



## SPREAD OF *CITRUS TRISTEZA VIRUS* IN GREECE AND CHARACTERISATION OF EXTANT ISOLATES WITH CLASSICAL AND MODERN TECHNOLOGIES (NGS)

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### Introduction

*Citrus tristeza virus* (CTV), the most important quarantine virus of citrus trees, was first reported in Greece 17 years ago and the country was declared an EU “Protected Zone”. Although phytosanitary measures undertaken by the national competent authorities appeared to contain CTV for more than a decade, during the last few years new virus foci and isolates emerged (Figure 1). The Prefectures of Argolida (Peloponnese) and Chania (Crete) do not belong to the “Protected Zone”, since 2011 and 2014, respectively. Extensive surveys (Table 1) are carried out every year in the frame of a national survey programme and results are communicated to the EU Authorities.

Extant CTV isolates were characterized serologically and molecularly and they belong to three out of the six currently identified virus phylogenetic groups (Harper, 2013), i.e. T30 (T386) (Varveri *et al.*, 2006), VT “Western type” (Varveri *et al.*, 2014), and Resistance Breaking “RB” (Owen *et al.*, 2014).

In the year 2016 CTV was for the first time identified in an “Ortanique” mandarin grove of Lakonia Prefecture (Peloponnese). The obtained M423GR isolate was positive to the MCA13 monoclonal antibody, indicating a probable severe phenotype. Following EFSA’s recommendations (EFSA PLH Panel, 2014), it was essential to proceed with the full genome identification of M423GR to obtain an accurate isolate genotype characterisation. NGS was the most appropriate method to obtain rapidly the information needed.



**Fig.1.** Map of CTV spread in Greece showing the location and year of identification.

Green colour represents eradicated foci.

**Table 1.** The National Survey project for CTV

Year	No of trees tested	CTV positive
2009	12.285	8
2010	12.886	144
2011	24.013	278
2012	19.198	110
2013	20.964	126
2014	22.675	491
2015	19.905	495
2016	29.935	667

### Materials and methods

#### Plant Material and siRNA extraction

Leaf tissue (midribs) from a CTV (M423GR) infected “Ortanique” mandarin from Lakonia Prefecture was used for siRNA extraction using mirPremier microRNA isolation kit (Sigma)

#### NGS technology

Illumina (GenXPro, Frankfurt, Germany)

#### Bioinformatics

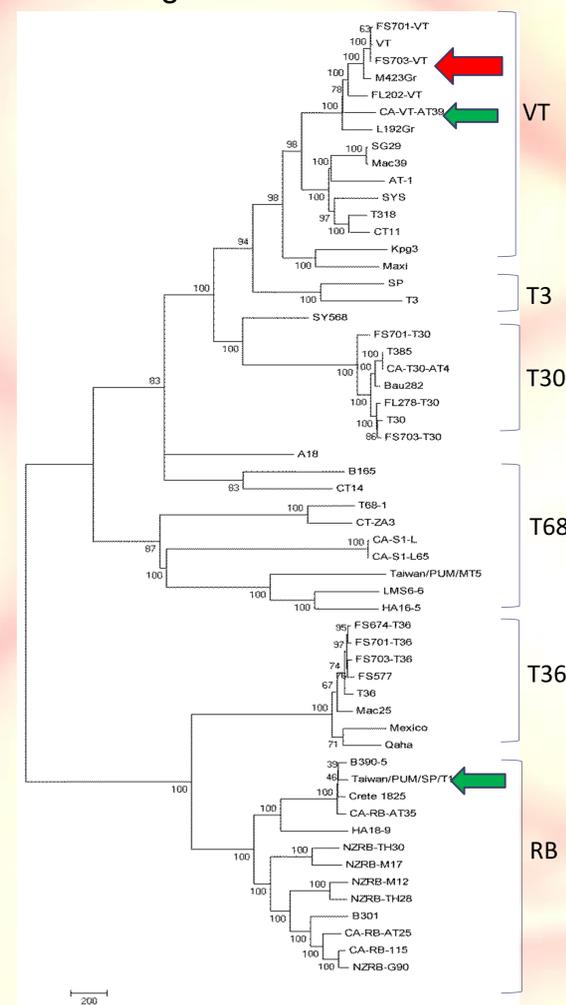
NGS data were analysed using Velvet, AssemblyAssembler and VirusDetect 1.6. Results were confirmed using Geneious 1.10.

### Results

The NGS analysis of M423GR provided a total of 16,1 Mb (reads) of which 90% were of high quality (> Q20). BLAST analysis of the total read data with quality >Q25 showed that 87,5% aligned to the reference CTV sequence (GenBank acc. no. EU937519). A total of 690.000 nodes of 27-30 nt were assembled and the contigs obtained permitted construction of the complete CTV genome of 19.254 nt. The M423GR CTV isolate genomic sequence was deposited in the GenBank database under the acc. no. MF595989.

Sequence analysis showed that the complete genome of M423GR shared maximum sequence identity of 99% with the mild American isolate FS2-2 (GenBank acc. no. EU937519) and 98,7% with the mild Greek isolate L192GR (Varveri *et al.*, 2014).

NGS data were in total agreement with those obtained through classical Sanger partial sequencing of the CP and P20 virus genes.



**Fig. 2.** Neighbour-joining phylogenetic tree obtained by full genome analysis of M423GR (MF595989, red arrow), other two Greek isolates (L192GR, Crete 1825, green arrows) and reference CTV isolates.

### Conclusions

• A CTV isolate identified in a new focus in Greece was readily fully sequenced by NGS following EFSA’s recommendations for CTV genotype characterisation.

• It is highly likely that it is a mild VT isolate, since it shares 98,7% sequence identity with the mild fully characterised L192GR, from which it might have derived through natural or artificial transmission.

• CTV is spreading in Greece in new areas, new isolates are identified and eradication measures do not seem to be able to contain the virus. Therefore, new strategies have to be implemented.