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Detection of hibiscus chlorotic ringspot virus, citrus exocortis viroid and citrus viroid VI in China rose from Italy using high-throughput sequencing

Giuseppe PARRELLA[1,*], Chantal FAURE[2], Armelle MARAIS[2], Elisa TROIANO[1,4], Thierry CANDRESSE[2]

[1] *Institute for Sustainable Plant Protection of National Research Council (IPSP-CNR), 80055 Portici, Italy*
[2] *University of Bordeaux, INRAE, UMR BFP, CS 20032, 33882 Villenave d'Ornon CEDEX, France*
[3] *University of Naples "Federico II", Department of Biology, 80126, Naples, Italy*
[4] *Enbiotech s.r.l., 84012, Angri (SA), Italy*
*Corresponding author. E-mail: giuseppe.parrella@ipsp.cnr.it

Summary. In Spring 2024, a symptomatic plant of China rose (*Hibiscus rosa-sinensis*) showing leaf yellowing and deformation, located in the province of Naples (South Italy), was re-sampled for further investigations. Total RNAs were purified from leaves and subjected to Illumina HTS analysis. Results confirmed that the plant was infected by hibiscus chlorotic ringspot virus (HCRSV), citrus exocortis viroid (CEVd), and citrus viroid VI (CVd-VI). The two viroids had not been previously detected in China rose. HTS results were confirmed by RT-PCR in the re-sampled plant, and in three nearby China rose plants with the same symptoms, using specific primer pairs for the three pathogens, followed by Sanger sequencing and BLAST analysis of the sequences. Different results obtained can be due to differing sensitivity and specificity in HTS-based detection of plant viruses. This is the first report of multiple infections by HCRS, CEVd and CVd-VI in China rose, which is also a new host for both of the viroids.

Keywords. HCRSV, CEVd, CVd-VI, mixed infections, NGS

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ORCID:
GP: 0000-0002-0412-4014
AM: 0000-0003-2482-1543
ET: 0000-0001-7755-4915
TC: 0000-0001-9757-1835

Hibiscus rosa-sinensis Linn. (*Malvaceae*), also known as China rose, is a popular ornamental shrub in Italy, and is frequently found near private and public buildings and in parks and gardens. China rose is affected by several diseases that are significant factors in reducing yield and quality. This host can be infected by viroids and viruses from several families, and previous reports have highlighted the phytosanitary status of Chine rose in Italy (De Stradis *et al*., 2008; Luigi *et al*., 2013; Parrella and Mignano, 2024). Recently, an siRNA HTS analysis applied to *H. rosa-sinensis* leaves with generalized yellowing revealed the association of hibiscus chlorotic ringspot virus (HCRSV) infection with these symptoms (Parrella and Mignano, 2024).

Nevertheless, since the symptoms observed were different from those attributed to HCRSV (Pourrahim *et al.*, 2013), other viruses or viroids, not previously identified, were suspected to be present in the same plant (Figure 1). A new HTS analysis was carried out on the plant, using leaf tissues collected in spring, with the aim to determine if additional pathogens were present.

Total RNAs were purified from leaves of the previously analysed symptomatic plant (Parrella and Mignano, 2024), and following a ribodepletion step, these were subjected to Illumina HTS (2 × 150 nt). After quality trimming, a total of 20,902,880 reads were obtained and assembled *de novo* (CLC Genomics Workbench 21, Qiagen). Contigs were annotated by Blastn and BlastX analyses against the GenBank database, resulting in the identification of single contigs for citrus exocortis viroid (CEVd), citrus viroid VI (CVd-VI) and HCRSV.

The single identified HCRSV contig was a near complete genome. It integrated 414,901 reads (2.0% of total reads), with a 13,614x average coverage, and had greatest nucleotide similarity (99.4%) with the Ita-1 isolate (GenBank OR891792) previously identified from the same plant (Parrella and Mignano, 2024). The single identified CEVd contig represented a complete genome and integrated 425 reads (0.002% of total reads) for a 158x average coverage. This had greatest nucleotide similarity (99.2%) with a severe variant from *Gynura aurantiaca* in the United States of America (isolate CEVdg-S; GenBank AF298177). The single identified CVd-VI contig also corresponded to a complete genome. It integrated 175 reads (0.0008% of the total reads) for a 70x average coverage, and had greatest nucleotide similarity (91.3%) with the Kaki13-5 isolate from *Diospyros kaki* in Japan (GenBank AB366017). Therefore, the identified contig was quite divergent from all previously identified CVd-VI isolates, as confirmed by phylogenetic relationships among sequences of the different isolates available in GenBank (Figure 2).

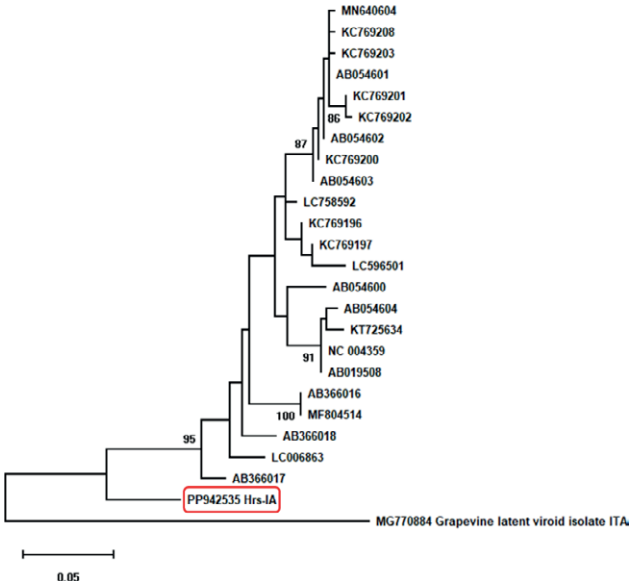

Figure 2. Phylogenetic relationships among the whole genomes of citrus viroid VI isolates obtained from GenBank (n = 23), based on a maximum-likelihood analysis and the Kimura-2 parameter method implemented in Mega X (Kumar *et al.*, 2018). The tree had the greatest log likelihood (-1382.63) generated using 1000 bootstrap replicates. The sequence of the grapevine latent viroid genome (Acc. No. MG770884) was used as the outgroup. Bootstrap values >70% are indicated near the branches. The position of the Hibiscus isolate of citrus viroid VI (named Hrs-1A; Acc. No. PP942535) is highlighted by a red box.

Figure 1. Yellowing symptoms observed on the leaves of the *Hibiscus rosa-sinensis* analysed in the present study.

The HCRSV contig and the complete genomes of the CEVd and CVd-VI isolates have been deposited in GenBank (respective Acc. Nos. PP942537, PP942536 and PP942535). In addition, the presence of these three pathogens in China rose was confirmed by the RT-PCR analysis in the original plant and in three additional symptomatic plants from the same location. HCRSV was detected using the primer pairs HCRSV-2F/HCRSV-3R (Parrella and Mignano, 2024); CVd-VI was detected using the CVd-VI-F/CVd-VI-R primers described by Cao *et al.* (2017), and CEVd was detected with primers CEVd-F/CEVd-R described by Abualrob *et al.* (2024).

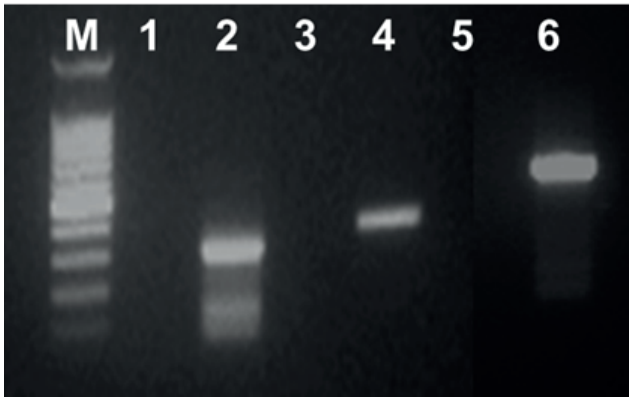

Figure 3. Validation of the HTS results by RT-PCR using a specific primer pairs for CVd-VI (lane 2; 326 bp), CEVd (lane 4; 371 bp), and HCRSV (lane 6; 697 bp) infections in the original and re-analyzed *Hibiscus rosa-sinensis* plant. Lanes 1, 3 and 5 are the negative controls for each RT-PCR. M is the 100 bp ladder.

Amplicons obtained by RT-PCR were further sequenced from both ends at Macrogen (Milano, Italy), and the sequences obtained were found to be 100% identical to the respective contigs obtained by HTS (Figure 3).

Different sensitivity and specificity in HTS-based detection of plant viruses have been recently reported. In particular, RNA-Seq approaches demonstrated better performance than small RNA-Seq (Di Gaspero *et al*., 2022) This could explain why, in the first siRNA-based HTS analysis of the China rose leaf sample, the two viroids were not identified (Parrella and Mignano, 2024). Alternatively, it is possible that the analysis of samples collected in two different periods (late summer, by Parrella and Mignano (2024) and spring, in the present study) may have contributed to the different results. This was likely to be due to differences in accumulations in plant tissues of the two viroids during the two different seasons.

With the increased use of HTS analyses for plant virome characterization, multiple infections by viruses and/or viroids have become increasingly evident, particularly in perennial plants. The present study detected multiple infections in China rose plants, involving HCRSV (already reported, Parrella and Mignano, 2024), and the two viroids CEVd and CVd-VI, for all of which China rose is a new host. In addition, these results are the first detection of CVd-VI in Italy.

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