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Identification and characterization of two isolates of Bougainvillea chlorotic vein banding virus (*Badnavirus venabougainvilleae*) in bougainvillea plants from Italy

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Summary. Ornamental plants can be reservoirs of plant viruses, facilitating virus dissemination through international trade. In this bougainvillea study, 58 samples collected from six ornamental nurseries in Apulia (southern Italy) were screened for the presence of known bougainvillea-infecting viruses, using PCR assays. Among the viruses tested, only Bougainvillea chlorotic vein banding virus (BCVBV) was detected, occurring in five (8.6%) of the samples, whereas Impatiens necrotic spot virus (INSV), Clerodendron yellow mosaic virus (ClYMV), tomato yellow ring virus (TYRV), and cucumber mosaic virus (CMV) were not detected. High-throughput sequencing (HTS) of one BCVBV-positive sample (O54) enabled reconstruction of a complete viral genome, designated BCVBV Mini-Thai (GenBank accession no. PQ682517). A second HTS analysis of a pooled set of PCR-negative samples revealed a genetically distinct BCVBV isolate that was not detected by PCR using previously published primers. The complete genome of this isolate was reconstructed through contig assembly and gap-filling PCR using newly designed primers, and was named BCVBV Apulia 1 (PQ682518). The genome of Mini-Thai was 8,822 nucleotides long, and had high nucleotide (98.5%) and amino acid (98.7%) similarities with the Malaysian isolate BCVBV-UKM. In contrast, BCVBV Apulia 1 (PQ682518) was 8,694 nucleotides long and had greatest nucleotide similarity (81.6%) with the Brazilian isolate BCVBV UNB-01, with 84.0% amino acid similarity in the polyprotein. Both isolates had typical *Badnavirus* genome organization, with four open reading frames, and clustered in distinct phylogenetic clades. Further screening of the 58 collected samples using primers designed on the Apulia 1 isolate detected seven (12.1%) additional infected samples. A primer set targeting conserved genomic regions was also developed, enabling detection of both isolates. This report is the first detection of BCVBV in Italy and Europe, and also reveals the presence of a genetically divergent isolate of this virus.

Keywords. BCVBV, HTS, *Badnavirus*, genetic diversity, Apulia.

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INTRODUCTION

Bougainvillea is a perennial ornamental plant genus that is widely cultivated and traded. *Bougainvillea* (*Nyctaginaceae*) includes 18 species, of which three are of major commercial importance: *Bougainvillea spectabilis* Willd., *B. glabra* Choisy, and *B. peruviana* Bonpl. (Abarca-Vargas and Petricevich, 2018). Numerous hybrids and cultivars have arisen from interspecific crosses or spontaneous hybridizations where these species are co-cultivated, such as in East Africa, India, the Canary Islands, Australia, North America, and the Philippines (Datta *et al*., 2021).

Despite its global distribution, relatively few viral diseases have been reported in *Bougainvillea*, likely reflecting limited research attention rather than true resistance. The most studied virus is Bougainvillea chlorotic vein banding virus (BCVBV; *Badnavirus venabougainvilleae*), a putative *Badnavirus* first described in Brazil on *B. spectabilis* (Chagas *et al*., 2001) and subsequently confirmed at the molecular level by Rivas *et al.* (2005). A related *Badnavirus* infecting *B. glabra*, associated with chlorotic spot and mild mosaic symptoms, was also reported in Brazil (Yamashita *et al*., 2004). A complete genome sequence of this virus, initially named Bougainvillea spectabilis chlorotic vein-banding virus, was characterized in Taiwan by Wang and coworkers in 2007 (NC_011592). Similar pathogens were later described in Taiwan, India, and Malaysia (Tsai *et al*., 2008; Baranwal *et al*., 2010; Yusop *et al*., 2019), and were subsequently assigned to BCVBV by the International Committee on Taxonomy of Viruses (2026). Another *Badnavirus*, showing 75% similarity to BCVBV and named Bougainvillea glabra chlorotic spot virus-Egypt (BgCSV-E), was reported in Egypt (Abdel-Salam, 2020).

The BCVBV genome consists of a circular dsDNA molecule of 8,759 bp containing four ORFs. Molecular analyses of Brazilian and Asian isolates have indicated the presence of at least three novel *Badnavirus* species or strains infecting *Bougainvillea* (Alexandre *et al.,* 2016). Typical symptoms caused by these viruses include leaf mottling, vein banding, chlorosis, and distortion, and plant stunting (Bhat *et al*., 2016).

Other viruses reported in *Bougainvillea* include: Clerodendron yellow mosaic virus (ClYMV, *Begomovirus clerodendronflavi*) infecting *B. peruviana* in India (Nehra *et al.,* 2014); Impatiens necrotic spot virus (INSV, *Orthotospovirus impatiensnecromaculae*) in Iran (Shahraeen *et al*., 2002); tomato yellow ring virus (TYRV, *Orthotospovirus tomatanuli*) (Ghotbi *et al*., 2005); and cucumber mosaic virus (CMV, *Cucumovirus CMV*) on *B. spectabilis* (Shahmohammadi *et al*., 2015).

The present study investigated occurrence of viruses in *Bougainvillea* spp. in ornamental nurseries in Apulia (Southern Italy) and outlines the first detection of Bougainvillea chlorotic vein-banding virus in *Bougainvillea* spp. in Italy.

MATERIALS AND METHODS

Plant material

From April to June 2022, 58 leaf samples were collected from symptomatic (chlorotic leaf spots and mottling) and asymptomatic bougainvillea plants, in six ornamental nurseries in Apulia (Southern Italy), located in Terlizzi (n = 16), Valenzano (n = 8), Sammichele di Bari (n = 6), Monopoli (n = 25), and Muro Leccese (n = 3) (Supplementary Table S1).

DNA and RNA extractions

DNA was extracted using a CTAB-based protocol (Doyle, 1991). Approximately 50 to 80 mg of midribs and petioles from each leaf sample were homogenized in CTAB buffer (1M Tris-HCl, NaCl, 0.5M EDTA, 2% CTAB, 2.5% PVP40), then incubated at 65 °C, purified with chloroform: isoamyl alcohol (24:1), precipitated with isopropanol, and then resuspended in sterile water for storage at –20 °C. Total RNA was extracted according to Foissac *et al*. (2001), using a guanidine thiocyanate-based buffer and silica particle purification. Nucleic acid quality and concentrations were assessed using a Qubit™ Fluorometer (ThermoFisher Scientific) and agarose gel electrophoresis (1.2% TBE). Purified DNAs were stored at –20 °C until further analysis.

Complementary DNA synthesis and PCR assays

cDNA was synthesized from 500 ng of total RNA, using 1 µL random hexamer primers (0.5 mg mL^{-1}) and M-MLV reverse transcriptase (200 U μL^{-1}), according to the manufacturer's instructions (Thermo Fisher Scientific).

PCR reactions were carried out using DreamTaq DNA Polymerase (5 U μL^{-1}), each in a final volume reaction of 25 µL, with 2.5 µL of cDNA as template. Different primer sets were used for each virus (Table 1). Cycling conditions each consisted of an initial denaturation at 94°C for 4 min, followed by 40 cycles each of 94°C for 30 s, annealing at 50–55°C for 35 s, and 72°C for 45–50 s, with a final extension at 72°C for 7 min. All

Table 1. List of primers used for RT-PCR detection of viruses in bougainvillea plants.

Virus	Primers	Sequence 5′- 3′	Amplicon size (bp)	References
Bougainvillea chlorotic vein banding virus (BCVBV)	BCVBV-1 BCVBV-2	ACAGCGATTTCATTGCTGTG GACTAGTTCTCGATCAGAAGG	413	Tsai *et al.,* 2008
Clerodendron yellow mosaic virus (ClYMV)	ClYMV-FP ClYMV-RP	GTGTGAATATTGGTTGCATCATGTGGG ACCCAGGCCTGTCTTCTTGTGACG	313	Akram *et al*., 2020
cucumber mosaic virus (CMV)	CMV-F4 CMV-F3	TGAGTCGAGTCATGGACAAA CTCCCAGTCTGATTCCGTGT	657	Lin *et al*., 2003
Impatiens necrotic spot virus (INSV)	INSV-F3 INSV-R2	GCAAAGATTACCAAGGAG TCCCAAAATCAATAGTAGC	763	Elliott *et al.,* 2009
tomato yellow ring virus (TYRV)	TYRV-F TYRV-R	ACTCATTAAAATGCATCGTTCT CTAAGTAAACACCATGGCTACC	912	Birithia *et al.,* 2012

amplifications were carried out in a Bio-Rad T100 thermal cycler. Amplified products were analysed on 1.2% agarose gels and were sequenced using Sanger sequencing by Eurofins Genomics, Germany.

High-Throughput Sequencing (HTS) using Illumina

Based on the results of the previous molecular analyses, one virus-infected sample was selected for high-throughput sequencing (HTS). In addition, HTS was used for a pooled set of 20 randomly selected and apparently negative samples, to investigate the possible presence of other, potentially unknown viruses, or of highly divergent isolates of known viruses that may not have been detected by the primers used in the initial molecular assays. A total of 500 ng of RNA was used for ribosomal RNA (rRNA) depletion, carried out by Eurofins Genomics, Ebersberg, Germany. Library preparation was subsequently carried out, followed by fragment amplification and purification. Illumina sequencing was then used on the reverse-transcribed templates, with a 2 × 150 bp paired-end sequencing run to obtain approx. 5 million sequence pairs (10 million reads per sample per pool) (Eurofins Genomics).

Sequence and library analysis

Raw reads were processed using Geneious Prime 2024.0. Quality assessment and trimming were carried out using the BBDuk package (k-mer length = 21, allowing one mismatch). Filtered reads were then error-corrected and normalized using BBNorm, targeting 40× coverage, while retaining regions with minimum depths of 6×. *De novo* assembly was carried out using SPAdes v3.15.5 (Bankevich *et al*., 2012). The resulting contigs were screened for sequence homology using BLASTx (e-value cut-off 10^{-6} to 10^{-2}) and BLASTn against the NCBI viral RefSeq database (accessed 6 May 2025). Complete virus genomes were reconstructed by reference mapping using the Geneious mapper, with the medium sensitivity / fast setting and associated default parameters, and remaining gaps were filled by designing additional primers. Preliminary phylogenetic relationships were inferred in Geneious, using the Maximum Likelihood method with 1,000 bootstrap replicates.

RESULTS

PCR detection of Bougainvillea viruses

The PCR assays carried out on the 58 bougainvillea samples detected presence of one virus, BCVBV, of the five viruses assessed. Five plants (P53, P54, P55, O54, O58), all of Mini Thai cultivar, were found to be infected (8.6% of the samples): two were from Terlizzi and three from Monopoli. None of the infected plants exhibited symptoms at the time of sampling. Contrary to the 413-bp amplicon size reported by Tsai *et al*. (2008), the same primers in this study amplified a 390-bp fragment, due to sequence differences between the virus isolates. Sequence analysis of 390 bp amplicons revealed 99.6 to 100% nucleotide similarities among them, and 97.2 to 99.6% similarities with the BCVBV UKM isolate from Malaysia (GenBank accession no. MK501794).

High-Throughput Sequencing (HTS) and subsequent PCR assays

The Illumina sequencing run carried out on RNA extracted from a single BCVBV-positive sample (O54) yielded 6.4 million paired-end reads, and 10.6 million paired-end reads from the pooled PCR-negative samples.

After quality filtering, these were each reduced to approx. 2.7 and 4.8 million high-quality reads. *De novo* assembly produced 13,454 contigs from sample O54 with contig lengths of 200 to 2,082 bp, and 32,126 contigs from the pooled samples with contig lengths of 160 to 1,452 bp.

BLASTn and BLASTx analyses revealed presence of BCVBV in both datasets. Using three contigs of varying lengths obtained from the BCVBV-infected sample (O54), the complete virus genome was successfully reconstructed. This isolate was tentatively designated as BCVBV isolate Mini Thai (PQ682517), as it was detected exclusively in the Mini-Thai cultivar. The assembled genome was 8,822 bp in length and had 98.5% nucleotide similarity with the BCVBV UKM isolate.

In the pooled sample, which had initially tested negative by PCR, several contigs were assembled. These were used for preliminary identification of virus sequences and showed greatest nucleotide similarities (from 70 to 90%) with the Brazilian isolate BCVBV UNB-01 (GenBank accession number MK473389). However, mapping against this reference genome revealed several gaps, indicating incomplete genome coverage.

To investigate the prevalence of this putative divergent variant, the specific primer pair BVs-ACAAGAAGAATCAGGAAGCT / BVas-CAATGGTGCAGAGGGTTT was designed to amplify a 197 bp fragment within the most divergent genomic region identified during mapping against the BCVBV Mini-Thai isolate. Of the 58 samples tested, seven (12.1%) were positive, including O109, O110, P47, P48, C4, O77, and O81, (Supplementary Table S1). None of the five plants infected with the Mini-Thai isolate tested positive with these primers.

To complete the virus genome, ten additional primer pairs were designed based on the newly assembled contigs and their alignments with the BCVBV UNB-01 reference genome, allowing all the remaining gaps to be bridged (Supplementary Table S2). PCR assays using these primers were carried out on a single positive sample (P47), and all target regions were successfully amplified. The resulting sequences were assembled to reconstruct the complete genome of the virus isolate, which was designated as BCVBV Apulia 1 (GenBank accession no. PQ682518).

A universal primer set was developed by aligning all available BCVBV sequences in GenBank together with those obtained in the present study. Primers (7bs- GACCTCACCACATGGAGATA and 7ba-AGGRTKATCTCYTCTTTGTCAA), targeting a highly conserved region, reliably amplified a 330 bp fragment within the polyprotein of the Mini-Thai and Apulia 1 isolates (Figure 1).

Characterization of BCVBV isolate genomes

Both BCVBV isolates identified in the present study retained the typical genome organization of badnaviruses. BCVBV Apulia 1 possessed a double-stranded DNA genome of 8,694 nt, in which four open reading frames (ORFs) were identified. The functions of ORF1, ORF2, and ORF4 remain unknown; these ORFs encode proteins of, respectively, 151, 145, and 197 amino acids (Figure 2 A). ORF3, the largest coding region, encodes a polyprotein of 2,214 amino acids, containing, in sequential order, motifs corresponding to a zinc-finger domain, a pepsin-like aspartic protease domain, a reverse transcriptase domain, and an RNase H-like domain. The genome is flanked by two non-coding regions (NCRs), measuring 537 nt at the 5′ end and 349 nt at the 3′ end.

The BCVBV Mini-Thai genome shared high nucleotide similarity (98.5%) with the Malaysian BCVBV

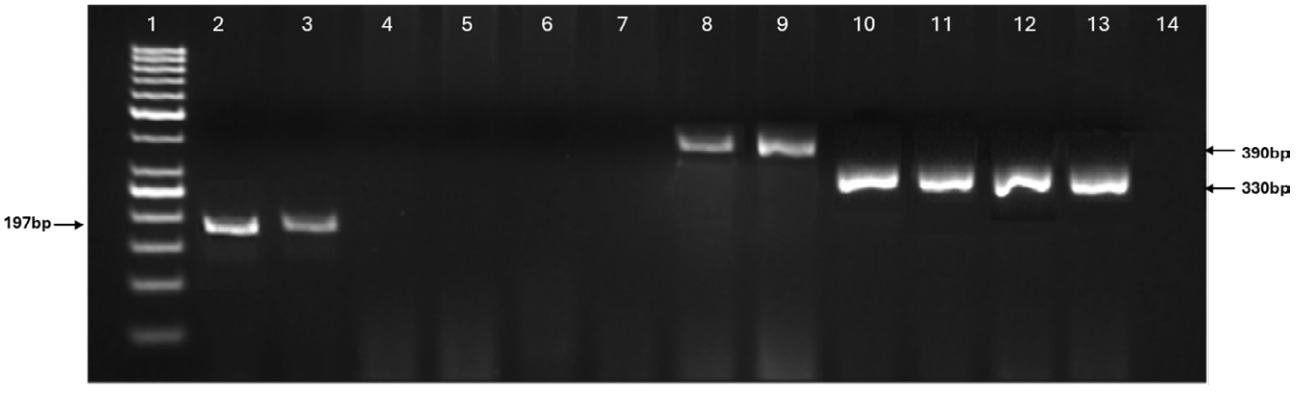

Figure 1. PCR amplifications of BCVBV isolates Apulia 1 and Mini-Thai, using isolate-specific and universal primers. Lane 1: 50 bp DNA ladder. Lanes 2 to 5: amplifications with primers BVs/BVa; lanes 6 to 9: amplifications with primers BCVBV-1/BCVBV-2; lanes 10 to 13: amplifications with universal primers 7bs/7ba. For each primer set, lanes 2 and 3, 6 and 7, and 10 and 11 correspond to isolate Apulia 1, while lanes 4 and 5, 8 and 9, and 12 and 13 correspond to isolate Mini-Thai. Lane 14 is the negative control.

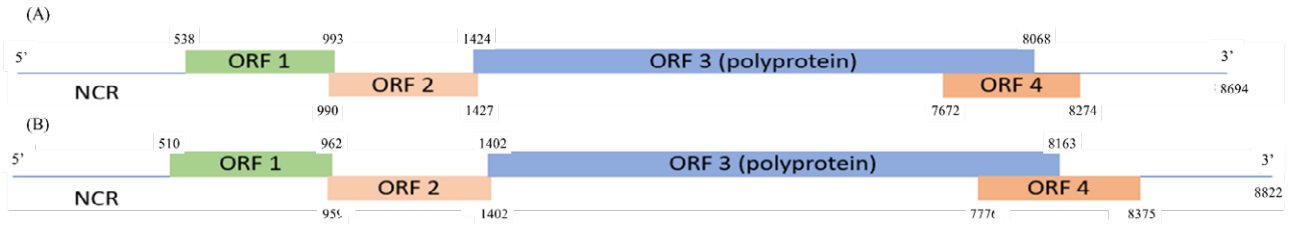

Figure 2. Genome organization of the two BCVBV isolates, showing the relative positions of the ORFs: (A) isolate Apulia 1; (B) isolate Mini-Thai.

UKM isolate. Its genome consisted of 8,822 nt and contained four ORFs: ORF1 encoding a protein of 150 amino acids, ORF2 of 147 amino acids, ORF3 (polyprotein) of 2,254 amino acids, and ORF4 encoding a protein of 199 amino acids (Figure 2B). The 5′ and 3′ non-coding regions (NCRs) were, respectively, of lengths 509 nt and 447 nt.

BLASTn analysis of the complete BCVBV Apulia 1 genome revealed greatest nucleotide similarity (81.6%) with the BCVBV UNB-01 isolate, whereas the least similarity (73.7%) was with the Taiwanese BCVBV isolate (NC_011592).

Detailed pairwise comparison of the polyprotein sequences between the two isolates identified in this study (Apulia 1 and Mini-Thai), previously reported as BCVBV isolates, and with other members of *Badnavirus*, confirmed the high divergence of Apulia 1 (Table 2). Sequence divergence ranged from 19.5% to 23.4% at nucleotide level, and from 15.4% to 19.0% at amino acid level. This comparison is particularly relevant, as this genomic region forms the basis for species demarcation criteria within *Badnavirus*, which is currently set at 20% nucleotide divergence.

Phylogenetic analyses based on the amino acid sequences of the polyprotein were carried out for the same viruses and isolates. Consistent with the results of the pairwise sequence comparison, two major clades were identified. Clade 1 comprised exclusively BCVBV isolates, with the Apulia1 isolate clustering in a subclade with the UNB-01 isolate, whereas the Mini-Thai isolate formed a separate subgroup with the Taiwanese and Malaysian (UKM) isolates. The remaining *Badnavirus* members included in the analysis clustered in a distinct, separate clade (Figure 3).

DISCUSSION AND CONCLUSIONS

Ornamental plants may serve as reservoirs of plant viruses, facilitating virus spread through international trade. Movements of infected plant material have repeatedly been associated with introductions of emerging pathogens into new areas, often with significant economic and environmental impacts. A notable example was introduction of *Xylella fastidiosa* into Italy via infected ornamental coffee plants, which resulted in severe out-

Table 2. Pairwise comparison matrix of polyprotein sequences from different BCVBV isolates and other *Badnavirus* members, based on nucleotide and amino acid sequences.

		% similarity of polyprotein sequences (nt)										
		Mini-Thai	UKM	TWN	Apulia 1	UNB-01	BVF	DBALV2	MBV1	RYNV	GVBaV	PVCV
% similarity of polyprotein sequences (aa)	Mini-Thai		**98.6**	99.3	**76.6**	**75.3**	43.8	42.0	38.7	41.4	39.3	37.3
	UKM	**98.7**		98.6	76.8	75.7	43.8	41.8	38.7	41.4	39.4	37.3
	TWN	98.5	98.7		76.7	75.5	43.9	42.0	38.8	41.5	39.4	37.3
	Apulia 1	**82.5**	83.3	82.7		80.5	43.0	42.1	38.9	41.3	39.1	35.7
	UNB-01	**81.0**	81.8	81.5	**84.6**		43.5	41.8	39.1	40.8	39.3	35.9
	BVF	35.8	36.0	36.1	36.2	36.0		40.6	39.1	38.9	38.9	25.3
	DBALV2	33.8	34.2	34.1	34.7	34.5	38.6		46.9	36.7	38.1	24.5
	MBV1	32.2	32.6	32.5	32.6	32.5	35.1	40.8		35.8	36.3	23.6
	RYNV	31.6	31.8	31.8	32.3	32.2	35.3	32.4	32.3		54.6	24.9
	GVBaV	33.8	34.1	33.9	33.7	34.3	36.9	34.1	34.3	50.4		23.3
	PVCV	16.3	16.4	16.3	15.9	16.2	15.2	14.2	13.6	12.7	13.8	

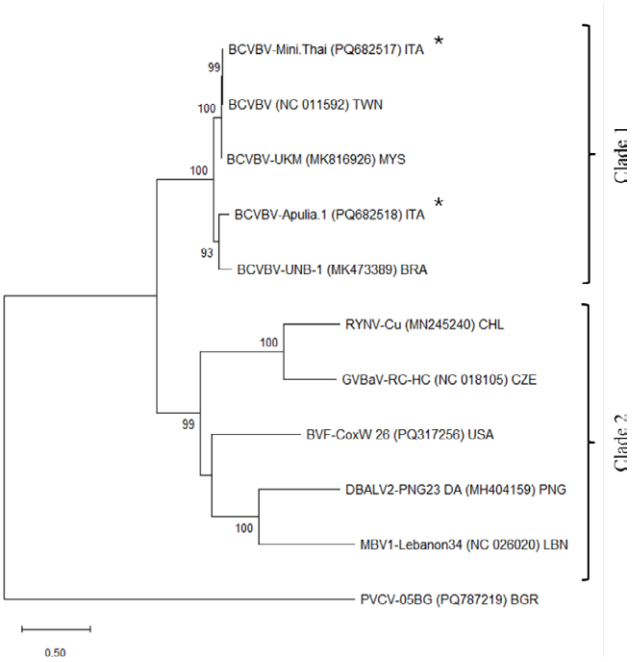

Figure 3. Maximum likelihood (ML) phylogenetic tree based on polyprotein amino acid sequences of BCVBV isolates found in this study (*), compared with homologous sequences from representative *Badnavirus* species. BCVBV, Bougainvillea chlorotic vein banding virus; RYNV, Rubus yellow net virus; GVBaV, Gooseberry vein banding virus; DBALV2, Dioscorea bacilliform AL virus 2; MBV1, Mulberry badnavirus 1; BVF, Blueberry virus F. Petunia vein clearing virus (PVCV) was used as an outgroup. GenBank accession numbers and countries of origin are indicated at the branch tips. The scale bar represents number of substitutions per site.

breaks of the bacterium infections in olive orchards in southern Italy (Saponari *et al*., 2013). These cases highlight the importance of effective phytosanitary measures and early pathogen detection systems.

In the present study, BCVBV was detected for the first time in Italy and in Europe. Initial RT-PCR screening associated the virus exclusively with the Mini Thai cultivar, with all infected plants (8.6% of those assessed) remaining asymptomatic at the time of sampling. Within the limits of this survey, no evidence of virus spread to other cultivars grown in close proximities was observed. Symptoms recorded during the surveys were not associated with any of the detected virus agents, suggesting that the symptoms may have been caused by physiological disorders or other biotic factors.

HTS analyses were essential for the detection of a previously undetected and genetically divergent isolate, designated BCVBV Apulia 1. This isolate had polyprotein nucleotide divergence ranging from 23.2 to 23.4% compared to other known BCVBV isolates, with the exception of isolate UNB-01, from which it differed by 19.5%. UNB-01, similarly to Apulia 1, also had divergence values exceeding 20% relative to other BCVBV isolates (Table 2). Current guidelines establish a 20% polyprotein nucleotide divergence as the species demarcation threshold within *Badnavirus* (Fauquet *et al*., 2005; International Committee on Taxonomy of Viruses, 2026). Therefore, Apulia 1, together with UNB-01, should be considered as a new species within *Badnavirus*, rather than BCVBV isolates, unless this demarcation threshold is increased. Subsequent targeted assays showed presence of BCVBV Apulia 1 in additional samples (12.1%) across different host cultivars, increasing the overall BCVBV infection rate to 20.7% when both isolates were considered. These results highlight the limitations of conventional PCR assays for genetically variable viruses, and the requirement for continuous refinement of virus diagnostic tools (Adams *et al*., 2013; Massart *et al*., 2014).

Development of a primer set capable of detecting both the Mini-Thai and Apulia 1 isolates is a diagnostic improvement, although further validation is required across a greater range of isolates than assessed in the present study. The absence or mildness of symptoms in infected plants indicates possible latent infections, possibly influenced by host age or environmental conditions. Latent infections would increase risks of spread of unnoticed viruses in ornamental production systems. Previous reports describing severe symptoms (Yamashita *et al*., 2004; Tsai *et al*., 2008; Bhat *et al*., 2016) indicate that pathogenicity of the isolates requires further assessment.

The present study has expanded knowledge of the distribution and genetic diversity of BCVBV, reporting its presence in Europe, and identifying a highly divergent BCVBV isolate. These results demonstrate the added value of integrating HTS with conventional diagnostic approaches to improve the detection and management of plant viruses in ornamental crops, thereby supporting effective phytosanitary strategies for virus disease management.

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