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The analysis of the virome associated with *Freesia refracta* plants with necrotic disorder sheds new light on the phylogenetic relationships in the *Konkoviridae* and *Yueviridae* families

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Abstract

Background The necrotic disorder of freesia, first described in 1970 in Northern Europe, is still affecting freesia cultivation globally. Although several viruses have been listed as possible causal agents, the etiology of the disease is still not clear and is possibly linked to a combination of different factors. In this study, a high-throughput sequencing (HTS) virome analysis was performed on total RNA extracts derived from symptomatic freesia leaves.

Methods Freesia leaves showing necrotic disorder symptoms were collected in the Liguria region (Northwestern Italy) in 2011, 2014 and 2022. Total RNA was extracted and sent to specialized companies for rRNA-depletion library construction and Illumina sequencing. *Ad hoc* bioinformatics and phylogenetic analyses were performed on HTS data in order to identify and characterize plant viral entities. The Serratus Project Database was used to explore publicly available metatranscriptomic datasets with the aim of expanding selected viral families.

Results Freesia konkovirus 1, a novel virus putatively belonging to the recently ratified *Konkoviridae* family was identified and characterized. The family was further expanded through public metatranscriptomic data analyses; its phylogeny was investigated, and new genera were proposed. Moreover, a novel virus, putatively belonging to the *Yueviridae* family, was partially characterized, and its phylogenetic position was discussed.

Conclusions This is the first untargeted HTS virome analysis of freesia necrotic disorder. Through a combined wet-lab and *in silico* approach, we identified unknown novel viruses, increasing the current knowledge of the diversity

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of viral agents that infect freesia, expanding and clarifying the phylogenesis of the very recently ratified families of *Konkoviridae* and *Yueviridae*, and highlighting possibly new actors in the freesia necrotic disorder.

Keywords Bunyaviricetes, Virome, Necrosis syndrome, Metatranscriptomics, Serratus project

Introduction

First described in the Netherlands more than 50 years ago and soon reported in Germany and England [1], the necrotic disorder of freesia (*Freesia refracta* hyb., family Iridaceae) caused, by the beginning of this century, considerable economic losses across Europe [2, 3]. Typical symptoms include chlorotic interveinal spots and streaks starting at the leaf tips, which varying depending on cultivar and climate (Fig. 1).

Despite its economic importance, the etiology of the syndrome remains unresolved. Indeed, although several viruses have been associated with the disorder over time, no single virus can be considered the sole causal agent. In the past, the syndrome was supposed to result from a mixed virus infection, mainly involving the potyvirus freesia mosaic virus (FreMV; *Potyvirus freesiae*) and a not yet well characterized varicosavirus [4]. Subsequently, the disease was widely linked to a then newly discovered ophiovirus named freesia sneak virus (FreSV; *Ophiovirus freesiae*) [2], belonging to the family *Aspiviridae* [5], to date reported only in freesia and lachenalia (*Lachenalia* hyb., Hyacinthaceae) [6]. However, even if the correlation between the presence of symptoms and FreSV detection is high, some uncertainty remains, and FreSV is considered one of the main agents involved in the syndrome but not the only one.

High-throughput sequencing (HTS) enables comprehensive and unbiased virus discovery, offering clear advantages over conventional detection methods. Thanks to its untargeted approach, this technique can effectively aid in identifying new etiological agents [7]. Here, an HTS-based analysis of total RNA extracted from symptomatic freesia leaves was performed to expand our knowledge of the virome associated with the necrotic disorder, and two new viral species representing putative new members of the *Konkoviridae* and *Yueviridae* families were identified.

The increasing availability of global open-science resources, such as public metatranscriptomic datasets, provides an unprecedented opportunity to expand virome discovery and uncover novel viral diversity. To this end, we employed the open-science viral discovery platform Serratus Project Database [8] to explore the virome associated with already available transcriptome sequencing data from millions of samples collected worldwide. This approach led to the identification of seven potential new members of the *Konkoviridae* family, thus expanding this newly created family. Based on phylogenetic analysis, we propose here the reorganization of the *Konkoviridae* family, suggesting the existence of two new genera and identifying a new *Konkoviridae* species isolated in freesia as representative of one of them.

Moreover, we partially characterized a novel virus, that, according to our phylogenetic analysis, represents a new member of the newly established *Yueviridae* family.

Materials and methods

Sample collection

Necrotic foliar tissue from freesia plants was collected in the Liguria region (Northwestern Italy) in 2011 (sample fr231211) and 2014 (sample fr140314), both in the Sanremo area in the same plots, and used for the first HTS analysis. More recently, 22 plants showing various degrees of necrotic symptoms were collected in 2022 in the same area; from these, six plants exemplifying the typical symptomatology were chosen and pooled (sample fr090322) for the second HTS analysis. All the samples were stored at −80 °C until processing.

Sample processing and illumina sequencing

Total RNA was individually extracted with a Spectrum™ Plant Total RNA Kit (Sigma Aldrich, St. Louis, MO, USA) following the manufacturer's instructions. After DNase treatment with the Turbo DNA-free kit (Invitrogen, UK), total RNA was sent to specialized companies for Illumina sequencing. Symptomatic samples collected from 2011 to 2014 were pooled and sent to Macrogen (Seoul, Republic of Korea; http://www.macrogen.com): Illumina TruSeq Stranded Total RNA with riboZero Plant ribosomal removal was used for library construction and Illumina NovaSeq 6000 100 bp paired-end reads was used for sequencing. Symptomatic samples collected in 2022 were pooled and sent to Novogene (Cambridge, United Kingdom; https://www.novogene.com/): Illumin a TruSeq Stranded Total RNA with riboZero Plant ribosomal removal was used for library construction and Illumina NovaSeq 150 bp paired-end reads was used for sequencing.

Virome annotation

The raw reads were processed with fastp v. 0.21.0 [9], using default parameters, for quality check and removal of adapters. Residual ribosomal sequences were discarded using bbduk script (default parameters) from bbmap v. 38.7 [10]. The reads were then assembled using metaspades v. 3.15.1 [11], with k-mer values of 71, 81 and 91, and the obtained contigs were further extended with CAP3 (VersionDate: 02/10/15) [12]. Contigs with a

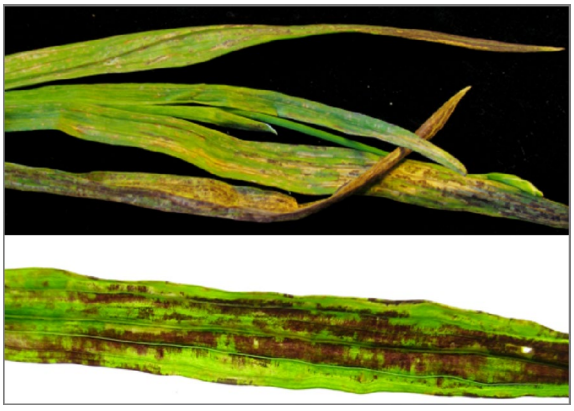

Fig. 1 Necrotic and deformed freesia leaves (sample Fr6 collected in 2022) showing typical symptoms of freesia necrotic disorder; details are shown in the lower panel

length above 600 nt were queried against the NCBI non-redundant protein database (https://www.ncbi.nlm.nih.gov/) using DIAMOND blastx with an e-value cutoff of $1e^{-5}$. To explore the taxonomical content, the results from DIAMOND blastx were uploaded into MEGAN v. 6.24.1. Viral contigs identified using DIAMOND blastx were further queried against the NCBI non-redundant nucleotide database (https://www.ncbi.nlm.nih.gov/) using BLASTN. ORFfinder (https://www.ncbi.nlm.nih.gov/orffinder/), with default parameters and a minimal ORF length of 300 nt, was used to identify open reading frames and encoded protein sequences.

Molecular detection, RACE–PCR and validation

The diagnostic, validation and RACE primers used in this study are listed in Additional file 1. Virus-specific primers were synthesized to match the sequences obtained by HTS analysis. cDNA synthesis was performed using High-Capacity cDNA Reverse Transcription Kit (Thermo Fisher Scientific, Waltham, MA, USA), and PCRs were carried out using Platinum II Taq Hot-Start DNA Polymerase (Thermo Fisher Scientific, Waltham, MA, USA) according to the manufacturers' instructions. RT–PCR was performed in some cases using the faster procedure of the One-step RT–PCR kit (Qiagen, Hilden, Germany) following the manufacturer's instructions. PCR-amplified fragments were visualized by electrophoresis using a 1.5% agarose gel, purified with the Zymoclean DNA Clean & Concentrator Kit (Zymo Research, CA, USA) and sent to a specialized company (BMR Genomics, Padova, Italy) for Sanger sequencing. For RACE-PCR, the 5′/3′ RACE Kit (Roche, Basel, Switzerland) was used according to the manufacturer's instructions.

Metatranscriptomic data mining

Palmprints associated with putative novel konkoviruses were identified by querying the Serratus Project Database using the RdRp protein sequence of freesia konkovirus 1 as input. SRA raw data associated with selected palmprints were downloaded from the NCBI-SRA database, preprocessed using fastp v. 0.21.0 and *de novo* assembled with metaspades v. 3.15.1. The obtained contigs were compared using BLASTx with the protein sequences of known konkoviruses. ORFfinder (https://www.ncbi.nlm.nih.gov/orffinder/) was used to identify open reading frames and encoded protein sequences. Alignments were performed using MUSCLE algorithm embedded in MEGA11 v11.0.11.

Phylogeny reconstruction

Viral sequences were retrieved from the NCBI protein database (https://www.ncbi.nlm.nih.gov/). The updated taxonomy (Virus Taxonomy: 2023 release) reported by the International Committee on Taxonomy of Viruses (ICTV Website, accessed on 10/24/2024) was used as a guide to retrieve sequences of each representative virus species. Sequences were aligned with the MUSCLE algorithm embedded in the MEGA11 software v11.0.11. IQ-TREE v3.0.1 [13] was used to model selection and construction of phylogenetic trees. FigTree v1.4.4 (https://tree.bio.ed.ac.uk/software/figtree/) was used for visualization of phylogenetic trees.

Results

Overview of the virome associated with the necrotic disorder of freesia

Freesia leaves showing necrotic disorder symptoms were collected in Liguria (Northern Italy) in 2011 and 2014 and used for virome analysis through total RNA Illumina sequencing. After cleaning, a total of 47,516,574 clean reads were obtained and *de novo* assembled into 14,746 contigs (contig length > 600 nt). By querying these contigs against the non-redundant protein NCBI database (https://www.ncbi.nlm.nih.gov/), a total of 8 contigs showing similarity (e-value < e^{-5}) with viral protein sequences were identified (Table 1A and Additional file 2). These contigs were further compared with nucleotide sequences available in the NCBI non-redundant nucleotide database. The contig c1_11/14 was the most abundant in terms of reads (2.36%) percentage and matched the potyvirus FreMV polyprotein. Four contigs (c2_11/14, c3_11/14, c4_11/14, c5_11_14, 0.05–0.29% reads) matched the ophiovirus FreSV RNA genomic segments. Two contigs (c6_11/14, c7_11/14) showed a certain level of similarity with the RNA-dependent RNA polymerase and the nucleocapsid protein of lachenalia phenuivirus-1 (LacPhV-1) (1.64% and 0.88% reads, respectively), a newly identified viral species similar to tulip streak virus (TuSV), the first recognized member of the recently ratified *Konkoviridae* family [14–16]. Considering the level of similarity of our contigs with

Table 1 Viral contigs identified in freesia leaves showing necrotic disorder symptoms collected in Liguria (Northern Italy) (A) from 2011 to 2014 and (B) in 2022

Contigs	Length (nt)	BlastX first hit (GenBank id.)	Coverage %	Identity %	Read abundance %
A					
c1_11/14	9376	polyprotein [Freesia mosaic virus] (WLJ60149)	98	99	2.36
c2_11/14	7635	RNA-dependent RNA polymerase [*Ophiovirus freesiae*] (WNZ99845.1)	89	100	0.15
c3_11/14	1962	54 kDa protein [*Ophiovirus freesiae*] (WNZ99840.1)	84	100	0.29
c4_11/14	1549	coat protein [*Ophiovirus freesiae*] (AWJ64329.1)	88	96	0.25
c5_11/14	1483	39 kDa protein [*Ophiovirus freesiae*] (WNZ99843.1)	65	100	0.05
c6_11/14	6014	RNA-dependent RNA polymerase [*Phenuiviridae* sp.] (XDI83325.1)	99	74	1.64
c7_11/14	1047	nucleocapsid protein [*Phenuiviridae* sp.] (XDI83326.1)	62	78	0.88
c8_11/14	3824	putative RNA-dependent RNA polymerase [*Fusarium culmorum* yue-like virus 1] [*Yueviridae*](XCO56027.1)	38	23	0.003
B					
c1_22	841	VPg [Freesia mosaic virus] (YP_003620389.1)	43	93	0.355
c2_22	1534	CI [Freesia mosaic virus] (YP_003620393.1)	32	98	0.311
c3_22	1410	polyprotein [Freesia mosaic virus] (BDA76646.1)	33	94	0.367
c4_22	724	NIb [Freesia mosaic virus] (YP_003620391.1)	67	96	0.162
c5_22	607	CI [Freesia mosaic virus] (YP_003620393.1)	82	98	0.314
c6_22	653	20 kDa protein [Ophiovirus freesiae] (WNZ99846.1)	44	100	0.019
c7_22	7471	RNA-dependent RNA polymerase [Ophiovirus freesiae (WNZ99845.1)	88	97	0.241
c8_22	1935	coat protein [Ophiovirus freesiae] (AWJ64329.1)	85	93	0.307
c9_22	3393	54 kDa protein [Ophiovirus freesiae] (WNZ99840.1)	48	99	0.214
c10_22	3649	RNA-dependent RNA polymerase [Phenuiviridae sp.] (XDI83325.1)	44	74	0.898
c11_22	2706	RNA-dependent RNA polymerase [Phenuiviridae sp.] (XDI83325.1)	95	70	1.743
c12_22	3877	putative RNA-dependent RNA polymerase [Fusarium culmorum yue-like virus 1] (XCO56027.1)	37	23	0.027
c13_22	725	polyprotein [Bean yellow mosaic virus] (UOF93143.1)	75	96	0.099
c14_22	3426	polyprotein [Bean yellow mosaic virus] (WCR76010.1)	78	99	0.688

LacPhV-1, we propose that they represent the genome of a distinct new viral species belonging to the *Konkoviridae* family, which will be discussed in detail in dedicated Sect. 3.2. Finally, contig c8_11/14 (0.003%) had no significant similarity at the nucleotide level and shared limited protein similarity with the putative RNA-dependent RNA polymerase of *Fusarium culmorum* yue-like virus 1 (FcYV1), which belongs to the *Yueviridae* family. Notably, the reads equally mapped on both plus and minus polarities (Additional file 3), suggesting an active viral replication. This contig is likely to represent the genome of a new virus belonging to the *Yueviridae* family and will be discussed in dedicated Sect. 3.4.

The presence of the viral entities identified in symptomatic freesia leaves was confirmed by an independent RNA-Seq analysis performed on a pool of symptomatic leaves collected from six symptomatic freesia plants in the same area in 2022. In this new RNA-Seq analysis, we obtained a total of 101,460,426 clean reads that were assembled in a total of 79.864 contigs (length > 600 nt); among them, we retrieved contigs associated with all the viruses identified in the freesia symptomatic leaves collected in 2011–2014 (Table 1B and Additional file 2). In detail, five contigs (from c1_22 to c5_22) were associated with FreMV, four contigs (from c6_22 to c9_22) corresponded to FreSV, two contigs (c10_22 and c11_22) showed similarity with LacPhV-1, and one contig (c12_22) had weak similarity with the putative RNA-dependent RNA polymerase of FcYV1 and corresponded to the new putative member of the *Yueviridae* family already identified in the previous RNA-seq analysis (see Additional file 2 for details). Furthermore, in these samples, we also identified two contigs (c13_22 and c14_22) with high similarity with the polyprotein of the potyvirus bean yellow mosaic virus (BYMV), a virus that was not present in the samples collected in 2011 and 2014. The presence of viral RNAs in symptomatic freesia samples collected in 2022 was confirmed independently in each of the six plants pooled for the RNA-Seq analysis by RT–PCR with specific primers followed by Sanger sequencing of the amplified PCR fragments (Additional file 4).

Overall, the analysis of the virome associated with the freesia necrotic disorder led us to identify the presence of five different viral entities. Three of them, i.e., FreMV, FreSV and BYMV, consist of viruses already known to infect freesia [17], whereas the remaining two represent

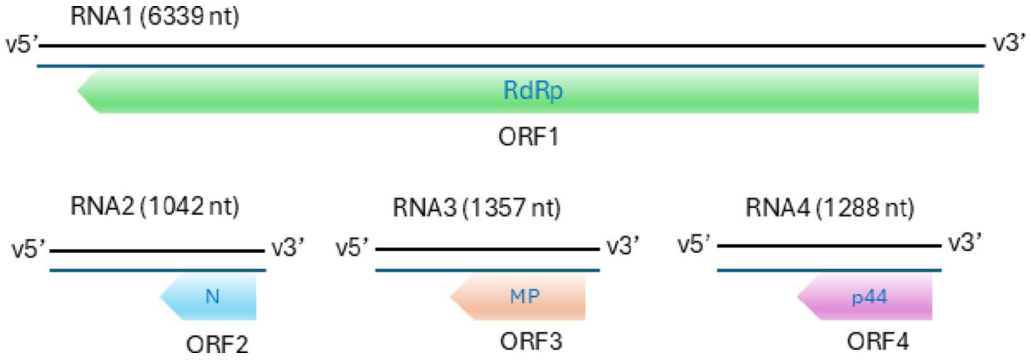

Fig. 2 Genome organization of FreKV-1; RdRp, RNA-directed RNA polymerase; N, nucleocapsid protein; MP, movement protein

Table 2 Full coding sequence organization of the four FreKV-1 genomic RNAs obtained in this study and statistics

Genomic segment	Length (nt)	*n*. of mapping reads	% of mapping reads	Average coverage	ORF sense	Protein function	Estimated molecular weight (kDa)/amino acids (aa)
RNA1	6339	792,887	1.67	12,479 X	vcRNA	RNA-dependent RNA polymerase	239.2/2069
RNA2	1042	417,904	0.88	39,964 X	vcRNA	Nucleocapsid protein	27.7/239
RNA3	1357	367,182	0.77	27,017 X	vcRNA	Movement protein	40.0/354
RNA4	1288	416,758	0.88	32,295 X	vcRNA	Unknown function	44.2/374

new viruses belonging to the recently ratified *Konkoviridae* and *Yueviridae* families.

Characteristics of a putative novel *Konkovirus* species

Some hints about this further putative actor in the necrotic disorder of freesia stage, came out for the first time following a Sequence Independent Amplification (SIA) procedure [18] on total RNA extracted from necrotic freesia leaves collected in Liguria (Northern Italy) during the 2011–2012 winter season. From this analysis, we obtained clones of unknown virus-like genome sequences [19]. This new virus was shown to be mechanically transmissible from symptomatic freesia leaves to the test plant *Nicotiana benthamiana* even if no symptoms were evident in this plant.

As reported in the previous section, RNA-Seq analysis allowed us to reconstruct two contigs (c6_11/14 and c7_11/14) corresponding to RNA1 and RNA2 of a putative new member of the *Konkoviridae* family (order *Hareavirales*, class *Bunyaviricetes*). Rapid Amplification of cDNA Ends (RACE-PCR) was performed to obtain the complete sequence of these genomic segments. The *Konkoviridae* family, ratified by the ICTV in 2023, currently lists a single genus (*Olpivirus*) represented by the negative-sense and soil-borne RNA virus TuSV originating from tulip (*Tulipa gesneriana* L.) and by the RNA virus lactuca big vein associated phlebovirus (LBVaPV, *Olpivirus lactucae*) isolated from lettuce [20] (ICTV website accessed on 03/05/2025). A putative third member provisionally named LacPhV-1 and showing similarity with TuSV and LBVaPV was recently identified in lachenalia [14]. Since the RNA genome of these viruses is composed by 4 RNAs, we decided to search our data for contigs that are similar to these RNAs, and we identified two more contigs corresponding to the full-length genomic segments RNA3 and RNA4. Since the new virus was identified in freesia plants, we propose the name Freesia konkovirus 1 (FreKV-1).Overall, the complete genomic sequence of the novel konkovirus FreKV-1, consisting of four viral RNA segments (RNA1-RNA4; GenBank accessions: PQ490803, PQ490804, PQ490805, PQ490806) that encode four virus-related proteins (Fig. 2; Table 2), was reconstructed.

These RNAs are similar to the four genomic RNAs of TuSV, with identity percentage values ranging between 72% and 64% (Table 3) and coverage percentages ranging between 58% and 44% (Additional file 5). We identified four ORFs encoding four proteins, i.e., the RNA-dependent RNA polymerase (RdRp), the nucleocapsid protein (N), the movement protein (MP) and the protein of unknown function p44 (Fig. 2), with identities ranging from 62% to 58% and coverage percentages ranging between 100% and 87% with the corresponding proteins encoded by TuSV (Table 3 and Additional file 5).

Interestingly, the RT-PCR with specific primers for RNA4 (Additional file 4), followed by Sanger sequencing, highlighted the existence of a defective RNA4 of 829 nt with an ORF coding for a shorter version of the p44 protein.

Table 3 (A) nucleotide identity among the RNA genome segments of FreKV-1 and the other members of the *Konkoviridae* family (TuSV, LBVaPV and LacPhV-1) either accepted or under evaluation; (B) amino acid identity among the protein sequences of FreKV-1 and the other members of the *Konkoviridae* family (TuSV, LBVaPV and LacPhV-1) either accepted or under evaluation; RdRp, RNA-directed RNA polymerase; N, nucleocapsid protein; MP, movement protein

A						
BlastN	**FreKV-1**	**TuSV**	**LBVaPV**	**FreKV-1**	**TuSV**	**LBVaPV**
	RNA1			**RNA2**		
TuSV	67.34%			72.08%		
LBVaPV	70.24%	75.25%		70.20%	74.98%	
LacPhV-1	72.54%	68.20%	70.93%	72.79%	67.01%	69.74%
	RNA3			**RNA4**		
TuSV	64.33%			63.93%		
LBVaPV	64.05%	74.82%		73.47%	69.26%	
LacPhV-1	73.21%	66.42%	65.06%	65.94%	65.26%	65.63%
B						
BlastP	**FreKV-1**	**TuSV**	**LBVaPV**	**FreKV-1**	**TuSV**	**LBVaPV**
	RdRp			**N**		
TuSV	58.15%			62.38%		
LBVaPV	58.00%	84.66%		63.98%	83.40%	
LacPhV-1	73.48%	57.47%	57.24%	73.53%	63.47%	62.73%
	MP			**p43/44**		
TuSV	49.29%			55.61%		
LBVaPV	50.28%	80.00%		54.01%	73.80%	
LacPhV-1	69.57%	49.86%	50.00%	57.75%	57.75%	58.02%

Since the family *Konkoviridae* has been ratified only recently, official species demarcation criteria are not yet available on the ICTV website. However, a taxonomy proposal recently approved in 2025 defines a species demarcation threshold of less than 95% identity for the RdRp protein sequence (https://ictv.global/). According to this criterion, the RdRp identity percentage observed between FreKV-1, TuSV, LBVaPV and the other putative konkovirus LacPhV-1 (Table 3), supports classification of FreKV-1 as a distinct new species belonging to the new *Konkoviridae* family. Interestingly, these four viruses were found in plants from four different families: *Iridaceae* (FreKV-1), *Hyacinthaceae* (LacPhV-1), *Liliaceae* (TuSV) and *Asteraceae* (LBVaPV), indicating that konkoviruses infect a phylogenetically diverse range of both monocot and dicot hosts.

The sequences of both the 5' and 3' ends of FreKV-1 genomic RNAs show characteristics typical of *Bunyaviricetes* [21–23].

Specifically, similar to other members of the *Konkoviridae* family, the 5' and 3' terminal regions can form a panhandle structure (Additional file 6 A) and have almost identical sequences, with 11 and 13 conserved nucleotides, respectively (Additional file 6B). The only exception is RNA4, which shows a reduced level of conservation at the 3' terminus.

A comparison of the terminal sequences of the four RNAs of FreKV-1 with those of the other konkoviruses revealed that RNA1 presents a relatively high level of conservation; indeed, the first 18 nt at the 5' terminus and the first 17 nt at the 3' terminus of RNA1 are conserved between FreKV-1 and LacPhV-1, and only one nucleotide at each termini differentiates these viruses from LBVaPV and TuSV, which, between them, share 100% identity at their RNA1 termini. All the other 5' and 3' termini are conserved for the first 11 to 14 nt, with the exception of RNA4, which presents a relatively lower level of conservation, particularly in the case of FreKV-1 (Additional file 6 C).

Metatranscriptomic exploration and phylogeny of the *Konkoviridae* family

Public databases contain a planetary collection of metatranscriptomic data that can be useful for reconstructing new viral genomes and extending our knowledge of viral families. Here, we took advantage of the cloud computing infrastructure Serratus [8] to search publicly available SRA-NCBI datasets (https://www.ncbi.nlm.nih.gov/sra/) for the presence of RdRp amino acid subsequences called 'palmprints' delineated by three essential motifs that together form the catalytic core in the RdRp structure. By querying the RdRp palmprint Serratus Database with the RdRp amino acid sequence of FreKV-1, we identified nine palmprints with an identity percentage greater than 70% (Fig. 3 - orange dots and Table 4). Among them, the palmprint u28439 corresponds to the RdRp of TuSV, whereas the remaining eight are representative of new putative RNA viruses and, given the level of similarity below 95%, are possibly representative of putative new members of the *Konkoviridae* family (https://ictv.global/).

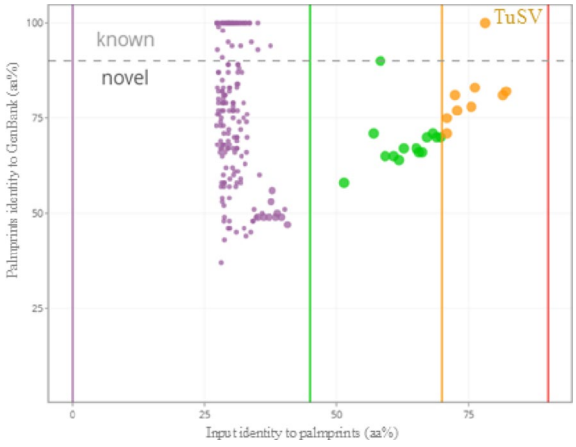

Fig. 3 Percentage identity of the RdRp FreKV-1 palmprint amino acid sequence used as the input sequence with the palmprints in the Serratus Database. The green, orange and red lines indicate the 45%, 70% and 95% identity thresholds, respectively. The dotted line corresponds to the 90% identity threshold and separates palmprints associated with known viruses (above the dotted line) and palmprints representing putative new viruses (below the dotted line). The figure was obtained via the Serratus Project Database tool

Table 4 Serratus palmprints showing an identity percentage value above 70% with the RdRp FreKV-1 palmprint aminoacidic sequence; the palmprint corresponding to TuSV is in italic

Serratus Palmprint id	% id	E-value	Associated SRA-NCBI dataset
u28439	*82.5*	*2,90E-83*	*DRS174919*
u172440	82.1	8.50E-90	SRR5237250
u77408	81.5	1.90E-86	SRR11565435
	81.5	1.90E-86	SRR11565175
	81.5	1.90E-86	SRR11565436
	81.5	1.90E-86	SRR11076227
u218371	76.2	5.00E-82	ERR690823
u272064	75.5	1.60E-85	SRR13263231
u174091	72.8	3.10E-78	SRR7030606
u272319	72.4	3.60E-75	SRR6787483
u288562	70.9	3.00E-76	ERR2040528
u78567	70.9	4.70E-73	SRR8286202

To reconstruct the genome of the novel konkoviruses identified by the palmprint similarity analysis, the raw reads of the selected SRA-NCBI datasets (Table 4) were retrieved, cleaned and *de novo* assembled following the same bioinformatic pipeline used for the previous analyses; the obtained contigs were then compared with the protein sequences of the four known konkoviruses (FreKV-1, TuSV, LBVaPV, and LacPhV-1) using blastx; contigs representing the viral RNA genomic sequences of the new putative konkoviruses were identified and checked for completeness. For seven out of the eight new putative konkoviruses, this approach allowed us to reconstruct the complete (or almost complete) CDS of RNA1, which codes for the RdRp protein, and RNA2, which codes for the N protein; for two of them, we were able to reconstruct the complete CDS of all four expected RNA genomic segments (Table 5). Notably, in the SRA-NCBI dataset SRR7030606, two different putative complete RNA1 CDSs were retrieved (blastx comparison: 53.27% identity-96% coverage and 43.95% identity-93% coverage with respect to the RdRp of FreKV-1), suggesting the existence of two different konkoviruses in this sample. In line with the hypothesis that these sequences correspond to new members of the *Konkoviridae* family and therefore are expected to infect plants, seven of them originated from SRA-NCBI datasets obtained from RNA extracted from plants belonging to different families that can be considered putative hosts: *Waitzia nitida* (*Asteraceae*), *Campanula rotundifolia* (*Campanulaceae*), *Lilium leucanthum* (*Liliaceae*), *Epipactis purpurata* (*Orchidaceae*), *Tripterocalyx crux-maltae* (*Nyctaginaceae*), *Lennoa madreporoides* (*Lennoaceae*), *Erythranthe pardalis* (*Phrymaceae*), and *Holcus lanatus* (*Poaceae*) (Table 5). The remaining palmprint originating from the SRA-NCBI datasets is associated with soil metatranscriptomic data, in line with the supposed soil-borne nature and fungus-mediated transmission of the viral members of the *Konkoviridae* family [16]. On the basis of the plants from which the SRA-NCBI datasets originated, for the novel konkoviruses, we propose the following names: Waitzia-associated konkovirus 1 (WaKV-1), Campanula-associated konkovirus 1 (CaKV-1), Lilium-associated konkovirus 1 (LilaKV-1), Epipactis-associated konkovirus 1 (EpaKV-1), Epipactis-associated konkovirus 2 (EpaKV-2), Tripterocalyx-associated konkovirus 1 (TaKV-1), Lennoa-associated konkovirus 1 (LeaKV-1), and Erythranthe-associated konkovirus 1 (EryaKV-1). For the new konkovirus identified in the metatranscriptomic soil, we propose the provisional name of soil-associated konkovirus (SaKV).

The representatives of all thirty-seven genera of viruses included in the eight families of the *Hareavirales* order were used to infer the phylogenetic relationships of the RdRp and N proteins obtained from the already known and newly identified putative konkoviruses. The maximum likelihood (ML) tree of the RdRp proteins (Fig. 4) revealed that all the konkovirus RdRp sequences grouped together in a monophyletic group, which was clearly separated from the other families of the *Hareavirales* order. The existence of this group is also highlighted by the identity percentage of the RdRp sequences shown in the similarity matrix (Additional file 7). The same group is also evident in the phylogenetic tree obtained with the N protein sequences and in the corresponding similarity matrix (Additional files 8 and 9). The phylogenetic analysis of the RdRp protein sequences may suggest the existence of at least three distinct genera in the *Konkoviridae* family: the official genus *Olpivirus*, represented by TuSV

Table 5 Novel putative konkoviruses identified by bioinformatic analysis. * indicates genomic sequences with partial CDSs. (-) indicates genomic segments that were not submitted to the GenBank NCBI database because they were too short or fragmented. [§]Putative host was inferred from the NCBI biosample origin

Novel konkovirus	Serratus Palmprint id	Associated SRA-NCBI dataset	Associated NCBI Biosample	Putative host [§]	Genomic segments recovered (length)	GenBank Acc.*N.*
Waitzia-associated konkovirus 1 (WaKV-1)	u172440	SRR5237250	SAMN06309684	*Waitzia nitida*	RNA1 (6381 nt) RNA2 (1324 nt) RNA3 (1587 nt) RNA4 (1307 nt)	BK070191 BK070192 BK070193 BK070194
Soil-associated konkovirus (SaKV)	u77408	SRR11565175	SAMN14514881	---	RNA1 (6356 nt) RNA2 (1152 nt) RNA3 (1003 nt) RNA4 (1342 nt)	BK070195 BK070196 BK070197 BK070198
Campanula-associated konkovirus 1 (CaKV-1)	u218371	ERR690823	SAMEA3157961	*Campanula* aff. *rotundifolia*	RNA1* (1514 nt) RNA2 (1010 nt) RNA3 (1294 nt)	BK070199 BK070200 BK070201
Lilium-associated konkovirus 1 (LilaKV-1)	u272064	SRR13263231	SAMN17082061	*Lilium leucanthum* var. centifolium	RNA1*(875-752-617-470 nt), RNA2*(295 nt) RNA3*(438 – 279 nt) RNA4* (222 nt)	- - - - -
Epipactis-associated konkovirus 1 (EpaKV-1) Epipactis-associated konkovirus 2 (EpaKV-2)	u174091	SRR7030606	SAMN08949893	*Epipactis purpurata*	RNA1 (6508 nt) RNA1 v2 (6306 nt) RNA2 (1165 nt)	BK070202 BK070203 BK070396
Tripterocalyx-associated konkovirus 1 (TaKV-1)	u272319	SRR6787483	SAMN07527256	*Tripterocalyx crux-maltae*	RNA1 (6322 nt) RNA2 (1038 nt) RNA3 (1375 nt)	BK070397 BK070398 BK070399
Lennoa-associated konkovirus 1 (LeaKV-1)	u288562	ERR2040528	SAMEA104170572	*Lennoa madreporoides*	RNA1 (5459 nt) RNA2 (778 nt)	BK070400 BK070401
Erythranthe-associated konkovirus 1 (EryaKV-1)	u78567	SRR8286202	SAMN10527072	*Erythranthe pardalis*	RNA1 (5087 nt) RNA2 (1053 nt)	BK070402 BK070403

(*Olpivirus tulipae*) and LBVaPV (*Olpivirus lactucae*); a new genus represented by the newly identified FreKV-1 (this study), LacPhV-1 [14], WaKV-1 (this study) and SaKV (this study); and a third genus, including LeaKV-1, EryaKV-1 and EpaKV-1, all identified in this study (Fig. 4).

Characteristics and phylogeny of a putative novel *Yueviridae* species

By analyzing the RNA-seq data, we also retrieved a contig of 3824 nt with the highest identity to the putative RdRp of *Fusarium culmorum* yue-like virus 1, belonging to the *Yueviridae* family (GenBank id. XCO56027.1) as the first BLASTX hit, with 22.71% protein identity and 38% coverage. The integration of this viral sequence in the genomic freesia DNA was excluded by PCR versus RT–PCR (data not shown). Furthermore, we verified the existence of HTS reads mapping both positive and negative sense of the viral genomic sequence (Additional file 3) as indicators of possible dsRNA accumulation, which is typical of viral replication. On the basis of these data, we concluded that this contig represents a replicating RNA segment corresponding to a novel yue-like virus, which constitutes the first member of this group reported in angiosperms. Accordingly, for this newly identified virus, we propose the provisional name of freesia associated yue-like virus 1 (FraYV-1; GenBank Acc. N. PV171508). *Yueviridae* (https://ictv.global/report/chapter/yueviridae/yueviridae) is a family of negative-sense RNA viruses belonging to the order *Goujianvirales* (*Yunchangviricetes* class, *Haploviricotina* subphylum); viruses of this family have bi-segmented negative single-stranded RNA genomes, with each segment possessing at least one ORF. The ORF in the L segment encodes a large protein corresponding to a RdRp and contains a GDP: polyribonucleotidyltransferase (PRNTase) domain related to that of viruses in the order *Mononegavirales*, suggesting a similar capping mechanism. The ORF in the S segment encodes a nucleocapsid protein structurally related to homologs encoded by other members of *Mononegavirales*, family *Paramyxoviridae*. The family has been very recently established, and several biological aspects remain to be elucidated. Currently, it includes a single genus with only two species, i.e., Běihǎi sesarmid crab virus 3 (BhSCV3; *Yuyuevirus beihaiense*) and Shāhé yuèvirus-like virus 1 (ShYLV1; *Yuyuevirus shaheense*), which have been found in unspecified sesarmid crabs and freshwater isopods, respectively [24]. However, several

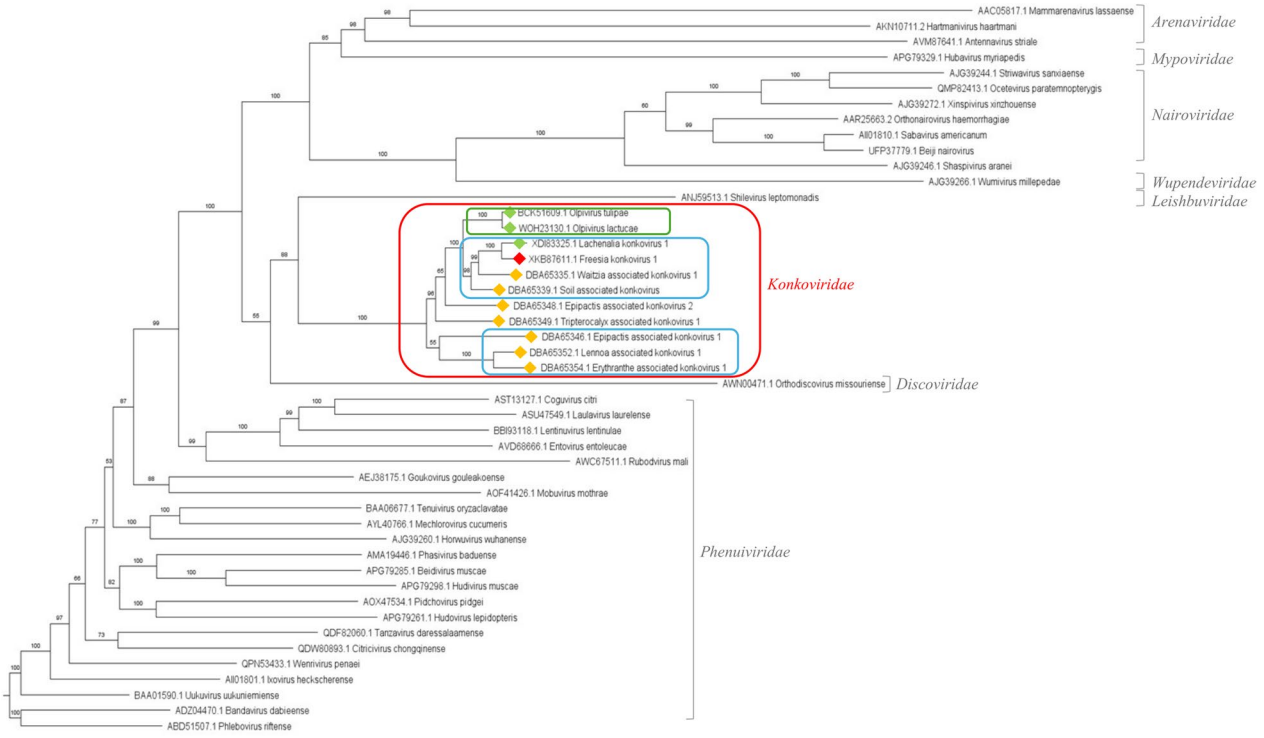

Fig. 4 Maximum likelihood phylogenetic tree inferred using IQ-TREE based on the RNA-dependent RNA polymerase amino acid sequences of selected viral species belonging to the *Hareavirales* order, the official species in the *Konkoviridae* family and the newly discovered species tentatively belonging to the *Konkoviridae* family (for which the RNA-1 full CDS has been obtained). Node values represent ultrafast bootstrap supports (UFBoot) calculated from 1,000 replicates, with values ≥ 70% considered well supported. The best-fit substitution model (Q.PFAM + F + I + R6) was automatically selected by ModelFinder. The red diamond indicates FreKV-1, the green diamonds indicate the already known konkoviruses, including LacPhV-1, which has not yet ratified as member of the *Konkoviridae* family, and the orange diamonds indicate putative new members of the *Konkoviridae* family identified through the Serratus Project Database and reconstructed by bioinformatic analysis. The blue rectangles indicate the proposed new genera in the *Konkoviridae* family; the green rectangle indicates the only official genus currently ratified in the *Konkoviridae* family according to ICTV

unclassified yue-like viruses, often represented only by the L segment, have also been reported to be associated with different hosts, such as crustaceans and insects [25], stramenopiles [26–28], tracheophytes [29], and unspecified macrophytes [30], thus suggesting that the diversity of *Yueviridae* is likely underestimated [31–33] (ICTV Website accessed on 10/23/2024). Notably, as previously mentioned, FraYV-1 is the first yue-like virus identified in angiosperms, thereby broadening the known host range of this viral family. The phylogenetic tree inferred from the RdRp amino acid sequences of the members of the yuevirus-like group highlighted the existence of two separate clades in the *Yueviridae* family; clade A includes the two yuevirus species defined in the ICTV taxonomy and is predominantly associated with invertebrates as hosts, whereas clade B includes FraYV-1 and yue-like viruses isolated from different oomycetes and from ferns (Fig. 5A). The catalytic domain in the conserved motif C of the RdRp proteins [34] belonging to clade A shows the canonical SDD sequence, whereas all the RdRp proteins belonging to clade B house the IDD sequence instead. In this motif, a conserved G is present after the DD in all the *Yueviridae* members (Fig. 5B). These data suggest the need for further investigation in order to evaluate if the *Yueviridae* family could be split into two different genera associated to clades A and B and to better understand the phylogenetic relationships of the members of this family.

Virus detection and symptomatology

To clarify the possible correlation between the identified viruses and the necrotic disorder of freesia, we further investigated the level of association between the identified viruses and the necrotic symptomatology. Twenty-one freesia plant samples (Fr1-21) showing different types of necrosis and an asymptomatic plant used as a negative control (Fr22), collected in 2022 in Northern Italy, were tested individually using specific primers to detect the viruses revealed via virome analysis (Fig. 6). No correlation between symptoms and virus presence was highlighted by Fisher's exact test (Additional file 10). Indeed, the potyvirus FreMV, which is not associated with necrosis in the literature [17], was present in all the samples, including samples Fr1 and Fr9, in which some necrosis was only slightly evident on the collected leaves, and in the asymptomatic sample Fr22. BYMV was detected in almost all the samples, including asymptomatic sample

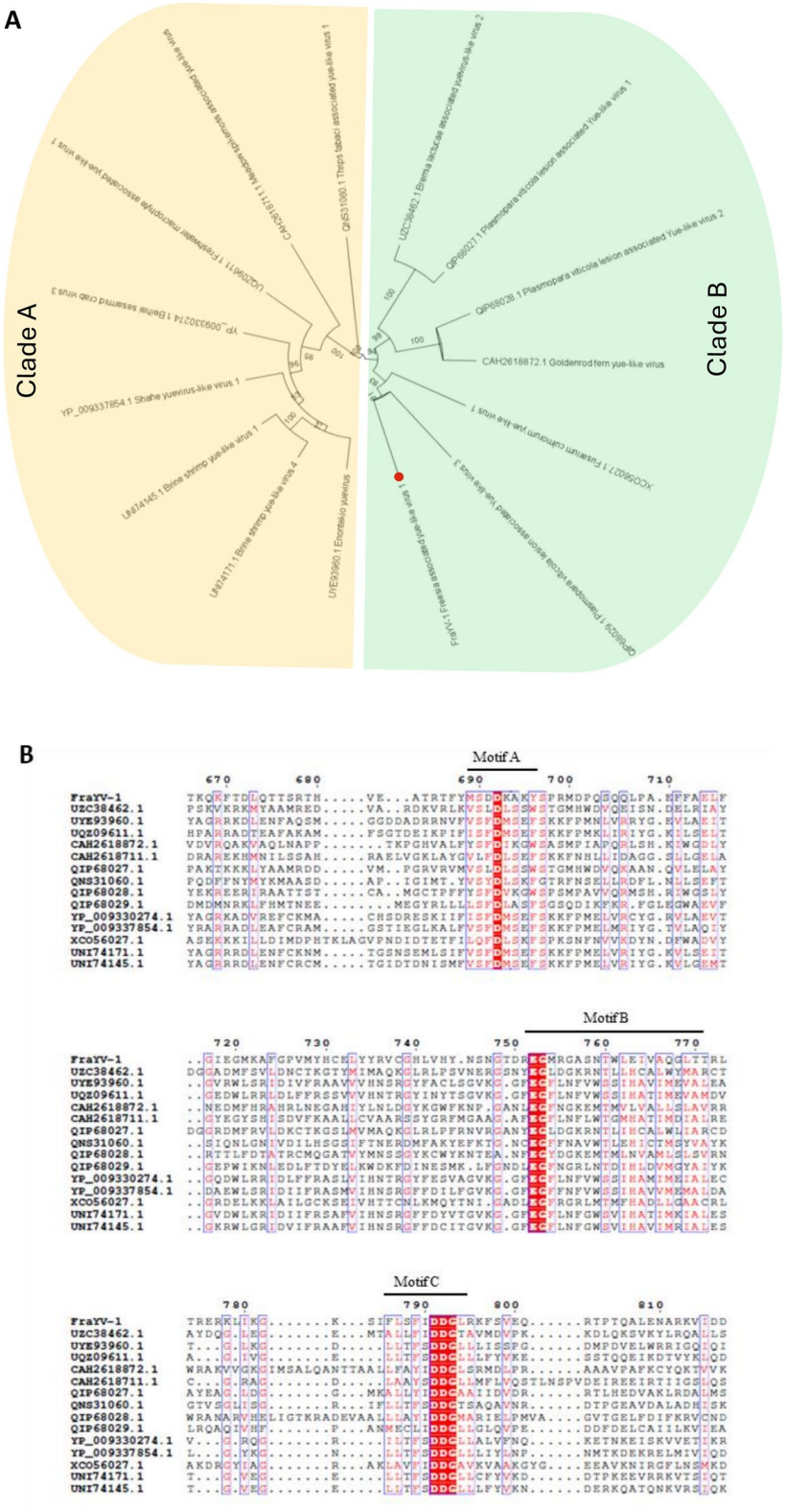

Fig. 5 (**A**) Maximum likelihood phylogenetic tree inferred using IQ-TREE based on the RNA-dependent RNA polymerase amino acid sequences of the members of the *Yueviridae* family according to the ICTV current release (https://ictv.global/; assessed on 14 October 2025). Node values represent ultrafast bootstrap supports (UFBoot) calculated from 1,000 replicates, with values ≥ 70% considered well supported. The best-fit substitution model (Q.PFAM + F + R4) was automatically selected by ModelFinder. The red circle indicates FraYV-1. (**B**) Alignment of the RdRp protein sequences of the members of the *Yueviridae* family

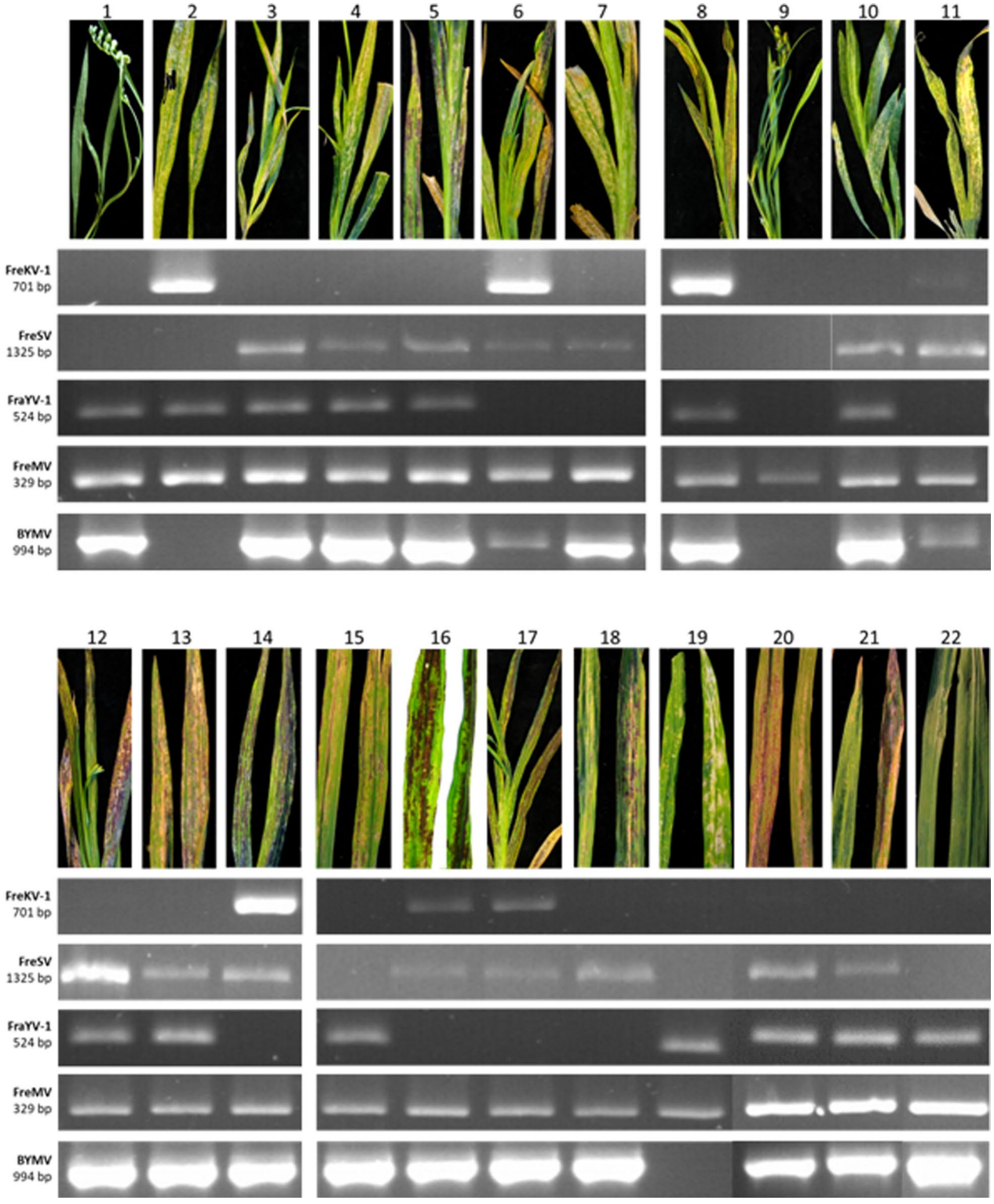

Fig. 6 PCR detection of FreKV-1, FreSV, FreYV-1, FreMV and BYMV in single freesia plants showing various degrees of necrotic disorder symptoms

Fr22, with the exception of samples Fr2, Fr9 and Fr19. Although this virus has been widely reported in freesia, it has been associated mainly with chlorotic leaves, corm necrosis and color breaks in flowers but has never been clearly associated with necrotic disorder. Our results indicate that although both FreMV and BYMV may be widely spread in freesia cultivations, they are not unequivocally correlated with the presence of necrotic symptoms and

therefore confirm that neither FreMV nor BYMV can be considered the etiological agent of the necrotic disorder of freesia. The ophiovirus FreSV, previously proposed as the causal agent but never convincingly associated with the disease, was detected in 15 out of 22 samples, and the newly identified FreKV-1 was detected in 6 out of 22; they were not diagnosed in the necrosis-free sample, nevertheless the results suggest that neither virus appears to be itself the causative agent of necrosis. The presence of a wavy appearance of the foliar edge is prominent in plants positive for FreKV-1 and might be a symptomatology linked to FreKV-1 infection; however, a wider statistical analysis and biological assays are needed to confirm this hypothesis. The newly discovered FraYV-1 was found in 15 out of 22 samples, including the little symptomatic samples Fr1 and Fr9 and the asymptomatic one Fr22, revealing a high percentage of infection in freesia but no clear association with necrotic symptomatology.

Discussion

In this study, the virome associated with symptomatic freesia plants was investigated. The plants exhibited varying degrees of necrosis, a disorder whose pathogenic agent remains largely unidentified. In an effort to identify the etiological agent of the disorder, by using high-throughput sequencing technologies, we identified viral sequences corresponding to two previously uncharacterized viral entities belonging to the *Konkoviridae* and *Yueviridae* families. Data on both families are very limited since they were recently ratified by the ICTV and, to date, are represented by only few members. Genomic and phylogenetic analyses revealed that FreKV-1 shares the four-segment organization typical of other konkoviruses and can be considered a fourth representative of this family, providing further insight into its genomic architecture, evolutionary relationships, and potential host range. An intriguing feature of FreKV-1 is the detection of a defective RNA4 segment encoding a truncated version of the p44 protein. Similar defective RNAs have been reported in several negative-strand RNA viruses and are known to influence replication dynamics, host adaptation, and symptom expression [35]. Future work should explore whether such defective molecules arise commonly in konkoviruses and how they might contribute to persistence or attenuation of disease.

We then leveraged the extensive amount of publicly available metatranscriptomic data made available through the Serratus Project Database [8], specifically related to RdRp palmprints of RNA viruses, to identify potential new members of the *Konkoviridae* family and thereby expand our understanding of this group, which was very recently reported and lists only one official genus with two species. This approach facilitated the reconstruction of genomes (partial or complete coding sequences) for seven putative novel konkoviruses, and the phylogenetic analysis of these sequences suggested the possible existence of at least three distinct genera within the family. The identification of novel konkoviruses in public datasets also broadened the host range of konkoviruses, suggesting their presence in a variety of plant species belonging to phylogenetically distant plant families, both monocots and dicots, among which valuable ornamental plants are listed.

The HTS approach also enabled the identification of a novel virus, provisionally named FraYV-1, with significant sequence similarity to members of the *Yueviridae* family. It is worth noting that FraYV-1 is the first report of a yue-like virus infecting the clade of Angiospermae, thus expanding the already very broad host range of this group of viruses. Phylogenetic analysis and conserved motif examination of the RdRps suggested that this family, which currently includes only two recognized members, may warrant future reevaluation of distinct clades, possibly representing separate genera, one comprising the two previously classified yueviruses and the other including yue-like viruses that infect oomycetes and plants, such as FraYV-1. The identification of additional yue-like viral sequences together with a better understanding of the biology of these viruses could help in deciphering their phylogenetic relationships.

The application of HTS once again underscored the complexity and underexplored nature of viral biodiversity. While this approach has been proven to be highly effective, it does not lead to definitively identifying the etiological agent of the necrotic disorder in freesia, likely because the symptoms are not solely attributable to a single pathogenic agent but rather are the result of the interplay of various biotic and abiotic factors that remain to be fully elucidated. Further experiments such as inoculation or co-infection trials in controlled conditions, although challenging to implement, would be crucial to provide critical insights into the causal role of the identified viruses in the development of the necrotic disorder.

Conclusions

In summary, this work focused on the complex characterization of the virome associated with the freesia affected by the necrotic disorder, resulting in an interesting analysis of unknown viruses. Through a combined wet-lab and *in silico* approach, we improved the current knowledge of the diversity of viruses infecting freesia and contributed to expanding and clarifying the phylogenesis of the *Konkoviridae* and *Yueviridae* families, two clades that were ratified very recently and whose biodiversity is still largely underestimated. These results highlight the importance of integrating HTS with open-science databases for uncovering novel plant viruses.

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s12985-026-03075-8.

Supplementary Material 1

Supplementary Material 3

Supplementary Material 2

Supplementary Material 6

Supplementary Material 5

Supplementary Material 4

Supplementary Material 9

Supplementary Material 8

Supplementary Material 10

Supplementary Material 7

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Author contributions

LM, AMV, and JH conceptualized and supervised the study. LM and AMV acquired funding and resources. MM, SR, FF, RP, LM, and AMV performed experiments. AMV and LM developed and provided specific methodologies. SR, LM, and AMV were responsible for data curation. LM, SR, MM, FF, RP, AMV, and JH analysed and validated data. LM and AMV wrote the first draft of the manuscript. All authors have contributed to reviewing and editing the manuscript and agree on the final version of this study.

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Data availability

The raw reads of HTS related to this work are deposited in the Sequence Read Archive (SRA) of NCBI (https://www.ncbi.nlm.nih.gov/sra) with BioProject ID PRJNA1014396 and BioProject ID PRJNA1189045.The genomic sequences of known and new viruses identified in this study are deposited at NCBI with the following accession numbers: PV159347 (FreMV), PV171508 (FraYV-1), PQ490803 (FreKV-1, RNA1), PQ490804 (FreKV-1, RNA2), PQ490805 (FreKV-1, RNA3), PQ490806 (FreKV-1, RNA4). GenBank accession numbers of putative new konkovirus sequences identified in this study are reported in Table 5.

Declarations

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Competing interests

The authors declare no competing interests.

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