, the significance of this finding is unclear.

TC- and Havel-narna-like viruses

Recently, Dinan et al. distinguished two clades of narna-like virus, which they named "alphanarnaviruses" and "betanarnaviruses" [37]. The "alphanarnavirus" group is proposed to be comprised of two species, "*Narnavirus saccharomajor*" and "*Narnavirus saccharominor*", as well as a number of unclassified viruses, including five viruses from the Havel River and Teltow Canal. As the members of this clade have been detected in fungal and non-fungal hosts, it is unclear whether the narna-like viruses of the Teltow Canal and Havel River are mycoviruses. Interestingly, a few "alphanarnaviruses" have an aligned, uninterrupted complementary reverse-frame ORF (ambigrammatic sequence) [37–40]. This interesting feature requires further investigation, as it is still unclear whether the encoded protein is really expressed or has a function, but the avoidance of CUA, UUA, and UCA codons, which correspond to translation termination codons in the complementary strand, suggest that the rORF encodes a protein [37, 38]. Narna-like viruses with an rORF have been named "mycoambinarnaviruses" [40], but the phylogenetic analyses of Dinan et al. [37], DiRisi et al. [39], and our study (Supplementary Fig. S3) do not indicate that these viruses belong to a single monophyletic clade.

TC-Zyba-like amalgaviruses

Both TC-zyba-like viruses exhibit the characteristic zybavirus gene layout with fusion of ORFs 1 and 2 by a

programmed frameshift and cluster with zyba-like viruses collected from various sources (Fig. 3). TC-zyba-like virus 1 groups closely with *Antonospora locustae* virus 1 and *Tetrodontophora bielanensis* associated virus 1, which were recently suggested on the basis of sequence identity scores to comprise a new genus of the family *Amalgaviridae* [51]. Other viruses of this clade were associated with unspecified diptera (KX884149 [50]), beetles (MZ209909), mosquitoes (OL700164), and insectivorous bats (OR871395). The oomycete *Antonospora locustae* is a pathogen of grasshoppers, and it seems plausible that the remaining zyba-like viruses of this clade also infect oomycetes, which are pathogenic to insects. Even the bat-associated zyba-like virus might have found its way into the *Rhinolophus* intestine via a dietary route rather than infecting bats. Another clade of zyba-like virus sequences includes a yeast-infecting virus of the species *Zybavirus bailii*, two viruses of plant-infecting fungi (MZ079606, MZ079609), and viruses obtained from water (MW784004) and soil (MN034945) samples. *Conidiobolus rugosus* virus 1 (MZ600508) was obtained from a soil sample has been associated with the fungus *Capillidium rugosum* (*Conidiobolus rugosus*). Chen et al. have associated their Xisha Islands zybavirus (MW784004) from an environmental water sample with putative plant/fungal/bacterial hosts [48]. TC-zyba-like virus 2 from a Teltow Canal water sample matches this correlation.

TC-Sclerophthora macrospora B-like virus

This virus shows similarity to oomycete-infecting SmBV but also to the Quinjiang sobemo-like viruses 4, 5, 28 (accession nos. PQ055480, PQ055481, and PQ055506) from crabs and a virus sequence from grassland soil (MN033517) (Figs. 4 and 5). The availability of only partial sequences, sparse information on host range, and a lack of virus isolates, however, hamper further classification attempts.

Overall, the great divergence of several botourmia-, *Ghabrivirales*-, mito-, and narna-like viruses from the Teltow Canal and Havel River reflects the fast-growing numbers of new, unassigned mycoviruses and may require the creation of further taxa to integrate them in the present virus taxonomy. Undoubtedly, further sampling will uncover more viruses related to those presented here and contribute to a coherent view of riverine viromes.

Supplementary Information The online version contains supplementary material available at <https://doi.org/10.1007/s00705-025-06496-y>.

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Author contributions Conceptualization, R.Z. and H.-C.S.; sampling, transport, large-scale virus enrichment, H.-C.S.; RNA preparation, R.Z.; sequencing and sequence data processing, M.G.; data curation, bioinformatic analysis and phylogenetic analysis, M.G., L.S., and R.Z.; manuscript preparation, R.Z. and H.-C.S. All authors have read and agreed to the published version of the manuscript.

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Data availability The datasets used and analyzed in the current study are available at NCBI: BioProject: PRJNA1174387; Biosamples: SAMN44339963, SAMN44339964; Short Read Archive: SRR31035838, SRR31035839; GenBank accession numbers: PX098516-PX098770.

Declarations

Ethical approval This article does not contain studies with humans or animals.

Conflicts of interest The authors declare that there are no conflicts of interest.

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