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Virology

A Polyvalent Tool for Detecting Coguviruses in Multiple Hosts Allowed the Identification of a Novel Seed-Transmitted Coguvirus Infecting *Brassicaceae*

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Abstract

The genus *Coguvirus*, a recently established genus in the family *Phenuiviridae*, includes several species whose members infect both woody and herbaceous hosts, suggesting a broader host range and wider distribution than previously. To gain insights into the epidemiology and biology of coguviruses, a polyvalent reverse transcription-PCR assay using degenerate primers was developed. The specificity of the assay for coguviruses was confirmed by testing citrus and apple plants infected by previously reported coguviruses and/or several unrelated viruses. The expected 236-bp amplicon was obtained from citrus, apple, pear, watermelon, and several species of the family *Brassicaceae*. Sequencing of the PCR amplicons allowed the identification, for the first time in Italy and/or Europe, of several coguviruses in multiple hosts, confirming the effectiveness of the assay. Moreover, a new virus, tentatively named Brassica oleracea Torzella virus 1 (BoTV1), was detected in several plants of Torzella cabbage. The complete +genome of BoTV1, determined by high-throughput sequencing and 5′ rapid amplification of cDNA ends, revealed that it has the typical molecular features of coguviruses and fulfils the current criteria to be classified as a member of a new species, for which the tentative name *Coguvirus torzellae* is proposed. The same polyvalent assay was also used to investigate and confirm that BoTV1 is transmitted through seeds in black cabbage, thus providing the first evidence on the relevance of this natural transmission mode in the epidemiology of coguviruses.

Keywords: microbe-genome sequencing, molecular, pathogen detection, techniques, virology

The genus *Coguvirus* of the family *Phenuiviridae* (order *Bunyavirales*) was created in 2019 (Abudurexiti et al. 2019) to classify citrus concave gum-associated virus (CCGaV, species *Coguvirus citri*) and citrus virus A (CiVA, species *Coguvirus eburi*). These viruses were initially discovered in citrus hosts by using high-throughput sequencing approaches (Navarro et al. 2018a, b). CCGaV was closely associated with concave gum, an old citrus graft-transmissible virus-like disease (Fawcett 1936) whose etiological agent had remained unidentified for over 80 years. Since 2018, CCGaV has been reported to naturally infect apple in several areas of the world where it seems not to be associated with any disease (Diaz-Lara et al. 2022; Minutolo et al. 2021; Wright et al. 2018). Shortly after CCGaV discovery, CiVA was identified in citrus in southern Italy, although it was not associated with any noticeable disease symptoms (Navarro et al. 2018b). Later, CiVA was also detected in Greece (Beris et al. 2021) and South Africa (de Bruyn et al. 2022), where it was reported to be associated with symptoms of citrus impietratura in sweet orange (*Citrus sinensis* L.) and/or grapefruit (*Citrus paradisi* Macfad.), respectively. Impietratura is a disease that induces spotting and gumming in the rind and albedo of citrus fruits and has been known for a long time (Reichert and Hellinger 1930; Ruggieri 1955). CiVA has also been reported in asymptomatic pears (*Pyrus communis* L.) in France (Svanella-Dumas et al. 2019) and South Africa (Bougard et al. 2022). The same virus was identified in sweet orange trees in Australia, although the authors highlighted the need further research to conclusively define symptoms, if any, associated with CiVA infections in the field (Donovan et al. 2022). Two additional viruses, watermelon crinkle leaf-associated virus 1 and 2 (WCLaV1 and WCLaV2), infecting watermelon plants showing symptoms of leaf yellow mottling, chlorosis, and wrinkling, were initially found in China (Xin et al. 2017; Zhang et al. 2021) and recently classified within the genus *Coguvirus* as representative members of two new species (*Coguvirus citrulli* and *C. henanense*). Soon after, they were reported in watermelon plants cultivated in several locations of the United States (Texas, Georgia, and Florida) (Adeleke et al. 2022; Hendricks et al. 2022; Hernandez et al. 2021), as well as in Brazil (Maeda et al. 2022). In 2021, WCLaV1 was also identified in cantaloupe (*Cucumis melo* var. *cantalupensis* Naudin) grown in Georgia and showing yellow mottling and chlorosis of leaves (Adeleke et al. 2022). In 2023, they were reported, always associated with leaf crinkling, chlorosis, and/or mosaic, in several other hosts, including straightneck squash (*Cucurbita pepo* var. *recticollis*) (Jailani et al. 2023) and in zucchini (*Cucurbita pepo*) (Iriarte et al. 2023) in the United States and watermelon in Australia (Mulholland et al. 2023). Finally, three novel coguviruses, Brassica campestris chinensis coguvirus 1 (BCCoV1; Tang et al. 2021; species *Coguvirus chinense*), Yunnan Paris negative-stranded virus (Chen et al. 2020; species *Coguvirus yunnanense*), and Edgeworthia chrysantha mosaic-associated virus (Wang et al. 2022; tentative species *Coguvirus chrysanthae*, https://ictv.global/files/proposals/pending?fid=13580#block-teamplus-page-title) were identified in *Brassica campestris* L. subsp. *chinensis*, *Paris polyphylla* Sm., and the ornamental plant *Edgeworthia chrysantha* Lindl., respectively.

With the exception of grapevine-associated cogu-like virus 1 (Chiapello et al. 2020), whose classification is currently un-

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der revision (ICTV proposal 2023.007M.A.v1.Coguvirus_1nsp.docx, https://ictv.global/files/proposals/pending?fid=13580#block-teamplus-page-title), coguviruses have a bipartite genome composed of a negative-strand RNA1, encoding the viral RNA-dependent RNA polymerase (RdRp), and an ambisense RNA2, coding for the putative movement protein (MP) and the nucleocapsid protein (NP), with the two open reading frames (ORFs) separated by a self-complementary intergenic region (IR) that assumes a hairpin conformation (Chen et al. 2020; Navarro et al. 2018a, b; Tang et al. 2021; Wang et al. 2022; Zhang et al. 2021).

The discovery of several new coguviruses infecting various host species since the first identification suggests that these viruses may have a broader host range than initially believed. Furthermore, they may have been largely overlooked in several crops so far. Leveraging the growing collection of data on coguvirus genomes available in databases, we have designed degenerate primers targeting a conserved region of the coguvirus gene encoding the RdRp. They were used to develop a simple and cost-efficient polyvalent reverse transcription (RT)-PCR assay capable of detecting coguviruses at the genus level. This method, employed for screening many potential hosts, not only contributes to a more comprehensive understanding of the host range of several already known coguviruses but has also allowed the detection of two coguviruses, BCCoV1 and WCLaV2, for the first time in Italy. Additionally, it allowed the discovery of a new coguvirus infecting *Brassica oleracea* L., tentatively named Brassica oleracea Torzella virus 1 (BoTV1). This work reports the molecular and biological features of this new virus, including its transmission through seeds, as well as data supporting its classification as a novel species within the genus *Coguvirus*.

Materials and Methods

Plant material

Leaves from one CCGaV- and one CiVA-infected sweet orange, one CiVA-infected mandarin, and one CCGaV-infected apple tree were used as coguvirus-positive controls. Leaves of three sweet orange trees singly infected with citrus tristeza virus, citrus vein enation virus, or citrus psorosis virus; leaves of one apple tree simultaneously infected with apple rubbery wood virus 1, apple chlorotic leaf spot virus, apple stem pitting virus, and apple stem growing virus; and leaves of sweet orange, mandarin, and apple trees not infected by the viruses reported above were used as negative controls. A total of 539 samples belonging to 62 species and 24 families (Supplementary Table S1) were collected in 2021 and 2022, regardless of the disease status of the plant, from various areas of Campania region (Southern Italy). The plant species selected covered some of the main crops cultivated in this region. The samples were stored at 4°C until they were used for RNA preparation and RT-PCR assays.

Seeds of 19 plant species belonging to the families *Amaranthaceae*, *Apiaceae*, *Asteraceae*, *Brassicaceae*, *Chenopodiaceae*, *Cucurbitaceae*, and *Ranunculaceae* were purchased in 2023 from various garden centers in Campania (Southern Italy). These seeds were sown in 14-cm-diameter pots containing a sterile mixture (1:1, vol/vol) of volcanic sandy soil and commercial substrate (Type S). Additionally, 30 seeds of black cabbage were sown in 10-cm-diameter Petri dishes, on moistened sterile filter papers. Both pots and Petri dishes were placed in a thermo-conditioned greenhouse under natural light conditions. Fifteen days post germination, the seedlings of each host species were visually inspected for symptoms, and leaf samples were then collected for RNA extraction.

Design of degenerate primers, RT-PCR, and 5′ rapid amplification of cDNA ends (RACE)

The coding sequences of the RdRp genes of classified and putative coguviruses were retrieved from the GenBank database, aligned using Clustal Omega (Madeira et al. 2022), and a highly conserved region was identified and used for designing degenerate primers Cogu_RNA1_1For, Cogu_RNA1_2Rev, and Cogu_RNA1_3For (Supplementary Table S2). The combination of Cogu_RNA1_1For and Cogu_RNA1_3For with Cogu_RNA1_2Rev in an RT-PCR assay was expected to amplify a cDNA of 236 and 227 nucleotides (nt), respectively.

For polyvalent RT-PCR with degenerate primers, total nucleic acids (100 ng) extracted from fresh leaves (200 mg) were reverse transcribed as reported previously (Minutolo et al. 2020). The optimal conditions of the polyvalent PCR were determined by testing different annealing temperatures (in a gradient ranging from 50 to 60°C) and $MgCl_2$ and primer concentrations (ranging from 0.25 to 2.5 mM and from 0.2 to 0.4 mM, respectively). The PCR amplification assay was set up as follows: 2 µl of the cDNA reaction and 1.0 unit of GoTaq polymerase (Promega, Madison, WI, U.S.A.) in a reaction volume of 25 µl, containing a final concentration of 0.3 mM of each primer and 1.5 mM $MgCl_2$. The cycling conditions were as follows: initial denaturation at 94°C for 3 min, followed by 32 cycles at 94°C for 30 s, 53°C for 30 s, 72°C for 30 s, and a final extension step at 72°C for 7 min. The reaction products were analyzed by electrophoresis on 1.5% agarose gels buffered in TAE (0.04 M Tris-acetate, 1 mM EDTA, pH 8) and visualized by UV light after ethidium bromide staining under a gel documentation system (Vilber, Collégien, France). The RT-PCR products of the expected size were excised, and their nucleotide sequences were determined via Sanger sequencing (Eurofins, Ebersberg, Germany). The identity of amplified sequences was ascertained by BlastN analysis of the sequenced cDNAs (Altschul et al. 1990). The same procedure was applied for the RT-PCR assays performed using specific primers (Supplementary Table S2), except for the final concentration of primers (0.2 mM) and the annealing temperature, which was always selected according to the specific primer pair.

High-throughput sequencing and bioinformatic analysis

The total RNA from leaves of a symptomless plant of Torzella cabbage (*Brassica oleracea* L. var. *acephala*) grown in Campania (Southern Italy) was obtained with a plant/fungi total RNA purification kit (Norgen Biotek, Ontario, Canada), and, after a DNase digestion with DNase turbo (Invitrogen, San Diego, CA, U.S.A.) and ribosomal RNA depletion with Ribo-Zero Plant (Illumina, San Diego, CA, U.S.A.), it was used for the generation of a cDNA library with the TruSeq stranded total RNA kit (Illumina). The cDNA library was pair-end sequenced (2 × 150) in a NovaSeq 6000 sequence system (Illumina). Raw sequenced reads were filtered for quality with the FASTX-Toolkit (http://hannonlab.cshl.edu/fastx_toolkit/) and processed to remove ambiguities, bases with a Q score less than 20, Illumina adapters, and sequences with a length shorter than 50 bp with cutadapt v 1.8 (Martin 2011). Reads selected with quality filtering were assembled using SPAdes software (version 3.15.5) (Bankevich et al. 2012) using a multiple K-mer approach (from 31 to 111 bp by 20). The resulting contigs were compared against the NCBI database using BlastN and BlastX to identify homologous viral sequences. The termini of the viral genomic RNAs were determined by 5′ rapid amplification of cDNA ends (RACE). Briefly, total RNA was reverse transcribed using a specific primer (Supplementary Table S2), and, after the addition of a poly(dG) tail, the tailed cDNAs were PCR amplified with the primer PolyGTail5RACE (5′-CGCGGATCCCCCCCCCCC) and a specific primer nested to that used for the cDNA synthesis (Supplementary Table S2).

Sequence and phylogenetic analysis

The potential ORFs and conserved protein domains were predicted by ORFfinder at NCBI and Pfam analysis (Finn et al. 2016), respectively.

The RdRp phylogenetic analysis was performed using the conserved core amino acid sequence in the proteins encoded by representative members of all the genera in the family *Phenuiviridae* and some recently reported putative coguviruses. Multiple alignments were performed with Clustal Omega (Madeira et al. 2022) and used for generating a phylogenetic tree inferred by the maximum-

likelihood method (1,000 bootstrap replicates) based on the protein best-fit model LG+F+G+I. The phylogenetic analysis was implemented in MEGAX (Kumar et al. 2018).

Results

Detection of coguviruses by RT-PCR using degenerated primers

To develop a polyvalent RT-PCR detection method for coguviruses, a highly conserved region was identified within the RdRp gene of the coguviruses known at the beginning of this study, and degenerated primers were designed (Fig. 1; Supplementary Table S2).

These primers were used to perform RT-PCR assays on RNA extracts from several host species infected with CCGaV and CiVA, including one apple and one sweet orange tree infected by CCGaV (isolates CE-c and CGW2, respectively) and one sweet orange (isolate W4) and one mandarin (isolate HE1) infected by CiVA, identified in previous studies (Minutolo et al. 2020, 2021; Navarro et al. 2018a, b). RNA preparations from several negative controls of the same plant species were included in these preliminary assays. The degenerated primers Cogu_RNA1_1For/Cogu_RNA1_2Rev were effective to detect CCGaV and CiVA in all the respective infected samples, as shown by the amplification and sequencing of the expected cDNA fragment of 236 bp (Fig. 2).

The RT-PCR amplicons obtained from isolates CE-c3 and CGW2 displayed 100% sequence identity with the previously determined CCGaV sequences (GenBank accession numbers OK495690 and KX960112, respectively) from the same isolates (Minutolo et al. 2021; Navarro et al. 2018a). RT-PCR amplicons from isolates HE1 and W4 showed 99.7 and 100% sequence identity with the CiVA sequence of isolate W4 (accession number MG764565; Navarro et al. 2018b). Therefore, the degenerated primers were able to detect CCGaV and CiVA in different host species. No amplicons were generated when RNA extracted from negative controls was tested (Fig. 2). In this assay, negative controls (CCGaV- and CiVA-free) also included three sweet orange trees singly infected by citrus tristeza virus, citrus vein enation virus, or citrus psorosis virus and one apple tree simultaneously infected with apple rubbery wood virus 1, apple chlorotic leaf spot virus, apple stem pitting virus, and apple stem growing virus (Fig. 2). Altogether, these results showed the absence of cross-reactivity of the degenerate primers with RNAs from the host plants and other viruses, thus encouraging the use of the same assay to perform field surveys. The combination of the Cogu_RNA1_2Rev primer with another forward primer designed in a slightly downstream region (Cogu_RNA1_3For; Fig. 1) was similarly effective in amplifying cDNAs of the expected size. Only the primer pair Cogu_RNA1_1For/Cogu_RNA1_2Rev was selected for further studies.

Field survey of coguviruses in several host species

A total of 539 samples belonging to 24 families and 61 species (Supplementary Table S1) were collected during 2021 and 2022 from fields of different crops in the Campania region (Southern Italy) and tested using the developed polyvalent RT-PCR assay. Samples of cabbage (*Brassica oleracea* L.), turnip (*Brassica rapa* L.), and pear (*Pyrus communis* L.) tested positive in the assay (Table 1; Supplementary Table S1). Specifically, among the 18 tested samples of Torzella cabbage (*Brassica oleracea* L. var. *acephala*) 15 (83%) tested positive, whereas the incidence in other *Brassicaceae*, including Catozza turnip (*Brassica rapa* L. var. *rapa* DC) and Friariello turnip (*Brassica rapa* L. subsp. *sylvestris* var. *esculenta*), ranged from 22 to 36% (Table 1). Only 1 out of 54 pear trees generated the expected amplicon (Table 1). All other tested species, including various vegetables and stone and pome fruit trees, tested negative (Supplementary Table S1).

Sequencing of amplified cDNAs, followed by BlastN searches in databases, showed that RT-PCR products from the positive Friariello and Catozza turnips displayed high similarity to BCCoV1,

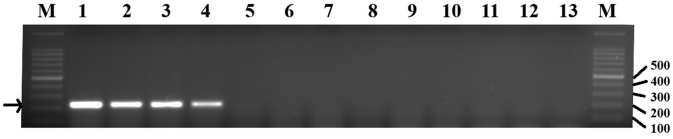

Fig. 2. Reverse transcription-PCR assay performed with degenerate primers Cogu_RNA1_1For and Cogu_RNA1_2Rev, designed for the detection of coguviruses. The expected amplified cDNA of about 236 nt, corresponding to a fragment of the coguvirus RNA-dependent RNA polymerase gene, is indicated by the arrow on the left. Lane M, 100-bp DNA ladder (Promega, Madison, WI, U.S.A.) with fragment sizes (bp) reported on the right; lane 1, sweet orange infected by citrus concave gum-associated virus (CCGaV); lane 2, apple infected by CCGaV; lane 3, sweet orange infected by citrus virus A (CiVA); lane 4, mandarin infected by CiVA; lanes 5 to 13, negative controls: lanes 5 to 7, sweet orange, mandarin, and apple trees not infected by citrus viruses; lanes 8 to 10, sweet orange trees infected by citrus vein enation virus, citrus tristeza virus, and citrus psorosis virus, respectively; lane 11, apple tree infected by apple chlorotic leaf spot virus, apple rubbery wood virus 1, apple stem growing virus, and apple stem pitting virus; and lanes 12 and 13, water controls.

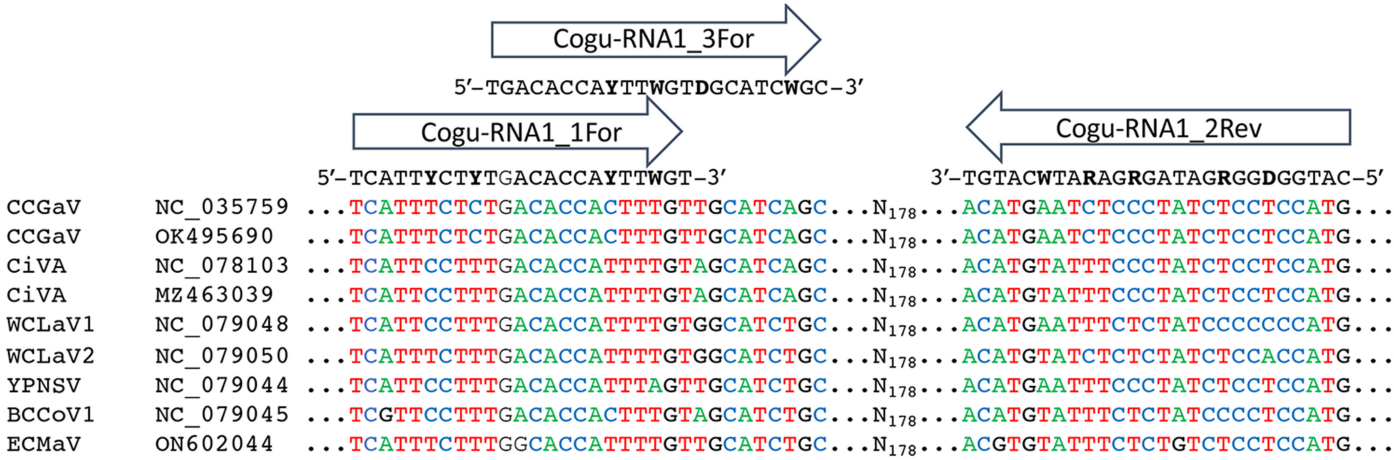

Fig. 1. Multiple sequence alignment of the RNA-dependent RNA polymerase nucleotide sequences showing the conserved regions among several coguviruses used for designing the degenerated primers. Based on the sequence alignment, two forward primers (Cogu_RNA1_1For, TCATTYCTYTGACACCAYTTWGT, and Cogu_RNA1_3For, TGACACCAYTTWGTDGCATCWGC) and a reverse primer (Cogu_RNA1_2Rev, CATGGDGGRGATAGRGARATWCATGT) were designed. The degenerated bases were R for A or G, D for A or G or T, Y for C or T, and W for A or T.

with nucleotide identity ranging from 96.26 to 98.4% and 100% query coverage. Conversely, the closest BlastN hit of the amplicon from the pear tree was the previously reported CiVA isolate (97.86%, query coverage 100%) (Table 1). Altogether, these data indicate that the designed primers successfully detected various coguviruses previously described in both woody and herbaceous hosts. Additionally, this study marks the first-time detection of BCCoV1 in Italy and is also the first report of this virus in Europe. Moreover, CiVA was identified for the first time in a pear tree in Italy.

It is worth noting that the amplicon sequence from the Torzella cabbage showed the highest similarity with BCCoV1 from China (accession number MW291944). However, in this case, the sequence identity with this virus was lower (81.6 to 82.2%, coverage 97%) than in the Friariello and Catozza turnip plants (Table 1), suggesting that the detected virus in Torzella cabbage might differ from BCCoV1. This possibility gained further support from the negative results of RT-PCR assays performed on the same samples using the primer pair CoVMNF/CoVMNR (Supplementary Table S2) designed by Tang et al. (2021) to specifically detect RNA2 of BCCoV1 (Supplementary Fig. S1). Therefore, the potential presence of a novel coguvirus infecting Torzella cabbage plants was considered and further investigated.

Molecular characterization of a novel coguvirus infecting Torzella cabbage

To obtain the complete genome sequence of the putative novel coguvirus infecting Torzella cabbage, a cDNA library from one of the cabbage plants that tested positive in the polyvalent RT-PCR assay was generated and sequenced by high-throughput sequencing. A total of 45,251,065 high-quality paired-end reads were assembled by SPAdes in 76,150 contigs. BlastN analysis of these assembled contigs identified two coguvirus contigs, NODE_147 of 6,764 nt (51.8× coverage) and NODE_1721 of 3,084 nt (114.4× coverage). These contigs share nucleotide identities of 79 and 78% with the RNA1 (ID: MW291944.1) and RNA2 (MW291945.1) of BCCoV1 isolate DC303, respectively. Additional RACE analyses allowed sequencing the complete genome of a putative new virus that was provisionally called Brassica oleracea Torzella virus 1 (BoTV1). This virus has a bipartite genome consisting of a negative-strand RNA1, 6,765 nt long (accession number OR513471), and an ambisense RNA2, with a size of 3,089 nt (accession number OR513472) (Fig. 3A). The 5′ and 3′ most terminal 20 to 21 nt of BoTV1 RNA1 and RNA2 are almost identical to each other and to those found in all previously described coguviruses (Fig. 3B), thus allowing for the formation of a panhandle structure reported in genomic RNAs of the other negative-strand RNA viruses and likely involved in the virus replication (Bouloy 2011).

BoTV1 RNA1 codes for a putative protein (2,200 amino acids [aa], 251.98 kDa) that shares the highest amino acid identity (88.5%) with the RdRp of BCCoV1 and contains the RdRp conserved motifs (premotif A and motifs A through E) found in other negative-strand RNA viruses, including coguviruses (Supplementary Fig. S2). A putative cap-snatching motif (H_{70} $D_{82}PD_{99-100}$ $ExA_{111-113}$ K_{130}), reported to be involved in the genome expression strategy of segmented nsRNA viruses of the order *Bunyavirales* and family *Orthomyxoviridae* (Decroly et al. 2012; Reguera et al. 2010), is present at the N-terminal region of this protein. Similar to BCCoV1, it contains an ExA motif instead of the ExG motif, typical of all other coguviruses, or the ExK motif typical of most bunyaviruses (Supplementary Fig. S3). The amino acid doublet RY, also conserved in most bunyaviruses' RdRp (Müller et al. 1994), was mapped at positions 601 to 602. These features support the conclusion that the protein encoded by BoTV1 RNA1 is very likely the RdRp of this virus.

Similar to other coguviruses, BoTV1 RNA2 contains two ORFs, one in each polarity strand, separated by an intergenic region of 99 nt that adopts a stem loop secondary structure in both polarity strands (Fig. 4). This hairpin structure resembles the hairpin (92-nt-long) formed in the RNA2 IR of BCCoV1, another coguvirus infecting *Brassicaceae*. In both BoTV1 and BCCoV1, such a hairpin occupies almost the entire IR sequence and is composed of 55% adenine-uracil (AU) base pairs. These IRs differ from those of coguviruses infecting other host species, such as CCGaV, CiVA, WCLaV1, WCLaV2, Edgeworthia chrysantha mosaic-associated virus, and Yunnan Paris negative-stranded virus, where only a portion of a longer (334 to 510 nt) IR forms the hairpin that ranges in size from 136 to 166 nt and is rich in AU (68.7 to 76.4%) in its central and basal part (Fig. 4). Although a terminal transcription signal resembling the one previously reported in several phleboviruses (Albariño et al. 2007) was observed in most coguviruses, such a motif was not found in the hairpin formed by the RNA2 of both BoTV1 and BCCoV1.

ORF2a in the viral strand of RNA2 encodes a putative protein (416 aa, 46.72 kDa) with the highest identity (82.89%) with the predicted putative MP of BCCoV1. The putative protein of 543 aa (58.63 kDa) encoded by ORF2b in the viral complementary strand of BoTV1 shares 62.92% identity with the putative NP of BCCoV1. Notably, in both BoTV1 and BCCoV1, the N-terminal end of this protein contains an additional 180 aa compared with the NP encoded by all other known coguviruses infecting hosts belonging to different plant families (*Rutaceae*, *Roseaceae*, *Cucurbitaceae*, *Melanthiaceae*, and *Thymelaeaceae*).

BoTV1 is a new species in the genus *Coguvirus*

The availability of the complete genome sequence of BoTV1 allowed further studies aimed at investigating its phylogenetic relationships with other coguviruses and negative-strand RNA viruses. In a maximum-likelihood phylogenetic tree generated with the RdRp core region of BoTV1, all other coguviruses and representative members of the genera in the family *Phenuiviridae*, BoTV1 clusters within the same clade as the other coguviruses. high bootstrap values provide robust statistical support to this clustering (Fig. 5).

TABLE 1. Positive samples assayed by polyvalent reverse transcription-PCR for detecting coguviruses from field plants

Host family	Host species	Host common name	Number of tested plants	Number of positive plants	Sequenced virus isolate(s)	BLAST best hit[a]	Nucleotide identity (%)[b]
Brassicaceae	*Brassica oleracea* L. var. *acephala*	Torzella cabbage	18	15	TC-1, TC-2, TC-3	BCCoV1 NC_079045	81.97
Brassicaceae	*Brassica rapa* L. var. *rapa* DC	Catozza turnip	11	4	T-1, T-2	BCCoV1 NC_079045	96.26–97.33
Brassicaceae	*Brassica rapa* L. subsp. *sylvestris* var. *esculenta*	Friariello turnip	18	4	FR-1, FR-2, FR-3	BCCoV1 NC_079045	97.86–98.4
Rosaceae	*Pyrus communis* L.	Pear	54	1	P-1	CiVA OM272943	97.86
	Total		101	24			

[a] Best BLAST hit of the sequenced amplicon; BCCoV1, Brassica campestris coguvirus 1; and CiVA, citrus virus A.
[b] Nucleotide identity between the amplicon and the BLAST best hit.

The RdRp amino acid sequence identity of BoTV1 with the closest coguvirus BCCoV1 is 88.5% (Supplementary Table S3). This value falls below the threshold of 95%, which is currently used as the identity threshold demarcating species within the genus *Coguvirus* (Sasaya et al. 2023). Altogether, these data strongly support the conclusion that BoTV1 represents a new coguvirus that should be classified as a new species, for which the name *Coguvirus torzellae* is proposed.

Survey of coguviruses in seedlings from commercial seeds using the polyvalent RT-PCR

As coguviruses represent a group of relatively recently discovered infectious agents, their epidemiology remains largely unknown. Considering their relatively high incidence in vegetables, which are generally sown, and leveraging the polyvalent RT-PCR detection method developed in this study for coguviruses, we investigated the possibility of some coguviruses being transmitted through seeds. To this aim, seedlings germinated in soil from commercial seeds of plant species of the families *Asteraceae*, *Brassicaceae*, *Chenopodiaceae*, *Cucurbitaceae*, and *Ranunculaceae* were tested. In a first assay, leaf samples were collected from four seedlings of each host species 15 days post germination and pooled to extract RNA for testing. For each species, between two and seven pooled RNA samples were prepared, generating a total of 89 samples that were assayed by the polyvalent RT-PCR (Supplementary Table S4). Amplicons of the expected size were exclusively obtained from samples of some *Brassicaceae* and *Cucurbitaceae* species. Specifically, all mixed samples from seedlings of black cabbage (*Brassica oleracea* L. var. *acephala sabellica*) and watermelon (*Citrullus lanatus* Thunb. 'Crimson sweet' and 'Sugar Baby'), as well as some mixed samples of Catozza and Friariello turnip plants and of Black head 2 red cabbage (*Brassica oleracea* L. var. *capitata* f. *rubra*), tested positive (Supplementary Table S4). These findings suggest the presence of at least one coguvirus in the tested seedlings and are consistent with the hypothesis that these infecting coguviruses may be seed-transmitted or soilborne pathogens.

To assess virus prevalence in the seedlings and to gain further insights into the infecting coguviruses, seeds from the same seed lots of host species that tested positive in the initial assay were resown. In contrast to the previous experiment, RNA extracts from each germinated seedling were individually tested by the polyvalent RT-PCR assay. In *Brassicaceae* seedlings, the infection rate was relatively high, ranging from 28.6 (in Black head 2 red cabbage) to 73.3% (in the Torzella cabbage), with the exception of Catozza turnip, which displayed a lower infection rate of only 0.04% (Table 2). Similarly, the infection rates among the tested watermelon varieties were also relatively high, ranging from 46.1 to 57.8% (Table 2).

Sequencing of representative amplicons from each tested species revealed that, besides Torzella cabbage, black cabbage and Black head 2 red cabbage are also natural hosts of the newly reported BoTV1 (Table 2). Moreover, we confirmed the presence of BCCoV1 in several turnip varieties, including some local Italian varieties such as Friariello and Catozza (Table 2). Amplicons from Sugar Baby and Tonda F1 watermelon showed 100% identity with WCLaV1 and WCLaV2, respectively. To the best of our knowledge, these viruses were identified for the first time in Italy and in Europe in this study. No symptoms were observed in the tested samples, although it cannot be ruled out that the young developmental stage of the tested plants might be incompatible with symptom expression in the infected hosts. However, in the case of 10 Torzella cabbage plants infected by BoTV1, no symptoms were observed during 6 months of observation in the greenhouse.

To further discriminate whether coguviruses are transmitted through seeds or are soilborne, 30 seeds of black cabbage, from the same commercial lot that previously tested positive for BoTV1, were sown in sterilized soil or on layers of moistened sterile filter papers. In three independent trials, similar germination rates (ranging from 86.7 to 93.3%) and BoTV1 prevalence (ranging from 65.4 to 67.8%) were recorded in the seedlings, regardless of whether they were germinated in sterile soil or on filtered paper. Altogether, these data strongly support the conclusion that BoTV1 is a seed-transmitted virus.

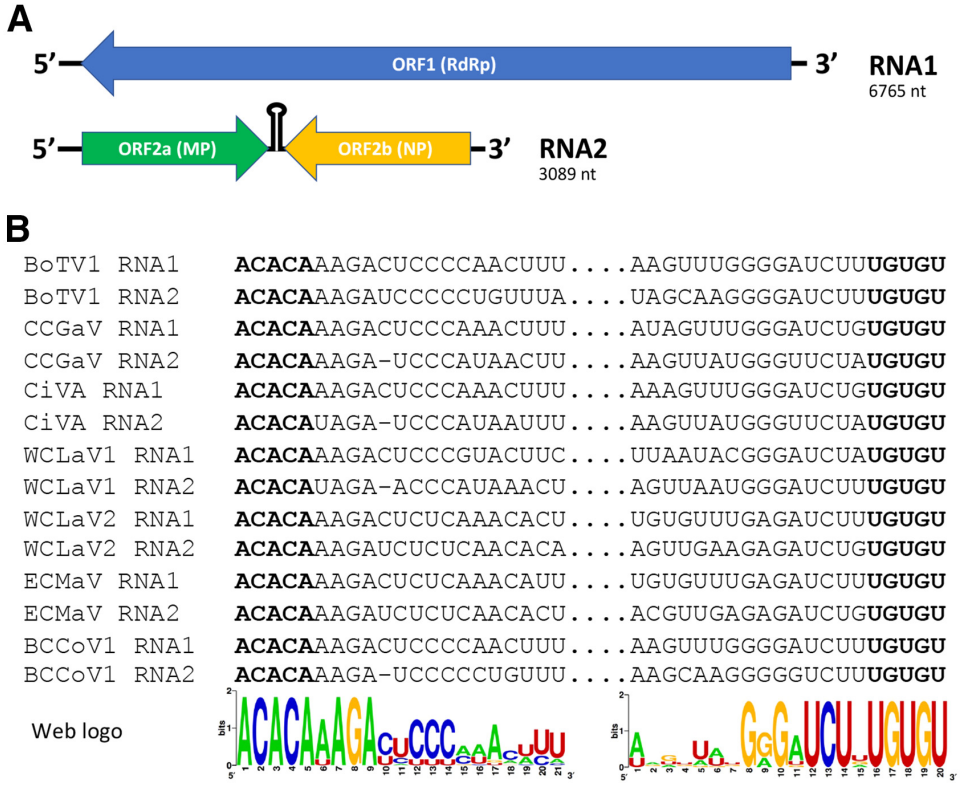

Fig. 3. A, Schematic diagram of the Brassica oleracea Torzella virus 1 (BoTV1) genome. RNA1 contains in the negative polarity the open reading frame (ORF) 1, which encodes the putative RNA-dependent RNA polymerase (RdRp); RNA 2 is bicistronic, with the ORF2a and ORF2b in opposite orientations and separated by an intergenic region forming a hairpin. **B,** Multiple alignments of the 5′ and 3′ terminal ends of coguviruses, with the consensus sequence obtained by WebLogo (https://weblogo.berkeley.edu/logo.cgi), analysis reported at the bottom. Citrus concave gum-associated virus (CCGaV), citrus virus A (CiVA), watermelon crinkle leaf-associated virus 1 and 2 (WCLaV1 and WCLaV2), Edgeworthia chrysantha mosaic-associated virus (ECMaV), and Brassica campestris chinensis coguvirus 1 (BCCoV1).

Discussion

The discovery of CCGaV and the publication of its genome (Navarro et al. 2018a) were almost immediately followed by the identification of six other new coguviruses infecting both woody and herbaceous hosts (Chen et al. 2020; Chiapello et al. 2020; Navarro et al. 2018b; Tang et al. 2021; Wang et al. 2022; Xin et al. 2017; Zhang et al. 2021), thus indicating that coguviruses are likely widely distributed in nature and possibly overlooked in several crops. Here, we have developed a cost-effective and fast polyvalent RT-PCR-based detection method to perform large-scale surveys of coguviruses in several economically relevant woody and herbaceous hosts. This method was initially shown to be effective in detecting CCGaV and CiVA using RNA preparations from both the citrus and pome fruit hosts they may infect. These preliminary tests also confirmed the specificity of the method because no cross-reaction with other viruses, in the presence or absence of co-infecting coguviruses, was observed. In a subsequent step, an extensive survey was carried out on several host species, testing a total of 539 samples. This field survey, combined with sequencing of the amplicons from the hosts testing positive, led to the identification of (i) BCCoV1 in Catozza and Friariello turnips with a relatively high prevalence (26 to 36%), which, to the best of our knowledge, is the first report of this virus in Europe; (ii) CiVA in pear, which was found for the first time on this host in Italy; and (iii) the novel coguvirus BoTV1 infecting Torzella cabbage. All these data support the reliability of the method for detecting both known and previously unreported coguviruses in field surveys addressing both woody and herbaceous plants.

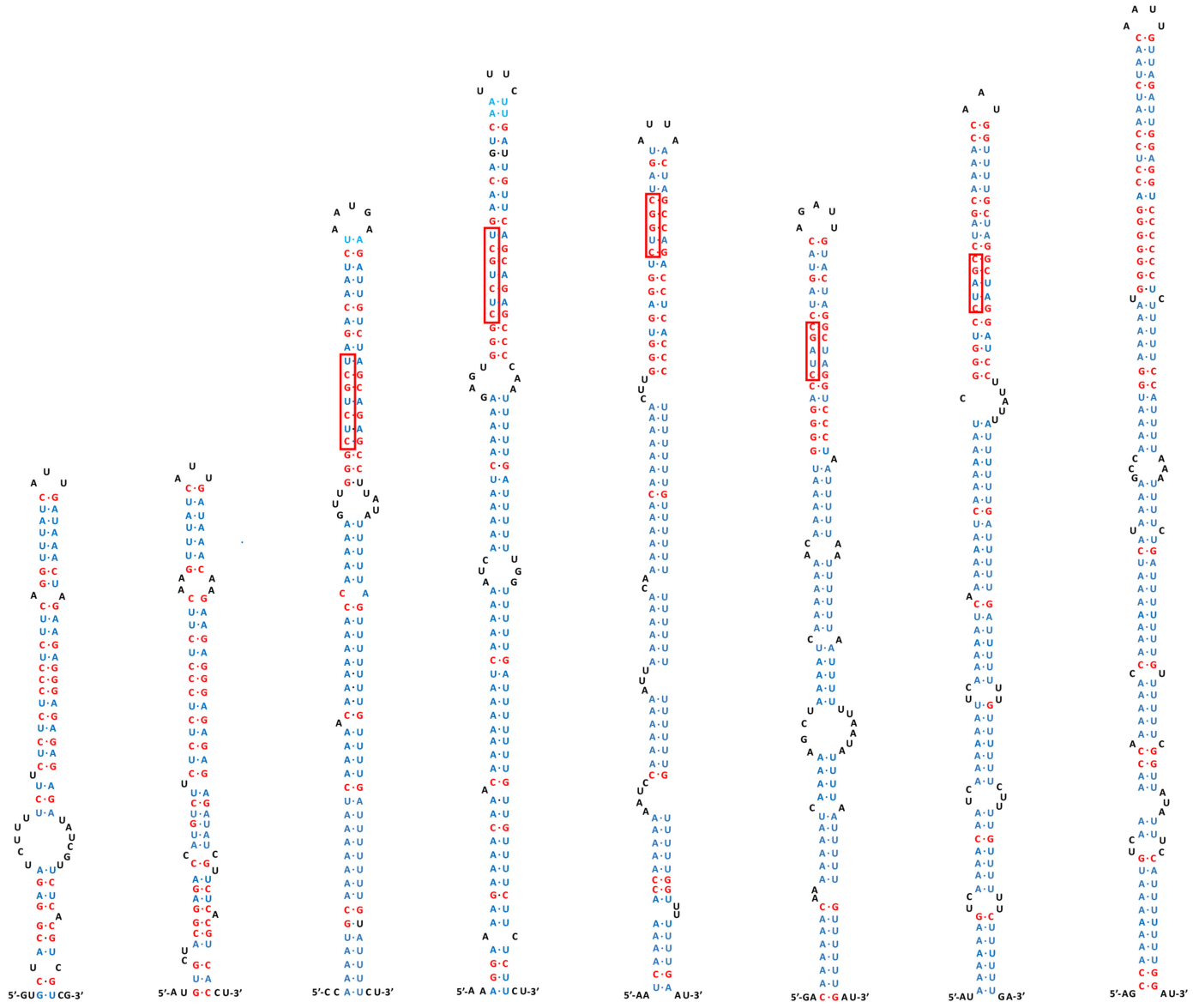

Fig. 4. Hairpin conformation adopted by nucleotide sequences of the intergenic region of RNA2 of coguviruses. The adenine-uracil (AU) content (%) of each hairpin is reported at the bottom. AU and guanine-cytosine base pairs are depicted in blue and red, respectively, with unpaired nucleotides marked in black. Terminal transcription signals in some coguviruses, resembling those found in some phleboviruses, are boxed. Brassica oleracea Torzella virus 1 (BoTV1), Brassica campestris chinensis coguvirus 1 (BCCoV1), citrus concave gum-associated virus (CCGaV), citrus virus A (CiVA), watermelon crinkle leaf-associated virus 1 and 2 (WCLaV1 and WCLaV2), Edgeworthia chrysantha mosaic-associated virus (ECMaV), and Yunnan Paris negative-stranded virus (YPNSV).

The same polyvalent RT-PCR method was used for testing coguvirus infections in seedlings of several herbaceous hosts, including plant species of the families *Amaranthaceae*, *Apiaceae*, *Asteraceae*, *Brassicaceae*, *Chenopodiaceae*, *Cucurbitaceae*, and *Ranunculaceae*. This survey and the following sequencing of the amplification products showed that, apart from Torzella cabbage, other species within the *Brassicaceae* family, such as black and red cabbages, are natural hosts of BoTV1, with a relatively high virus prevalence (up to 73%). Moreover, the Catozza and Friariello turnips were identified as additional natural hosts of BCCoV1. Finally, seedlings of various watermelon cultivars were also found to be naturally infected by the coguviruses WCLaV1 or WCLaV2, with a relatively high prevalence. As far as we know, this is the first report in Europe of these two coguviruses that were first reported in China (Xin et al. 2017), then in the United States (Hendricks et al. 2022; Hernandez et al. 2021) and Brazil (Maeda et al. 2022), and more recently, in Australia (Mulholland et al. 2023). Altogether, these data demonstrate that infection of seedlings by coguviruses is quite common among the tested *Brassicaceae* and *Cucurbitaceae* species. Although this experiment allowed us to extend the natural host range of several coguviruses and their geographic distribution, it did not conclusively clarify whether the coguviruses identified in the tested seedlings were transmitted through seeds or were soilborne. This epidemiological issue was further investigated here by testing seedlings of the black cabbage sown in sterilized soil or on layers of moistened sterile filter paper. The observed BoTV1 prevalence in the seedlings, especially the infection of seedlings germinated on filter paper, conclusively showed that BoTCV1 is seed-transmitted. At the same time, these findings suggest that the other coguviruses shown to naturally infect seedlings in this study (Supplementary Table S4) are very likely seed-transmitted. In this respect, the detection of WCLaV1 and 2 in watermelon seedlings by the polyvalent RT-PCR method reported here is consistent with the suggested seed transmission of these viruses advanced by Mulholland et al. (2023) based on circumstantial observations but without providing experimental data in support. The assessment of seed transmission is crucial to prevent the further spread of coguviruses, especially those causing or associated with diseases, such as WCLaV1, WCLaV2 (Mulholland et al. 2023), and the coguviruses infecting woody hosts. Whether coguviruses infecting woody plants

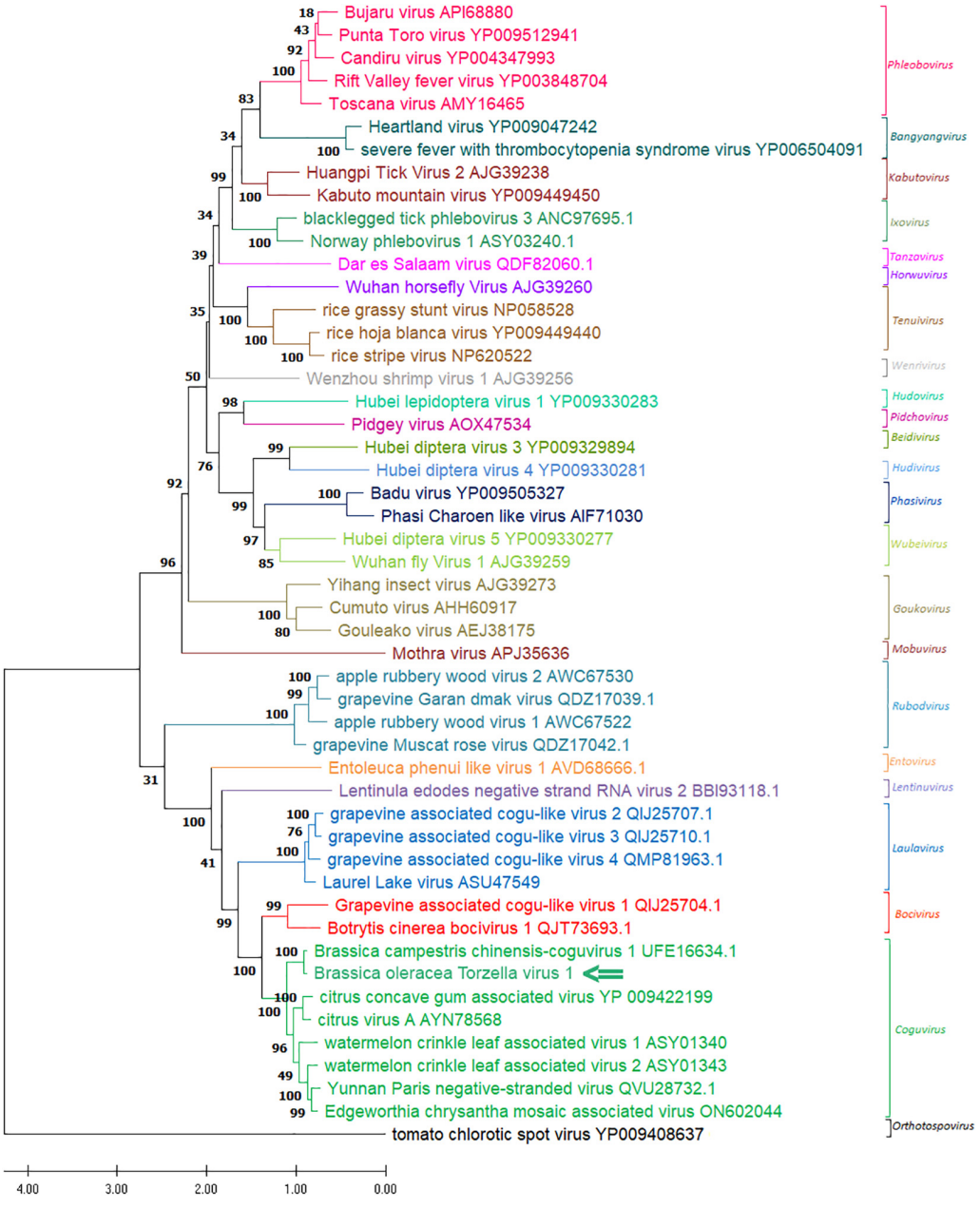

Fig. 5. Phylogenetic tree of the RNA-dependent RNA polymerase (RdRp) conserved core domain of Brassica oleracea Torzella virus 1 (BoTV1) and representative viruses of the family *Phenuiviridae*. The maximum-likelihood method adopting the LG+F+G+I amino acid substitution model was used to infer the phylogenetic tree. Bootstrap probability values (1,000 replicates) are shown at the branch nodes. Tree branches are proportional to the genetic distances, with the scale bar indicating substitutions per amino acid site. The names of the viruses and the accession numbers are reported. The arrow indicates BoTV1.

TABLE 2. Seedlings tested by reverse transcription-PCR with generic primers for detecting coguviruses

Family	Species name	Common name	Tested seeds	Positive seeds	Sequenced virus isolate	BLAST best hit	BLAST identity (%)	Coverage
Brassicaceae	*Brassica oleracea* L. var. *acephala*	Torzella cabbage	30	22 (73.3%)	TC-3	BoTV1 OR513472	100	100
Brassicaceae	*Brassica oleracea* L. var. *acephala sabellica*	Black cabbage	53	26 (49.0%)	BC-1, BC-2, BC-3	BoTV1 OR513472	100	100
Brassicaceae	*Brassica oleracea* L. var. *capitata* f. *rubra*	Black head 2 red cabbage	42	12 (28.6%)	RC-1	BoTV1 OR513472	100	100
Brassicaceae	*Brassica rapa* L. var. *rapa* DC	Catozza turnip	50	2 (0.04%)	T-3, T-4	BCCoV1 NC_079045	96.26–96.79	100
Brassicaceae	*Brassica rapa* L. subsp. *sylvestris* var. *esculenta*	Friariello turnip	29	14 (48.2%)	FR-4	BCCoV1 NC_079045	97.86	100
Cucurbitaceae	*Citrullus lanatus* Thunb.	Crimson sweet watermelon	16	7 (43.7)	WCS-1, WCS-2	WCLaV1 OM763699	100	100
Cucurbitaceae	*Citrullus lanatus* Thunb.	Sugar Baby watermelon	19	11 (57.8%)	WSB-1	WCLaV1 OM763699	100	100
Cucurbitaceae	*Citrullus lanatus* Thunb.	Tonda F1 watermelon	13	6 (46.1%)	WT-1	WCLaV2 LC636073	100	100

are also seed-transmitted was not investigated here and remains an interesting issue to be addressed in future studies.

An additional result of our surveys based on the polyvalent RT-PCR method is the detection of the previously unknow BoTV1. The complete genome of this virus showed the typical molecular features of coguviruses, including conserved terminal ends of the two genomic RNAs, a negative-sense RNA1 encoding a putative RdRp, and a bicistronic and ambisense RNA2 containing two ORFs, encoding the putative NP and MP, separated by a hairpin. In contrast to most coguviruses, the hairpin in the RNA2 of BoTV1 is not rich in AU and is relatively short, structurally resembling the hairpin formed in the RNA2 of BCCoV1. Moreover, in both cases, a terminal transcription signal previously reported in the other coguviruses was not present. These two viruses also share some other features distinguishing them from other coguviruses infecting different host species, including a longer (additional 180 aa) N-terminal of the NP and a peculiar cap-snatching signal containing an ExA motif instead of the ExG typical of all the other coguviruses or of the ExK reported in most bunyaviruses (Decroly et al. 2012; Reguera et al. 2010). Whether all these structural features conserved in *Brassicaceae*-infecting coguviruses are host-dependent is currently not known. The close relationship between BoTV1 and BCCoV1 is further supported by phylogenetic analyses based on the core domain of RdRp, where all coguviruses, including the novel BoTV1, cluster together, with BoTV1 and BCCoV1 forming a clear subclade. Interestingly, in the same phylogenetic tree, grapevine associated cogu-like virus 1, currently classified in the genus *Coguvirus* (Kuhn et al. 2022), clusters together with Botrytis cinerea bocivirus 1 in a different clade, further supporting the recent proposal that these two viruses with a tripartite genome should be considered representative members of the new tentative genus *Bocivirus* (Ruiz-Padilla et al. 2021). All the molecular and phylogenetic data reported in this study, including the RdRp amino acid sequence identity values with the other coguviruses, which are below the threshold of 95% established by ICTV as a species demarcation criterion, support our proposal of classifying BCTV1 as a representative member of a novel species named *Coguvirus torzellae*.

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