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New or Unusual Disease Reports

First report of camellia ring spot associated virus 3 in *Camellia japonica* in Europe

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Summary. Numerous viruses have been described in camellia, often found in association, and a variety of symptoms have been associated with these infections. In this study, a plant with chlorotic spots, including complete yellowing of the leaves, was subjected to high-throughput sequencing (HTS). Three viral contigs were identified by HTS, and complete genome sequences of a new isolate of camellia ring spot associated virus 3 (CRSaV-3) were determined using nested PCR to bridge the gap between discontinuous contigs. The new CRSaV-3 isolate shared the highest percentage of nucleotide identity (87.1%) with the American isolate CJ5_676. This represents the first report of CRSaV-3 in symptomatic camellia in Europe and, since only this virus was diagnosed, for the first time it was possible to reliably associate the symptoms observed in camellia to a specific CRSaV-3 isolate.

Keywords. CRSaV-3, High Throughput Sequencing, leaf variegation, chlorotic ringspots, virus detection.

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INTRODUCTION

Camellias (*Camellia* spp.) are important evergreen flowering plants in the *Theaceae* family, known for tea, oil camellia, and ornamental types. They are native to China and Japan and grows naturally in forests at altitudes of around 300–1,100 metres. There are over 250 species, with *C. japonica, C. reticulata, C. sasanqua*, and their hybrids being widely grown as garden ornamental plant, although *C. japonica* is preferred for its variety of flower colours, shapes, and blooming seasons (Macoboy and Mann, 1998). In Italy, the first specimen of the plant arrived in 1760 and was planted in the "English Garden" of the Royal Palace of Caserta. It was a gift, a token of love, from Admiral Nelson to Lady Emma Hamilton, the wife of the English ambassador to the Bourbon court of Naples (Monda, 2017).

Most of the new camellia cultivars come from crosses, but they are mainly propagated from cuttings, making them susceptible to accumulate viral infections. Symptoms of these infections varying from foliar mottle, mosaic, ringspots to foliar and flower variegations. The pattern of affected plant tissue also is irregular on the whole plant, since some branches show symptoms, others do not, and symptoms may not appear every year. Moreover, in the same plant different sizes and shapes of light-green and/or yellowish spots on the leaves are frequently observed. These foliar symptoms have always been associated with mixed infections involving two or more viral entities, making it difficult to attribute them to a single virus. Attempt to associated specific symptoms to a single viral entity were proposed in Zhang *et al.* (2020) based on comparative analysis of symptoms from several and multiple infected camellias. In 2018, a geminivirus, camellia chlorotic dwarf-associated virus (CaCDaV), was found associated to camellia chlorotic dwarf disease (Zhang *et al*., 2018), while in 2019, in USA, three betaflexivirus, camellia ringspot associated virus 1, 2 and 3 (CRaV-1, CRaV-2 and CRaV-3) were found in mixed infection and associated to chlorotic ringspot symptoms of *C. japonica* (Liu *et al*., 2019). Recent analysis of samples from affected plants from Italy using the high-throughput sequencing (HTS) technique revealed a complex group of viruses from the *Betaflexiviridae* and *Fimoviridae* families. Particularly, two new emaravirus species were identified, named camellia japonica associated emaravirus 1 and 2 (CjEV1 and CjEV2), were found only in symptomatic plants, while the betaflexiviruses identified were associated to camellia ringspot associated virus 1 and 2 (CRaV-1 and CRaV-2) (Peracchio *et al*., 2020). Using the same approach, new viruses were identified in China from camellias with various symptoms, provisionally named camellia chlorotic ringspot viruses (CaCRSVs, genus *Fimoviruses*), camellia yellow ringspot virus (CaYRSV, genus *Idaeovirus*), camellia-associated badnavirus (CaBaV), and camellia-associated marafivirus (CaMaV) (Zhang *et al*., 2020). In the same work, tea plant necrotic ring blotch virus (TPNRBV), previously detected in *C. sinensis* (Hao *et al*., 2018), has also been detected. Overall, a clear-cut picture correlating with precision, symptoms observed in *C. japonica* with a specific virus is still far to be complete. In the present study, we used HTS to analyse the virome of a *C. japonica* plant displaying yellow spots, yellow mottling up to yellowing in some leaves and colour-breaking of flowers (Figure 1). These symptoms

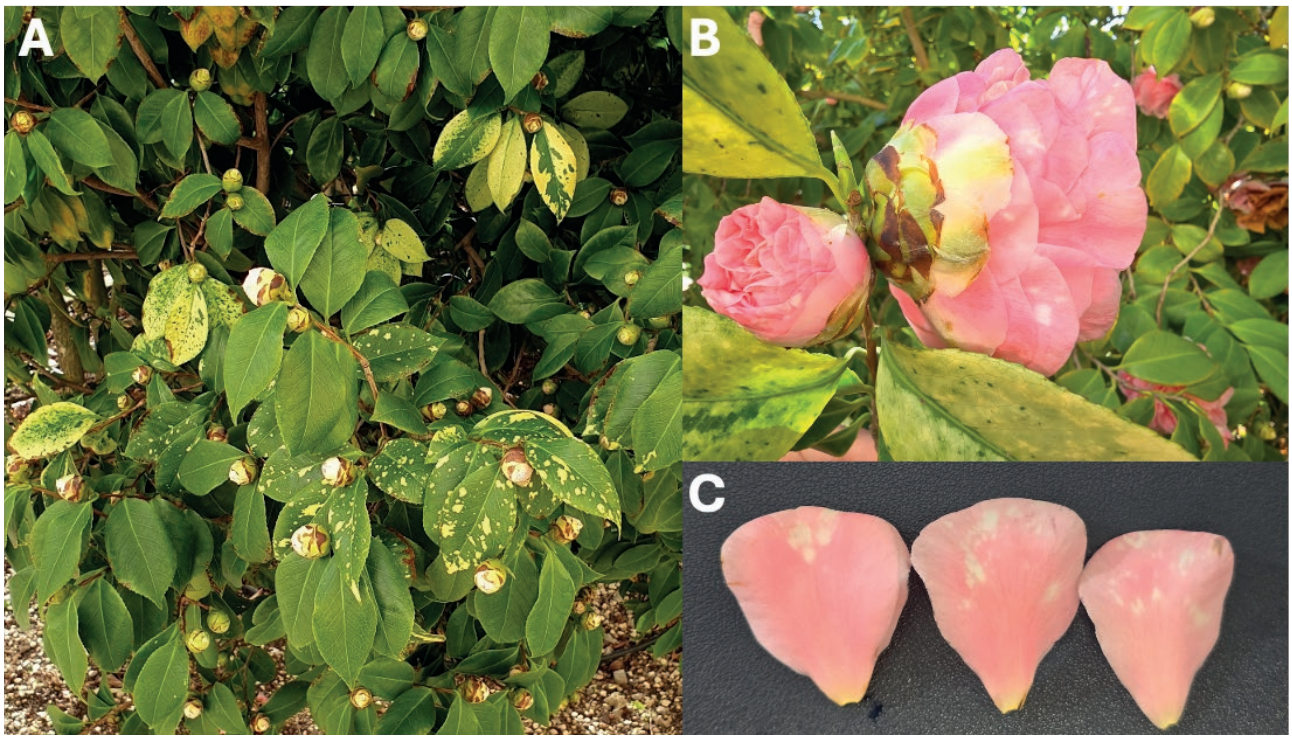

Figure 1. Camellia plant analysed in this study, showing leaf symptoms in some branch, consisting in yellow spots, yellow mottling up to yellowing (A) and colour-breaking of flowers (B and C).

were associated to only one virus for which a detection method was also proposed.

MATERIALS AND METHODS

Sample collection

In May 2020, during a tourist visit to the villa Campolieto, considered one of the most beautiful villas on the so-called "Golden Mile", an historic and cultural stretch of 18th-century Italian baroque architecture, located between Ercolano and Torre del Greco municipalities (Campania region, Italy), a camellia plant was noted for its evident viral leaf symptoms. Symptomatic leaf samples were kept in clean bags on ice and brought to the Portici section of the Institute for Sustainable Plant Protection of National Research Council (IPSP-CNR). Part of the fresh samples were used for RNA extraction and submitted to HTS, while the remaining part was annotated in the IPSP-CNR virus collection and kept in calcium chloride at 5 °C.

RNA isolation, library preparation and HTS analyses

Total RNA was extracted from 100 mg of fresh leaves (50 mg in the case of petals), by pooling 0.5 × 0.5 cm leaf pieces obtained from four different leaves of the same plant, using the NucleoSpin RNA Plant kit (Macherey-Nagel, Düren, Germany). The purified RNA (80 ng/ml) was used to construct cDNA libraries with the TruSeq Stranded Total RNA Library Prep Kit with Ribo-Zero Plant (Illumina, San Diego, CA, USA). The resulting cDNA libraries were sequenced on an Illumina NovaSeq 6000 platform, with sequencing services provided by Macrogen Inc. (Seoul, Republic of Korea). Raw sequencing data were processed using CLC Genomics Workbench v. 21 (Qiagen, USA). Reads were initially trimmed using the Trim Reads tool and reads with a quality score ≤ 0.05 (which corresponds to a Phred score of ~13) or a length shorter than 15 bp were discarded. The trimmed reads were then filtered against the *Camellia sinensis* reference genome (GenBank accession No. GCF_004153795.1; downloaded 03-30-2025) to remove host-derived sequences. The remaining reads were subjected to *de novo* assembly using CLC Genomics Workbench with the following parameters: word size of 19, bubble size of 50, and a minimum contig length of 200 bp. To identify viral sequences, assembled contigs were screened using tBLASTx against the viral RefSeq database, followed by BLASTn searches against the NCBI online database with a cutoff E-value of 10^{-5}. To obtain the longest possible viral genome sequences, contigs identified as viral were either extended at both termini and remapped to the most closely related complete viral genome sequences using the Map Reads to Reference function (length fraction: 0.5; similarity fraction: 0.8). Finally, the consensus viral genomes were deposited in NCBI GenBank and used for further analyses. The ORFs of viral nucleotide sequence and conserved domains in protein sequences inferred from ORFs were found using the NCBI ORF (https://www.ncbi.nlm.nih.gov/orffinder) and CD-search (https://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi) servers, respectively.

Development of a diagnostic method based on nested-PCR for HTS validation and virus detection

In this study, outer and internal primers were designed on viral contigs obtained by HTS and screened for detecting the virus identified in the symptomatic camellia from leaves and petal samples (Table 1). The cDNA was synthesized both from RNAs extracted from symptomatic leaves, used for HTS, and from symptomatic petals, using the ImProm-II reverse transcriptase system (Promega, USA) according to the manufacturer's instructions. Reactions were performed at 42 °C for 60 min, followed by incubation at 70 °C for 5 min. PCR amplification was performed using the GoTaq® DNA Polymerase (Promega, USA) under the following cycling conditions, used for both first round and second round PCR: initial denaturation at 94 °C for 4 min; 35 cycles of 94 °C for 30 s, 56 °C for 30 s, and 72 °C for 1 min; and final extension step at 72 °C for 10 min. First round of PCR was performed in 50 mL of total volume, using Cam_A/Cam_Br primers (Table 1). Five mL of the first round PCR were used as target in the second round PCR performed with four different primer pairs (Table 1). PCR products were visualized in 1.2% agarose gel, stained with ethidium bromide, and directly sequenced in both senses (Eurofins Genomics, Colonia, Germany).

RESULTS

HTS analysis

The HTS generated a raw dataset of 89,011,588 reads (101 bp paired end), totalling 8,990,170,388 bp, and have been deposited in the NCBI Sequence Read Archive (SRA) under BioProject accession number PRJNA1461450. A total of 15,174,063 reads were filtered out, resulting in a final set of trimmed reads with an average length of 90.46 bp. To isolate non-host sequences, the

Table 1. Primer pair used in first round and second round nested-PCR for CRSaV-3 detection.

Primer name	5'-sequence-3'	Interval (nt)	Size
First round			
Cam_A	GAGGCTCCATCTTTGAACAGGG	3038-3864	826 bp
Cam_Br	GAACCCACCCATCTTGAAGGTG		
Second round			
Cam_F	GATTCTGTGTGTCCAGAAGGG	3210-3820	610 bp
Cam_R	GAACCATCCTTCAATTCCCTG		
Cam_F1	CTGAATCCTATCTCTGAGCCC	3161-3712	551 bp
Cam_R1	CTCTGGCTTCTCCATCAGCAC		
Cam_F	GATTCTGTGTGTCCAGAAGGG	3210-3712	502 bp
Cam_R1	CTCTGGCTTCTCCATCAGCAC		
Cam_F1	CTGAATCCTATCTCTGAGCCC	3161-3815	654 bp
Cam_R2	CCTTCAATTCCCTGAACTCTC		

trimmed reads were mapped against the host reference transcriptome. A total of 64,470,046 reads were identified as host-derived and subsequently removed. The remaining filtered reads were then subjected to de novo assembly, which yielded 688 contigs. The assembled contigs were analysed using tBLASTx and BLASTn for plant virus identification. Three contigs were specifically annotated as plant viral sequences, all of which showed high similarity to camellia ringspot-associated virus 3 (CRSaV-3; family *Betaflexiviridae*, subfamily *Trivirinae*, genus *Capillovirus*; Gaafar and Ziebell, 2021). They were contig 30, of 3.482 bp, contig 5 of 2.932 bp and contig 44 of 833 bp, respectively. These contigs were aligned to the reference genome with the highest BLASTn score (GenBank accession no. MK050795) to determine their relative genomic positions and generate a consensus sequence (Figure S1A). After that, trimmed reads were remapped to this consensus sequences (Figure S1B), resolving the gap between contig 44 and contig 5 while gap between contigs 30 and 5 remain unresolved.

To bridge the remaining gap between the contigs 30 and 5 (Figure S1), specific primers were designed for gap filling, followed by PCR and Sanger sequencing (Table 1 and Figure 2). Through this integrative approach, the complete genome sequence of CRSaV-3 was successfully determined. Finally, the trimmed reads were re-mapped to this newly established complete sequence, with 11,234 reads uniquely mapped to the viral genome.

Genome analysis

The genomic sequence was determined to be 7402 nt (GenBank accession No. PZ012665), excluding poly(A) tail at the 3' end, and showed the highest percentage of nucleotide identity (87.1%) with the isolate CJ5_676 of CRSaV-3 (GenBank accession no. MK050795), identified in USA (Montgomery County Maryland) in 2016, in the CJ5 cultivar of *C. japonica* (Liu *et al*., 2019). According to the species demarcation criterion (< 72% identity at genomic sequence) for the family *Betaflexiviridae* (Adams *et al*., 2012), the virus identified in the present study should be considered a new isolate of CRSaV-3,

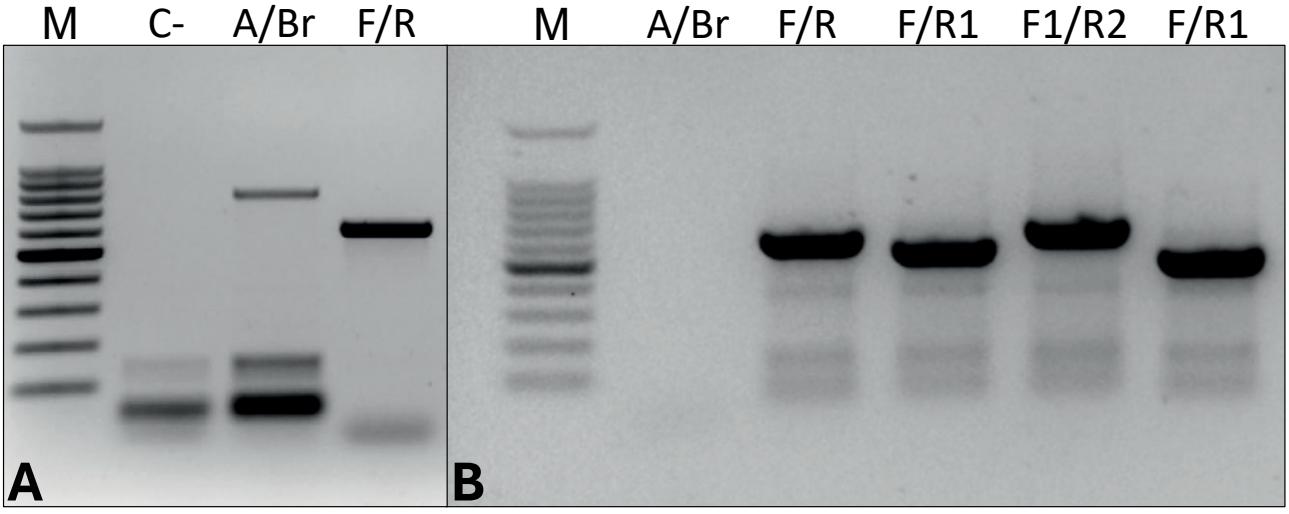

Figure 2. Results of the first round and second round nested PCR from RNA extracted from symptomatic camellia petals (A) and leaves (B), using primer pair listed in Table 1. M = 100 bp DNA ladder (Promega, USA); A/Br = first round primers Cam_A/Cam_Br; F/R = second round primers Cam_F/Cam_R; F1/R1 = second round primers Cam_F1/Cam_R1; F2/R2 = second round primers Cam_F2/Cam_R2; F/R1 = second round primers Cam_F/Cam_R1; C- = negative control.

Table 2. Percentage of identity of nucleotide (nt) and percentage of identity/similarity of amino acid (aa) sequences between the isolate 780_Campolieto and the two isolates CJ5_676 and CJ5_807 of CRSaV-3, obtained using BLASTN and BLASTP respectively (Altschul *et al*., 1997).

	Genome (nt)	Replicase (aa)	MP (aa)	CP (aa)	NBP (aa)
CJ5_676	87.1	84.4/89.0	92.9/96.0	99.4/100	91.7/94.0
CJ5_807	75.4	77.2/86.0	81.9/90.0	97.4/99.0	74.6/82.0

named 780_Campolieto, representing the first report of this virus in Europe in *C. japonica*.

The genomic organization and structure between the two CRSaV-3 isolates is similar: both genomes contain three ORFs, a large ORF 1, a nested ORF 2 and a small ORF 3 at the 3' region, with the ORF1 that encodes a polyprotein consisting of a methyltransferase, a viral RNA helicase, a RdRp domain and a Trichovirus coat protein (CP) domain; the ORF 2, nested with ORF 1, encoding a movement protein (MP); the ORF 3 that encodes a protein of 133 aa residues containing a nucleic acid binding protein (NABP) domain, as reported for some betaflexivirus (Liu *et al*., 2019, Supplementary material, Figure S2).

Genome comparison of 780_Campolieto with the two CRSaV-3 described in Liu *et al.* (2019) revealed that the 5' untranslated regions was at least of 59 nt for 780_Campolieto, 80 nt for CJ5_676 and 62 nt for CJ5_807; the 3'-UTR was at least of 7 nt for 780_Campolieto, 22 nt for CJ5_676 and 13 nt for CJ5_807. Summary results of the sequences pairwise comparison between 780_Campolieto and the two American isolates CJ5_676 and CJ5_807 of CRSaV-3 are reported in the Table 2.

Validation of HTS results and virus detection

The 780_Campolieto isolate of CRSaV-3 was easily detected in RT-PCR form RNAs extracted from symptomatic petals by using the Cam_A/Cam_Br primers. Amplicon of the expected size (826 bp) was obtained (Figure 2A) and sequence was 100% identical to the corresponding genome portion of the 780_Campolieto assembled by HTS analysis. Nevertheless, first round with these primers gave negative results when the RNAs extracted from fresh symptomatic leaves was used in RT-PCR. Thus, three nested primer pairs were designed to increase the detection sensitivity (Table 1). All of them gave expected amplicons (Figure 2B) and sequences were 100% identical among them and with 780_Campolieto. Overall, these primers were used to validate HTS results, to detect viruses in different plant tissues and to bridge gap between the two consecutive contigs 30 and 5.

DISCUSSION

As with many trees and shrubs, the multiplication of new camellia varieties is achieved through vegetative propagation from selected mother plants. This process, however, exposes the varietal germplasm to the risk of disease accumulation, especially those of viral origin. Numerous viruses have been described in *C. japonica*, often found in multiple associations, making it difficult to establish the contribution of each virus to the various symptoms observed in this plant (Liu *et al*., 2019; Peracchio *et al*., 2020; Zhang *et al*., 2020). Indeed, similar symptoms have been associated with different viral complexes, although some studies have proposed a correlation between the type of symptomatology and the prevalence of certain viral species (Zhang *et al*., 2020). Nonetheless, this approach appears to be highly empirical, failing to consider the possible multiple interactions at various levels between the plant and the viruses present. Here we report the identification of a new CRSaV-3 isolate in *C. japonica*, reported for the first time in Europe. Analysis conducted using HTS revealed that this was the only virus detected in the symptomatic plant analysed, and consequently, the symptoms observed, consisting in chlorotic spots, yellow mottling, and leaf yellowing (Figure 1), can be tentatively linked to the Italian CRSaV-3 isolate 780_Campolieto.

The complete or nearly complete genomic sequence of isolate 780_Campolieto has been produced and deposited (GenBank accession No. PZ012665). Comparison of the genomic characteristics with other CRSaV-3 isolates available in the database revealed some genetic divergence among these isolates, in the replicase, MP and NABP ORFs, unlike the CP. This would confirm previous supposition, namely that the CP plays an important role in maintaining the virion structure and plays an essential role in the virus life cycle (Liu *et al*., 2019). To validate the genomic sequence obtained by HTS, a RT-PCR was developed using primer pairs designed on the assembled sequence. This method was applied using RNA extracted from both symptomatic petals and leaves. Since camellia leaves are known to contain numerous substances that inhibit the Taq polymerase, including specific tannins such as camelliatannin D (Hatano *et al*., 1995; Uchii *et al*., 2019), and which are likely responsible for the failure of direct amplification in the first round of PCR. To increase the sensitivity of the RT-PCR on RNA extracted from leaves, due

to high content of tannins, a second round of PCR was used using internal primers (Table 1). This method was effective in amplifying CRSaV-3 (Figure 2) from leaves, which gave apparently negative results after the first round of PCR.

In conclusion, this virome analysis offers strong evidence about CRSaV-3 linked to observed symptoms, helping assess risks and manage known or new viruses of *C. japonica*.

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