Short Notes

Identification, full-length genome sequencing, and field survey of citrus vein enation virus in Italy

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Summary. Citrus vein enation virus (CVEV) was described in Spain and then it has been reported in several citrus growing areas of Asia, America and Australia. Here, the occurrence of CVEV in Italy has been documented for the first time. The full genome sequence of a CVEV Italian isolate (14Q) was determined by high-throughput sequencing and the presence of the virus was confirmed by RT-PCR and graft-transmission to indicator plants, from which the virus was recovered six-months post-inoculation. Phylogenetic analysis based on the full-length genome of CVEV isolates from different countries showed that they are phylogenetically related to each other based on their geographic origin, rather than on their host and that the Italian isolate is more closely related to the Spanish isolate than to the other ones. A field survey revealed the presence of CVEV in some areas of Campania region (southern Italy), prevalently infecting lemon trees. In the frame of this survey, kumquat was identified for the first time as a host of CVEV. No symptoms were observed in the field so far. The infection of asymptomatic hosts and the transmission by aphid species present in Italy increase the risk that the virus could further spread.

Keywords. Citrus virus, vein enation disease, woody gall disease, CVEV.

INTRODUCTION

Citrus vein enation virus (CVEV), a monopartite, single strand positive sense RNA virus (Luteoviridae, Enamovirus), has been proposed as the causal agent of citrus vein enation (VE) and woody gall (WG) (Vives et al., 2013). These two graft-transmissible citrus diseases were first described in United States of America in sour orange (VE) and in Australia in rough lemon (WG) (Wallace & Drake, 1953; Fraser, 1959; Moreno, 2000). Although CVEV may remain latent in several commercial citrus cultivars, it has been found associated with small outgrow in the underside tissues of leaves (enations), mainly in correspondence to secondary veins, of some susceptible citrus species, such as Mexican lime [Citrus aurantifolia (Christm.) Swingle], sour...
orange (C. aurantium L.), C. junos Siebold ex Tanaka, citron (C. medica L.), satsuma (C. x unshiu) and various local Chinese citrus varieties (Chen et al., 1992). Woody gall disease, characterized by galls on trunks and/or branches, has been reported in Rangpur lime (C. x limonia Osbeck), rough lemon (C. jambhiri Lush), and C. x volkameriana rootstocks (Fraser, 1959). Based on bioassays, it was shown that both VE and WG diseases were caused by the same transmissible agent (Wallace and Drake, 1960), now identified as CVEV (Vives et al., 2013). This virus is transmitted in a persistent manner by several aphid species, including Toxoptera citricidus, Myzus persicae and Aphis gossypii, (Hermoso de Mendoza et al., 1993; Maharaj and da Graca, 1989) and is considered a quarantine pest in several Mediterranean and American countries (https://gd.eppo.int/taxon/CVEV00/). The complete genome of twenty CVEV isolates from six countries (Spain, Japan, China, South Korea, United States of America and Australia) has been sequenced (Vives et al., 2013; Huang et al., 2015; Nakazono-Nagaoka et al., 2017; Wu et al., 2019; Yang et al., 2019). Although VE disease is widespread in Spain, it has not been reported in Italy (Catara and Davino, 1984). Spain is the only European country where the CVEV has been identified and characterized (Vives et al., 2013).

High-throughput sequencing (HTS) is the most effective technology for detection of viruses in plants (Maliogka et al., 2018). This has allowed identification of several novel viruses in citrus hosts (Loconsole et al., 2012a; Loconsole et al., 2012b; Roy et al., 2013; Vives et al., 2013; Harakava et al., 2017; Ramos-González et al., 2017; Chabi-Jesus et al., 2018; Navarro et al., 2018a; Navarro et al., 2018b; Chabi-Jesus et al., 2020; Wu et al., 2020; Xuan et al., 2020). The present study, based on HTS data, reports the first identification of CVEV in Italy. The complete genome sequence and variability of the Italian CVEV isolate is also presented, together with the results of graft-transmission bioassays. In addition, data are reported on the spread of this virus mainly in Campania region (Southern Italy), where lemons are important citrus crops.

MATERIALS AND METHODS

Plant material, RNA preparation and high-throughput sequencing of cDNA libraries

Fresh flushes, collected in 2018 from a non-symptomatic sweet orange tree (Citrus sinensis (L.) Osbeck), clementine (C. clementina Hortex Ex Tanaka), lemon [C. x limon (L.) Burm. f.], mandarin (C. reticulata Blanco), kumquat (C. japonica Thunb.), tangerine (C. x tangerina), grapefruit (C. x paradisi Macf.), sour orange, or citron (C. medica L.) trees were analyzed by RT-PCR (as outlined above) using the primer pair VE5f/VE15r. Bioassays were performed by grafting bark tissues from the Italian isolate to three sour orange indicator plants grown at 23-25°C in a greenhouse, which were sequenced (Macrogen).

RT-PCR detection, field survey and bioassays

Total nucleic acids (100 ng) were extracted and reverse transcribed as reported previously (Navarro et al., 2017). Two µL of the cDNA reaction were used for the PCR amplification in a reaction volume of 25 µL, using 1.25 units of GoTaq polymerase (Promega) and containing final concentrations of 0.2 µM of each primer. The two CVEV specific primer pairs VE5f/VE15r and VE16f/VE17r, previously reported by Vives et al. (2013) and targeting a CVEV genomic region coding for the polymerase and the coat protein, respectively, were used. The cycling conditions were as follows: initial denaturation at 94°C for 3 min, followed by 32 cycles at 94°C for 30 s, 55°C for 30 s, 72°C for 30 s, and a final extension step at 72°C for 7 min. The reaction products were analysed by electrophoresis on 2% agarose gels buffered in TAE (0.04 M Tris-acetate, 1 mM EDTA, pH 8) and visualized by UV light after ethidium bromide staining. Amplicons were purified, cloned in pGemT-easy (Promega) and sequenced (Macrogen).

A field survey was carried out in spring 2019 in 14 orchards (13 located in provinces of Campania region and one in Latina province of Lazio region). Leaves from fresh flushes from sweet orange [Citrus sinensis (L.) Osbeck], clementine (C. clementina Hortex Ex Tanaka), lemon [C. x limon (L.) Burm. f.], mandarin (C. reticulata Blanco), kumquat (C. japonica Thunb.), tangerine (C. x tangerina), grapefruit (C. x paradisi Macf.), sour orange, or citron (C. medica L.) trees were analyzed by RT-PCR (as outlined above) using the primer pair VE5f/VE15r. Bioassays were performed by grafting bark tissues from the Italian isolate to three sour orange indicator plants grown at 23-25°C in a greenhouse, which were
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then maintained under observation for two consecutive years (Roistacher, 1991).

**Phylogenetic analysis**

Sequence variability within the CVEV-14Q isolate was investigated by identifying the SNPs using Bowtie (Langmead *et al*., 2009). Multiple alignments of the full-genome, 5'UTR and 3'UTR nucleotide (nt) sequence, of the amino acid sequence of the putative proteins encoded by the Italian CVEV isolate, and with those available in databases were performed using Clustal Omega (Sievers and Higgins, 2014). A pairwise matrix was generated from each alignment. The multiple alignment of the genomic sequence was used to generate a phylogenetic tree by maximum likelihood method with the best-fit model of kimura-2+G and 1000 bootstraps inferred in the MEGA7 package.

**RESULTS AND DISCUSSION**

**Identification of an Italian isolate of CVEV**

To characterize citrus viromes in the Campania region (southern Italy), a cDNA library of total RNAs from a non-symptomatic sweet orange tree (isolate 14Q) was analyzed by HTS. A total of 24,800,000 high quality reads were obtained, and after removing those mapping was analyzed by HTS. A total of 24,800,000 high quality reads were obtained by HTS. These data confirmed the CVEV 6 months post-inoculation. No enations and no wood alterations were observed in the infected plants periodically inspected for symptom expression for more than 2 years after the graft-inoculation. However, additional bioassays with a greater number of tested plants and the other susceptible indicators rough lemon and Mexican lime must be performed to determine the pathogenicity of the CVEV-14Q isolate.

**Characterization of the full-length genome of an Italian isolate of CVEV**

Alignments of sequenced reads on the reference CVEV genome (VE-1 isolate, NC_021564) using the Bowtie software, allowed assembly of the consensus full-length genomic sequence (mean coverage depth = 33) of the first Italian CVEV isolate. This was named CVEV-14Q (Genbank ID: MW167082) (Figure 1a). The CVEV-14Q genome is 5983 nt long, with the same organization of the reference isolate VE-1, and consists of five ORFs surrounded by a 5' untranslated region (UTR) and a 3'UTR (Figure 1b). The overlapping ORF0, ORF1 and ORF2 were very likely directly translated from the genomic RNA, while the expression of ORF3 and ORF5 is proposed to occur through the synthesis of a 3' co-terminal subgenomic RNA (Vives *et al*., 2013). The abundance of this subgenomic RNA in the nucleic acid preparations used for generating the RNA seq library could justify the asymmetric distribution of the sequenced reads on the CVEV genome, with a prevalent coverage in the 3' region including ORF3 and ORF5 (Figure 1a). ORF0 (positions 219-1283) overlaps completely with ORF1, and encodes a putative protein of 39 kDa (P0) that likely is a suppressor of gene silencing (Vives *et al*., 2013). P0 contains the four conserved amino acids (LPxx(L/I)x10–13P) of a putative F-box-like motif (Huang *et al*., 2015). ORF1 (positions 208-2016) encodes for a putative peptidase of 100 kDa that contains the conserved serine proteinase domain characteristic of the S39 peptidase superfamily. The ORF2 (positions 2202-4148) is translated via a -1 ribosomal frameshift of ORF1, thus encoding a putative fusion protein of 148 kDa (P5) of 55 kDa, which is probably involved in aphid transmission,
symptom expression and virus accumulation (Vives et al., 2013).

Sequence variability within the CVEV-14Q isolate was investigated by aligning the HTS reads on CVEV-14Q consensus genome using Bowtie. This identified a total of 26 variable nucleotide positions in the CVEV genome of the 14Q virus isolate (Figure 1a; Table 1). Most of them were located between the positions 267 and 1651, which include the ORF0 overlapping with a fragment of ORF1 and a region of ORF1 not overlapping with other ORFs. Since in a sequence with overlapping ORFs one nucleotide change can differently affect the encoded proteins, we further considered the 10 mutations observed in the overlapping ORF0-ORF1 region. Most mutations were synonymous in P0 (six out of ten) and non-synonymous in the peptidase (nine out of ten). These data suggest that the nucleotide variability in this region has a greater effect on the amino acid variability in the peptidase than in the P0. Two other variable positions were identified in the replicase coding region and in the intergenic region. Although several of the mutations observed in the CVEV-14Q isolate were also identified in other CVEV isolates, most of the mutations are here reported for the first time (Table 1).

**Phylogenetic relationships between CVEV-14Q and other reported isolates**

Pairwise alignment of the CVEV-14Q full-length genome with genomes of the other 20 isolates that have been characterized from different geographic areas, revealed the greatest similarity (99.08% of nt identity) with the isolate VE-1 from Spain, and the least (97.09%) with the CVEV SM isolate from China (Figure 2). The 5’ UTR region of CVEV-14Q, showing the least similarity (92.75% of nt identity) with the Chinese SM iso-
Table 1. Sequence variability in the Italian CVEV isolate 14Q. Nucleotide variability and its effect at amino acid level are reported for polymorphic nucleotide positions.

| nt position | Nt in CVEV-14Q	extsuperscript{a} (number of reads) | Nt in reads showing variability (number of reads) | Nt in CVEV genomes from other isolates (number of isolates) | ORF | Variability in the codons (encoded amino acid) | Type of mutation | Overlapping ORF | Variability in the codons (encoded amino acid) | Type of mutation |
|-------------|--------------------------------------------------|------------------------------------------------|-----------------------------------------------------------|-----|-----------------------------------------------|----------------|---------------|-----------------------------------------------|----------------|
| 267         | A(7)                                             | T(4)                                           | A(0); T(20)                                           | Peptidase | ACA (Thr) to ACT (Thr) | Synonymous | P0          | ACT (Thr) to TCT (Ser) | Nonsynonymous |
| 290         | C(6)                                             | T(4)                                           | C(14); T(6)                                          | Peptidase | GCT (Ala) to GTT (Val) | Nonsynonymous | P0          | AGC (Ser) to AGT (Ser) | Synonymous |
| 1026        | T(6)                                             | G(3)                                           | C(16); T(4)                                          | Peptidase | TTC (Phe) to TTG (Leu) | Nonsynonymous | P0          | TTC (Ser) to GTC (Val) | Nonsynonymous |
| 1066        | A(7)                                             | T(6)                                           | A(20); T(0)                                          | Peptidase | ATT (Ile) to ATA (Ile) | Synonymous | P0          | TAT (Tyr) to TTT (Phe) | Nonsynonymous |
| 1118        | T(12)                                            | C(8)                                           | T(0); C(20)                                          | Peptidase | ATC (Ile) to ACC (Thr) | Nonsynonymous | P0          | CAT (His) to CAC (His) | Synonymous |
| 1121        | C(12)                                            | T(8)                                           | C(0); T(20)                                          | Peptidase | TCC (Ser) to TCC (Phe) | Nonsynonymous | P0          | CTC (Leu) to CTT (Leu) | Synonymous |
| 1135        | A(15)                                            | G(7)                                           | A(20); G(0)                                          | Peptidase | ACG (Thr) to GCG (Ala) | Nonsynonymous | P0          | GAC (Asp) to GGC (Gly) | Nonsynonymous |
| 1142        | A(18)                                            | G(7)                                           | A(0); G(20)                                          | Peptidase | AAG (Lys) to AGG (Arg) | Nonsynonymous | P0          | GAA (Glu) to GAG (Glu) | Synonymous |
| 1151        | C(22)                                            | T(7)                                           | C(0); T(20)                                          | Peptidase | GCT (Ala) to GGT (Val) | Nonsynonymous | P0          | GGC (Gly) to GGT (Gly) | Synonymous |
| 1277        | A(21)                                            | G(3)                                           | A(4); G(16)                                          | Peptidase | AAG (Lys) to AGG (Arg) | Nonsynonymous | P0          | GAA (Glu) to GAG (Glu) | Synonymous |
| 1374        | A(23)                                            | G(4)                                           | A(3); G(17)                                          | Peptidase | AAA (Lys) to AAG (Lys) | Synonymous |              |                                 |              |
| 1446        | T(25)                                            | A(7)                                           | A(1); T(19)                                          | Peptidase | ATT (Ile) to ATA (Ile) | Synonymous |              |                                 |              |
| 1509        | C(10)                                            | T(9)                                           | C(6); T(12); A(2)                                    | Peptidase | TCC (Ser) to TCT (Ser) | Synonymous |              |                                 |              |
| 1512        | G(10)                                            | A(9)                                           | G(1); A(9)                                           | Peptidase | AAG (Lys) to AAA (Lys) | Synonymous |              |                                 |              |
| 1525        | G(10)                                            | A(9)                                           | G(20); A(0)                                          | Peptidase | GTT (Val) to AGT (Ser) | Nonsynonymous |              |                                 |              |
| 1530        | G(11)                                            | A(9)                                           | G(20); A(0)                                          | Peptidase | GTG (Val) to GTA (Val) | Synonymous |              |                                 |              |
| 1533        | C(11)                                            | T(9)                                           | C(20); T(0)                                          | Peptidase | TAC (Tyr) to (TAT) Tyr | Synonymous |              |                                 |              |
| 1551        | T(11)                                            | C(5)                                           | T(19); C(1)                                         | Peptidase | GAT (Asp) to GAC (Asp) | Synonymous |              |                                 |              |
| 1557        | A(11)                                            | T(5)                                           | A(19); G(2)                                         | Peptidase | CTA (Leu) to CIT (Leu) | Synonymous |              |                                 |              |
| 1563        | G(11)                                            | A(5)                                           | G(20); A(0)                                          | Peptidase | TGG (Leu) to TTA (Leu) | Synonymous |              |                                 |              |
| 1611        | C(11)                                            | T(6)                                           | C(20); T(0)                                          | Peptidase | CTC (Leu) to CIT (Leu) | Synonymous |              |                                 |              |
| 1650        | T(11)                                            | C(6)                                           | T(3); C(17)                                         | Peptidase | TCT (Ser) to TCC (Ser) | Synonymous |              |                                 |              |
| 1651        | T(11)                                            | C(6)                                           | T(0); C(20)                                         | Peptidase | TTC (Phe) to CTC (Leu) | Nonsynonymous |              |                                 |              |
| 2454        | A(20)                                            | G(7)                                           | A(20); G(0)                                         | Peptidase | CGA (Arg) to CGG (Arg) | Synonymous |              |                                 |              |
| 3258        | T(13)                                            | C(12)                                          | T(3); C(17)                                         | Peptide | TGC (Cys) to CGC (Arg) | Nonsynonymous |              |                                 |              |
| 4214        | A(39)                                            | G(12)                                          | A(20); G(0)                                         | Intergenic |                                 |              |              |                                 |              |

\textsuperscript{a}Consensus sequence ID: MW16708
late, was more divergent than the 3'UTR from the other sequenced isolates. Amino acid sequence comparisons between homologous proteins (Figure 2) identified the putative peptidase protein and the replicase as the proteins of CVEV-14Q that mostly differed from the other isolates, especially from the Chinese isolate SM (96.78% aa identity with the putative peptidase protein and 96.83% aa identity with the replicase). The putative CP is the most conserved protein among the studied isolates, with the exception of the slightly divergent isolates SM from China (99.48% aa identity), IBK from Japan (99.48% aa identity) and JJ from Korea (98.95% aa identity). The other predicted proteins P0 and P5 also showed high sequence similarities with the respective proteins encoded by other isolates.

Although not yet associated with any biological roles, amino acid sequence changes in some CVEV isolates have been previously highlighted (Nakazono-Nagaoka et al., 2017). Four amino acid residues, at positions 83, 104 and 113 of ORF2 and position 41 of ORF5, have been proposed as differential residues among Spanish and Japanese isolates (Nakazono-Nagaoka et al., 2017). Like the Spanish isolates, the CVEV-14Q has a leucine and an arginine at positions 83 and 104 of the protein encoded in ORF2, whereas most of the other known CVEV isolates have a phenylalanine and a glycine at the same positions. The exception is isolate VE823, which also has an arginine at position 104 (Supplementary Figure 1). The amino acid at position 113 of ORF2 is a valine in most isolates, except in the Italian isolate 14Q (a threonine in this position), the Spanish isolate VE-1 (an isoleucine) and the Korean isolate JJ (an alanine). The serine at the position 41 of ORF5 remains specific of the Spanish isolates since in all the other CVEV characterized, including the Italian CVEV-14Q, there is an arginine at that position. Therefore, although closely related to other, the Italian and Spanish isolates had some diversity.

Relationships between CVEV isolate 14Q and virus isolates from different geographic areas and host species were investigated by generating a maximum likelihood phylogenetic tree using the available full-genomic sequences of the virus (Figure 3). The Italian 14Q and the Spanish VE-1 isolates clustered together. All CVEV isolates from the United States of America were grouped in the same clade, and those from Japan and South Korea clustered in the same phylogenetic subgroup. The CVEV isolate VE709 from Australia was related to the phylogenetic subgroup of American isolates, whereas the isolate from China was unrelated to the other CVEV clades. Therefore, CVEV isolates grouped according to their geographic origins, rather than their host species, indicating a minor role of hosts in the genetic diversity of CVEV isolates.

Field survey

Thirteen citrus growing areas of the Campania region and one on Latium region, at the northwest border of Campania (Suio, Latina Province), were surveyed. In Campania, the most common citrus species are sweet orange and lemon, which are widely grown in commercial citrus orchards as well as in private and public gardens. A total of 229 citrus trees of different species were tested by RT-PCR. The assays showed that 32 tree samples (14.0%) were infected by CVEV, with lemon trees showing the greatest incidence (26.4%) (Table 2). This is the first report of CVEV in Kumquat (https://gd.eppo.int/taxon/CVEV00), but only one of two plants analysed, collected from a household garden, tested positive. The tangerine, grapefruit, sour orange, and citron trees from Campania region analyzed in the survey tested negative for CVEV infections. However, in these cases, only very few plants of each host species were tested. None of the infected trees showed the typical symptoms of VE or WG that have been reported to be associated to CVEV in some citrus species. This agrees with previous data reporting symptomless infections of CVEV in lemon, mandarin and orange (Catara and Davino, 1984).

When the geographic distribution of infected samples was considered, the positive samples were only from some areas (mainly in Naples and Salerno province).
These data indicate that CVEV is not widespread in the surveyed area. However, since this virus is transmitted by several aphid species (Hermoso de Mendoza et al., 1993; Maharaj and da Graça, 1989), two of which, *Myzus persicae* and *Aphis gossypii*, are present in the surveyed area, the virus will likely be further disseminated. Asymptomatic infected citrus trees can also be sources of inoculum if used for vegetative propagation. Detection of CVEV in a symptomless kumquat tree is important. The trees are mainly grown in pots as ornamental plants, so they could favour long distance virus spread. Extension of CVEV surveys to a greater number of kumquat trees and to other ornamental citrus species would be worthwhile.

Inclusion of CVEV in the list of quality-affecting organisms in the Italian programs for certification of citrus propagation material is strongly recommended, as the present study has demonstrated that several citrus species can be infected by CVEV.

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Table 2. Survey of citrus vein enation virus in different orchards of Campania region and one orchard of Latina province. Number of CVEV infected trees /number of total trees analyzed is shown.

| Geographic area (Province) | Total citrus trees | Lemon | Sweet orange | Mandarin Clementine | Kumquat | Tangerin | Grapefruit | Sour orange | Citron |
|---------------------------|--------------------|-------|--------------|---------------------|--------|---------|------------|-------------|--------|
| Averno (Naples)           | 1/6                | 1/6   | -            | -                   | -      | -       | -          | -           | -      |
| San Antonio Abbate (Naples)| 3/19               | -     | 1/8          | 2/10                | 0/1    | -       | -          | -           | -      |
| Agnano (Naples)           | 0/2                | -     | -            | -                   | 0/2    | -       | -          | -           | -      |
| Casoria (Naples)          | 12/23              | 8/9   | 2/7          | 1/4                 | -      | 1/2     | 0/1        | -           | -      |
| Ercolano (Naples)         | 1/8                | 1/2   | 0/1          | 0/4                 | 0/1    | -       | -          | -           | -      |
| Napoli (Naples)           | 3/8                | 3/3   | -            | 0/1                 | 0/2    | -       | -          | 0/2         | -      |
| Ponticelli (Naples)       | 2/10               | 1/3   | 1/3          | 0/1                 | 0/3    | -       | -          | -           | -      |
| Maddaloni (Caserta)       | 0/16               | -     | 0/7          | 0/8                 | 0/1    | -       | -          | -           | -      |
| Sta Maria Capua Vetere (Caserta)| 0/10          | -     | 0/8          | 0/2                 | -      | -       | -          | -           | -      |
| Maiori (Salerno)          | 1/13               | 1/13  | -            | -                   | -      | -       | -          | -           | -      |
| Minori (Salerno)          | 8/26               | 8/26  | -            | -                   | -      | -       | -          | -           | -      |
| Eboli (Salerno)           | 0/47               | 0/15  | 0/26         | -                   | 0/2    | 0/2     | -          | -           | 0/2    |
| Pontecagnano (Salerno)    | 0/11               | 0/5   | 0/1          | 0/1                 | 0/2    | -       | -          | -           | 0/1    |
| Suio (Latina)             | 1/30               | 0/5   | 1/15         | 0/6                 | 0/4    | -       | -          | -           | -      |
| **Total**                 | **32/229**         | **23/87** | **5/76**   | **3/37**              | **0/18** | **1/2**  | **0/3**    | **0/2**              | **0/3** |

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