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Centro di ricerca
Difesa e Certificazione

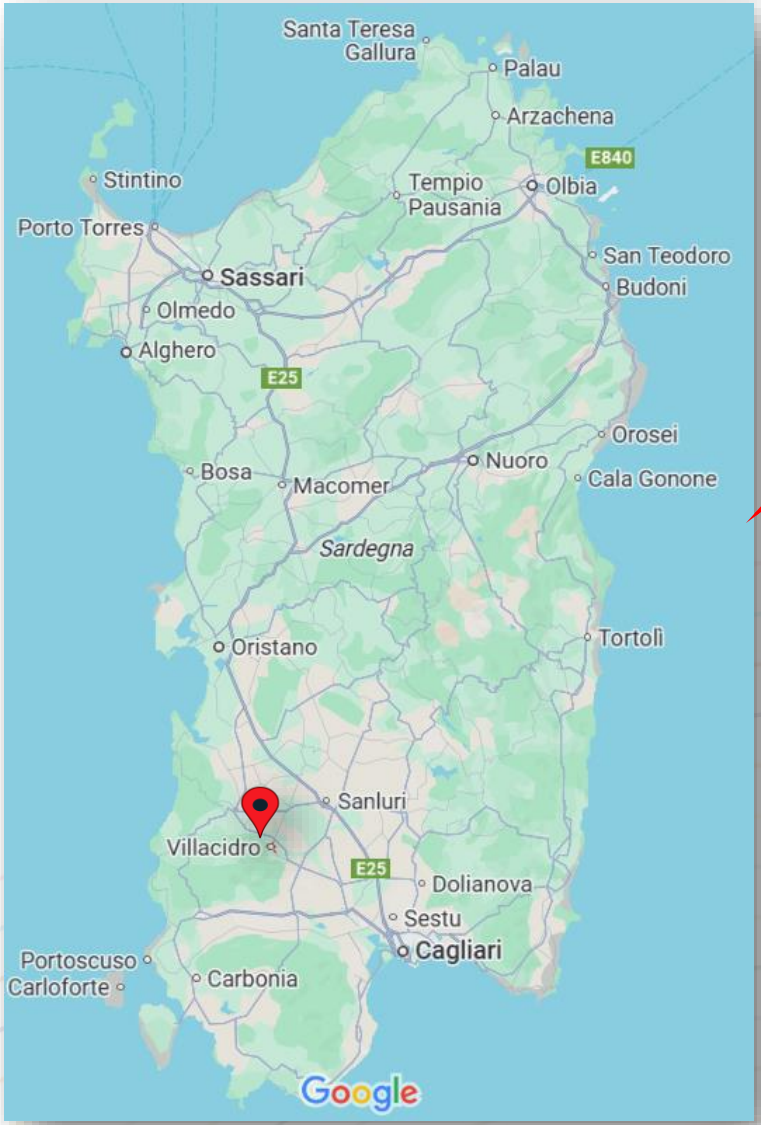
6th EPPO Workshop for Heads of Plant Pest Diagnostic Laboratories
Saku (EE), 2025-03-06/07

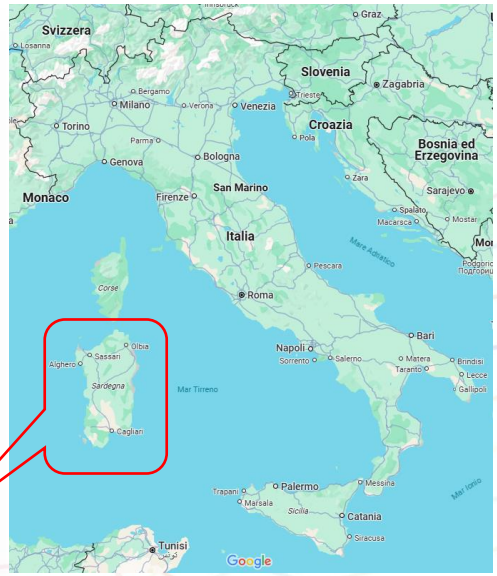
HTS sequencing and phylogenetic analysis of the non-EU CTV-resistance breaking (RB) isolates first identified in Italy

Anna Taglienti, Andrea Gentili, Marta Luigi, Livia Donati, **Luca Ferretti**

*CREA-Research centre for Plant Protection and Certification (**CREA-DC**),*
Via C. G. Bertero, 22 - 00156 Rome (Italy)

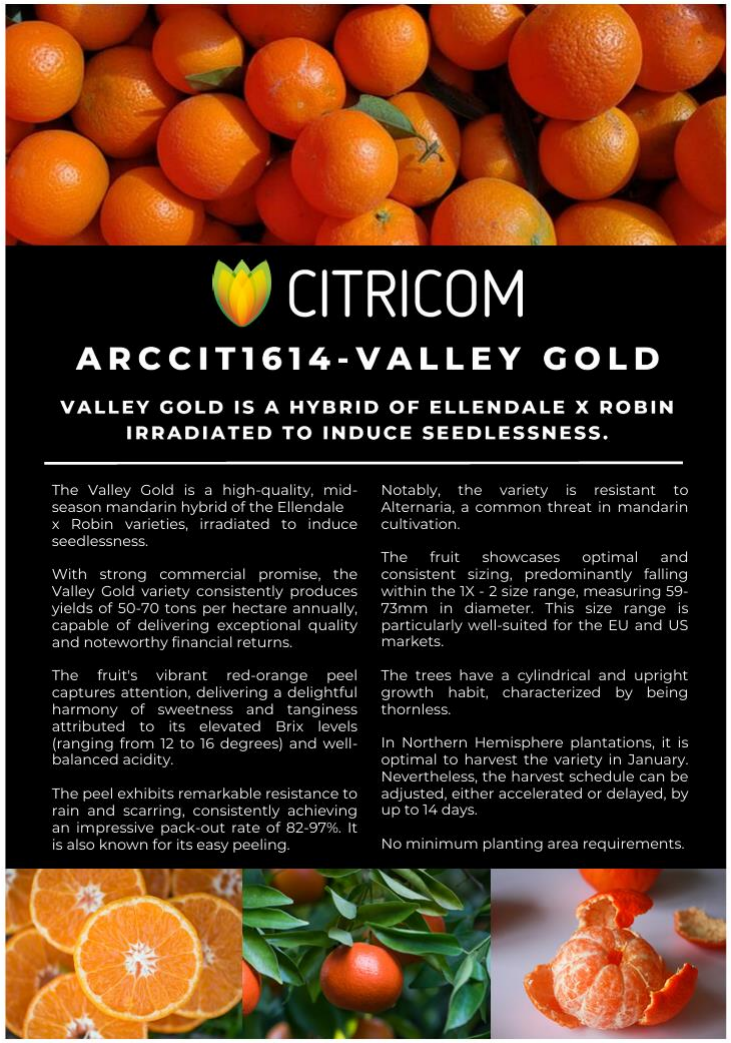
First identification of RB isolates in Italy

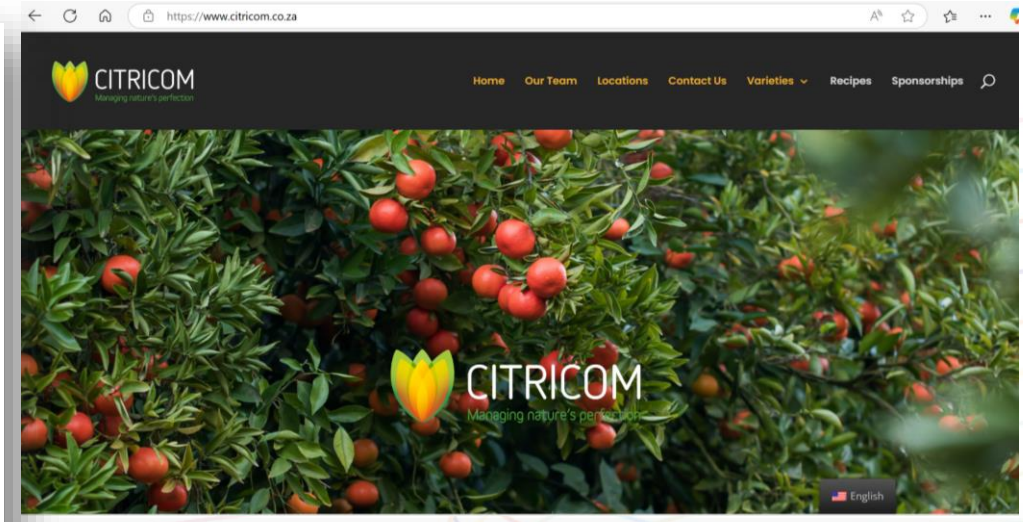




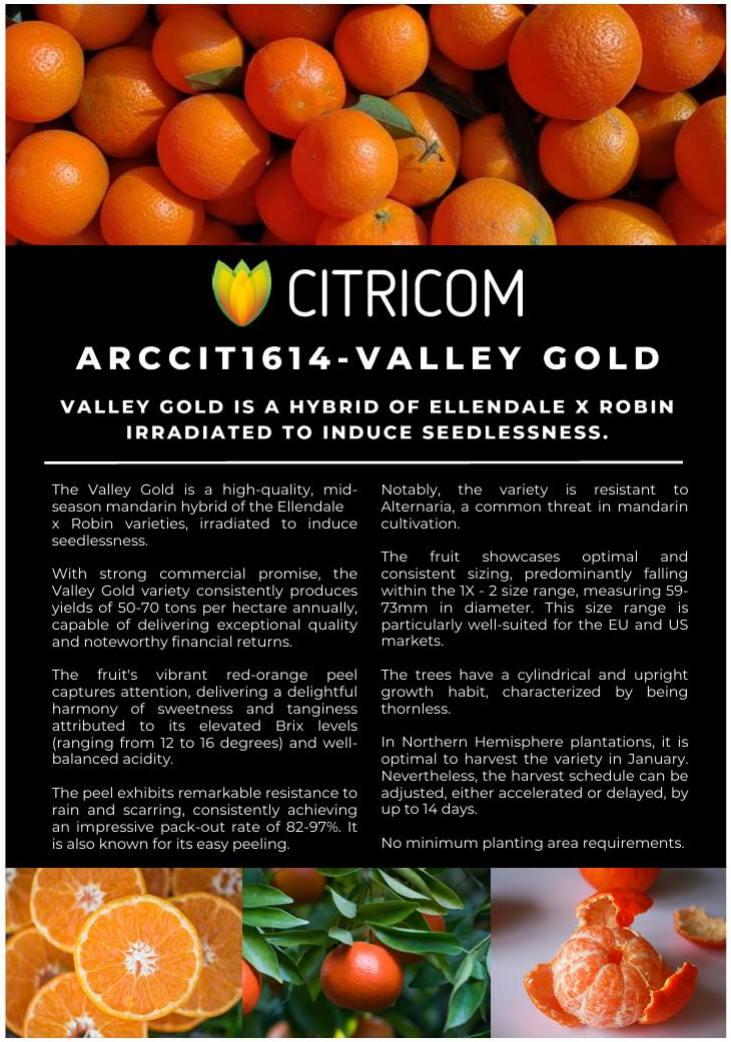
Mandarin cv Valley Gold®

Seedless mandarin (*Citrus reticulata*) hybrid patented by CITRICOM from South Africa



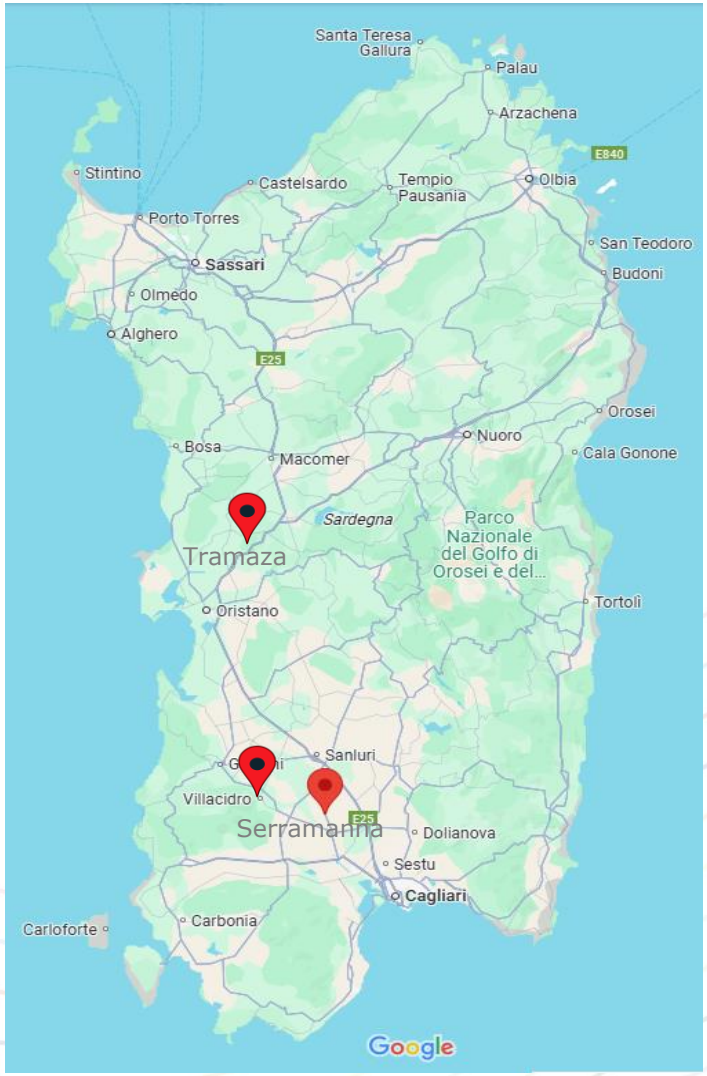


Mandarin cv Valley Gold®



- Based on our knowledge this variety was first introduced from South Africa to Spain
- In Italy, **only two nurseries authorized to propagate and commercialize plants of this variety**: one in Basilicata region (South Italy) and a second one in Calabria region (South Italy)

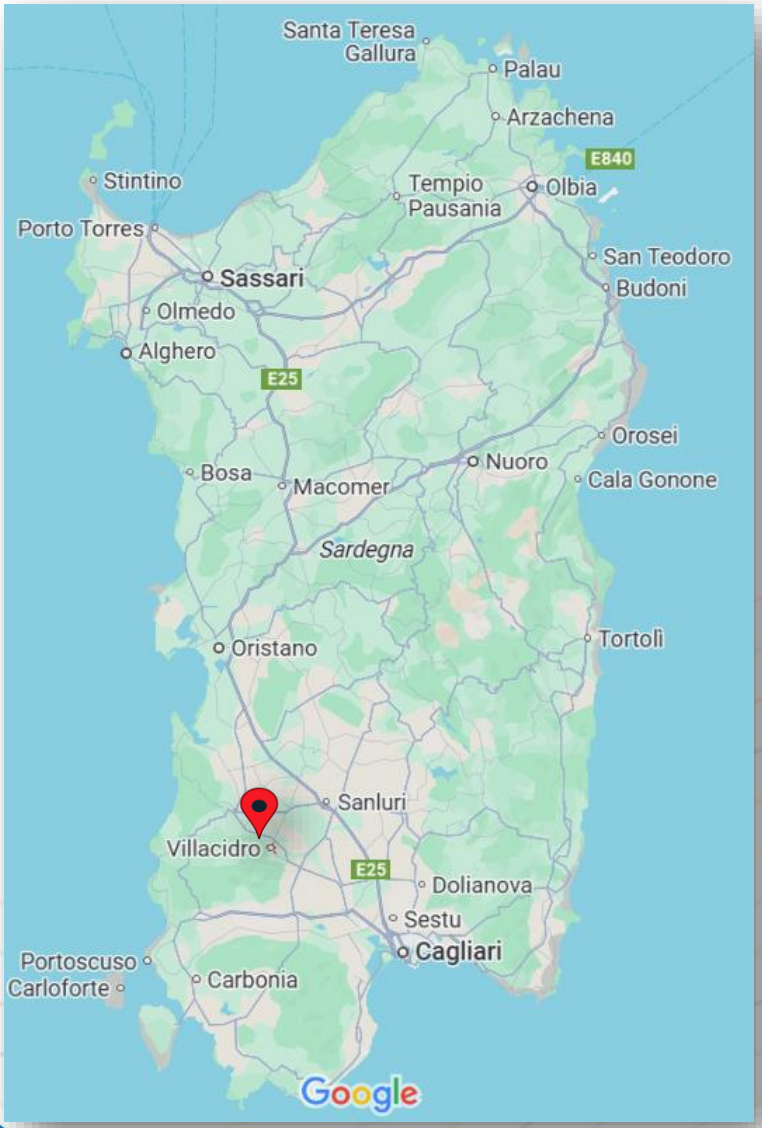
Mandarin cv Valley Gold® in Sardinia (Italy)



- In Sardinia **cv Valley Gold®** is cultivated **only in 3 farms** located in: **Tramaza** (OR), **Serramanna** (SU), **Villacidro** (SU)

- In these three sites, **all citrus groves** were established with plants of cv Valley Gold® **produced by the same nursery** located in Policoro (Basilicata region, Central Italy)

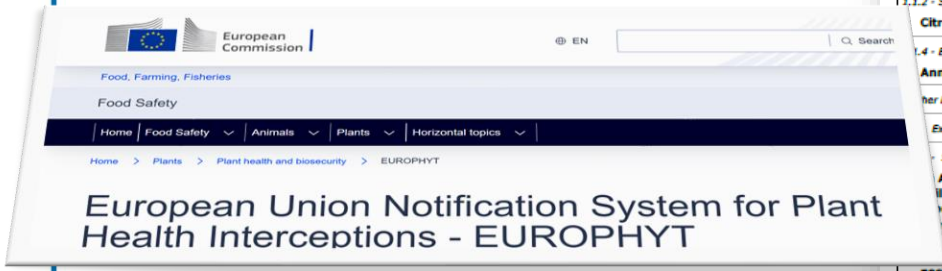
Identification of RB isolates in Sardinia: short history



- RB isolates were identified for the first time in **2024** on **4-years old, symptomless mandarin trees** (*Citrus reticulata*) cv Valley Gold® from the citrus grove located in **Villacidro** (SU), in a batch of samples **voluntarily subjected** to diagnostic analyses **by the grower** himself.

- Analyses were performed at **CNR – IPSP Bari Unit** (Bari, Italy) who promptly informed the Central Phytosanitary Service and the competent Regional Phytosanitary Service about the identification of non-EU CTV-RB isolates

Identification of RB isolates in Sardinia: short history



NOTIFICATION OF THE PRESENCE OF A PEST TO THE COMMISSION AND TO OTHER MEMBER STATES — Outbreak No. 2561

Member State:	2.1 - Notification from:	2.2 - Official contact:
IT - ITALY	SERVIZIO FITOSANITARIO CENTRALE	CARLO FRANCESCO CESARONI cf.cesaroni@politicheagricole.it +39.0646656193

Initial Notification date:	National reference number:
2024-04-18 10:26:16	IT/20/2024/3 Update No. & Date: 00/2024-04-18

1 - General Information

1.1 - Details of the identity of the pest

1.1.1 - Title:
First Presence (confirmed) of Citrus tristeza virus in ITALY (Sud Sardegna)

1.1.2 - Scientific name of the pest:	1.1.3 - EPPO preferred name:
Citrus tristeza virus	Citrus tristeza virus
1.4 - EU category of pest:	1.1.5 - EPPO category of pest:
Annex II A, Annex IV, Annex III	A2 list

...her information:

...Executive summary

...Short summary of the information submitted in points 3-7:

...April 2024, the presence of a non-EU isolate of Citrus Tristeza Virus was confirmed in a citrus grove located in Sardinia ...illacidro municipality). The Regional Phytosanitary Service carried out an official survey for CTV non-EU isolates, following a ...ort of the National Research Council of Bari. The citrus reticulata plants present in the field are 4 years old and are ...mptomatic. 22 samples were collected and 3 tested positive for Citrus Tristeza Virus non-EU isolates, RB (Resistance ...sking) genotype. The analyses were carried out by the official regional phytosanitary laboratory (test report AGRIS no.17 of 4 ...l 2024). The extracts of the positive samples were sent to the official national laboratory (CREA-DC) which confirmed the positivity (test report CREA-DC 16 April 2024).
The establishment of the demarcated area and the destruction of the three positive plants are underway.

1.2.3 - Reason for exceeding the 8 working day deadline from after official confirmation of the presence of a pest to notification (Article 32.1 of Regulation (EU) 2019/1715):

1.3 - Type of presence reported:
Presence (confirmed)

3 - Location of presence of pest

3.1 - Administrative region of the location of presence of pest

1 - NUTS year	2021
NUTS I	Isole (ITG)
NUTS II	Sardegna (ITG2)
NUTS III	Sud Sardegna (ITG2H)
Local Administrative Unit	
Other	

3.2 - Further information about location: "Please refer to boxes 6.1.4 and 7.3.2 where available"

4 - Reason for notification, pest status of the area, and the Member State concerned

4.1 - Reason for the notification
- (●) **First confirmed or suspected presence of the pest in the territory of the Member State concerned.**

4.2 - PEST STATUS of the AREA where the pest has been found to be present, AFTER the OFFICIAL CONFIRMATION.
- (●) **Present:**
  - [☑] **under eradication**

4.3 - PEST STATUS in the MEMBER STATE concerned BEFORE the OFFICIAL CONFIRMATION of the presence, or suspected presence, of the pest.
- (●) **Absent:**
  - [☑] **no pest records**

Identification of RB isolates in Sardinia: short history

- Without delay, the competent Regional Phytosanitary Services carried out an extensive **official field survey and sampling** on the same citrus grove in Villacidro: **no symptomatic plants were recorded**

- Molecular analysis based on **real time RT - PCR** technique (**Yokomi *et al.*, 2010**) was performed at the Official regional phytosanitary laboratory **AGRIS** and **3 samples tested positives with the T36NS probe** that recognizes the RB, S1 and HA16-5 phylogenetic groups

- **VT/T3 CTV isolates were also identified** by the same molecular test in some samples but **no mixed infection** with RB isolates were recorded

Identification of RB isolates in Sardinia: short history

- **Plant and RNA samples** from the three plants identified as RB-infected were sent by the Regional Phytosanitary Services to the **National Reference Laboratory (CREA-DC, Rome, Italy)** which **definitively confirmed** the presence of CTV – RB isolates by real time RT-PCR (**Yokomi *et al.*, 2010**) and RB-specific conventional RT-PCR (**Roy et al., 2013**)

Identification of RB isolates in Sardinia: short history

- Following the first identification of RB isolates in Villacidro, further **official field surveys and sampling** were carried out in the other two citrus groves located in: **Tramaza** (OR) and **Serramanna** (SU)

- **CTV-RB infected plants found** in the citrus grove located in **Serramanna** (SU), **less than 20 km away from Villacidro** but **not** in the **400 plants** of the citrus grove located in **Tramaza** (OR), **more than 60 km away** from Villacidro.

HTS sequencing

- **RNA** from **6** RB-infected plants (**3** from Villacidro and **3** from Serramanna) were submitted to **HTS sequencing**

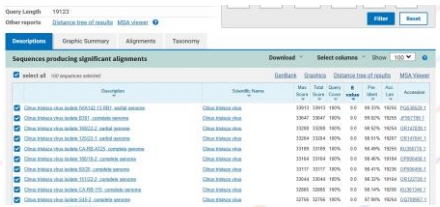
Total RNA
rRNA depleted
NextSeq 2000
2 x 150 bp

raw reads
(~ 20 M)

adapter & quality trimming, host filtering

de novo assembly
Geneious

BLASTn contigs
GenBank plant viruses



★ complete genome sequence

Taxonomic classification
ICTV demarcation criteria
Phylogenetic analysis

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- For all sec… **identity (… isolate IVI…** …nce …isch

Query Length 19237
Other reports Distance tree o…

Descriptions | Graphic Su…

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- Citrus tristeza virus isolate IVIA14…
- Citrus tristeza virus isolate B301…
- Citrus tristeza virus isolate 168/22…
- Citrus tristeza virus isolate CA-RE…
- Citrus tristeza virus isolate 125/22…
- Citrus tristeza virus isolate 60/20…
- Citrus tristeza virus isolate 186/18…
- Citrus tristeza virus isolate 151/22…
- Citrus tristeza virus isolate CA-RE…
- Citrus tristeza virus isolate S45-2…
- Citrus tristeza virus isolate NZRB…

Reset

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MSA Viewer

Accession
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KU356770.1
OR147841.1
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OP006458.1
OR122728.1
KU361340.1
OQ708957.1
FJ525432.1

Citrus tristeza virus isolate IVIA142.13.RB1, partial genome

GenBank: PQ538529.1

FASTA Graphics

Go to:

```
LOCUS       PQ538529               19255 bp    RNA     linear   VRL 26-NOV-2024
DEFINITION  Citrus tristeza virus isolate IVIA142.13.RB1, partial genome.
ACCESSION   PQ538529
VERSION     PQ538529.1
KEYWORDS    .
SOURCE      Citrus tristeza virus
  ORGANISM  Citrus tristeza virus
            Viruses; Riboviria; Orthornavirae; Kitrinoviricota; Alsuviricetes;
            Martellivirales; Closteroviridae; Closterovirus.
REFERENCE   1  (bases 1 to 19255)
  AUTHORS   Ruiz-Garcia,A.B. and Olmos,A.
  TITLE     Complete genome of Spanish RB1 genotype citrus tristeza virus (CTV)
  JOURNAL   Unpublished
REFERENCE   2  (bases 1 to 19255)
  AUTHORS   Ruiz-Garcia,A.B. and Olmos,A.
  TITLE     Direct Submission
  JOURNAL   Submitted (29-OCT-2024) Plant Protection and Biotechnology,
            Instituto Valenciano de Investigaciones Agrarias (IVIA), Ctra
            Moncada-Naquera km 4.5, Moncada, Valencia 46113, Spain
COMMENT     ##Assembly-Data-START##
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                                     Prime v. v.2023
            Sequencing Technology :: Illumina
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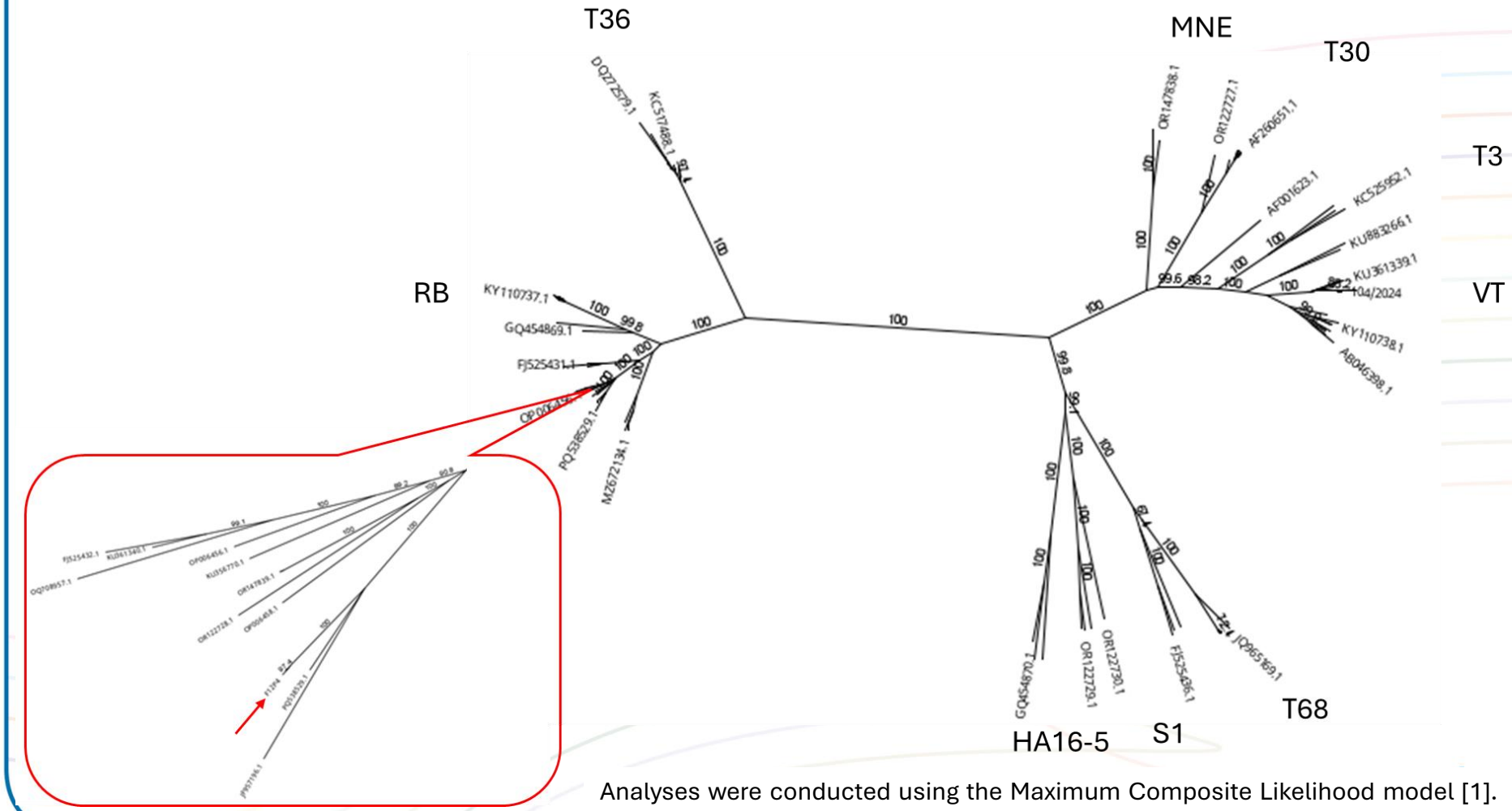
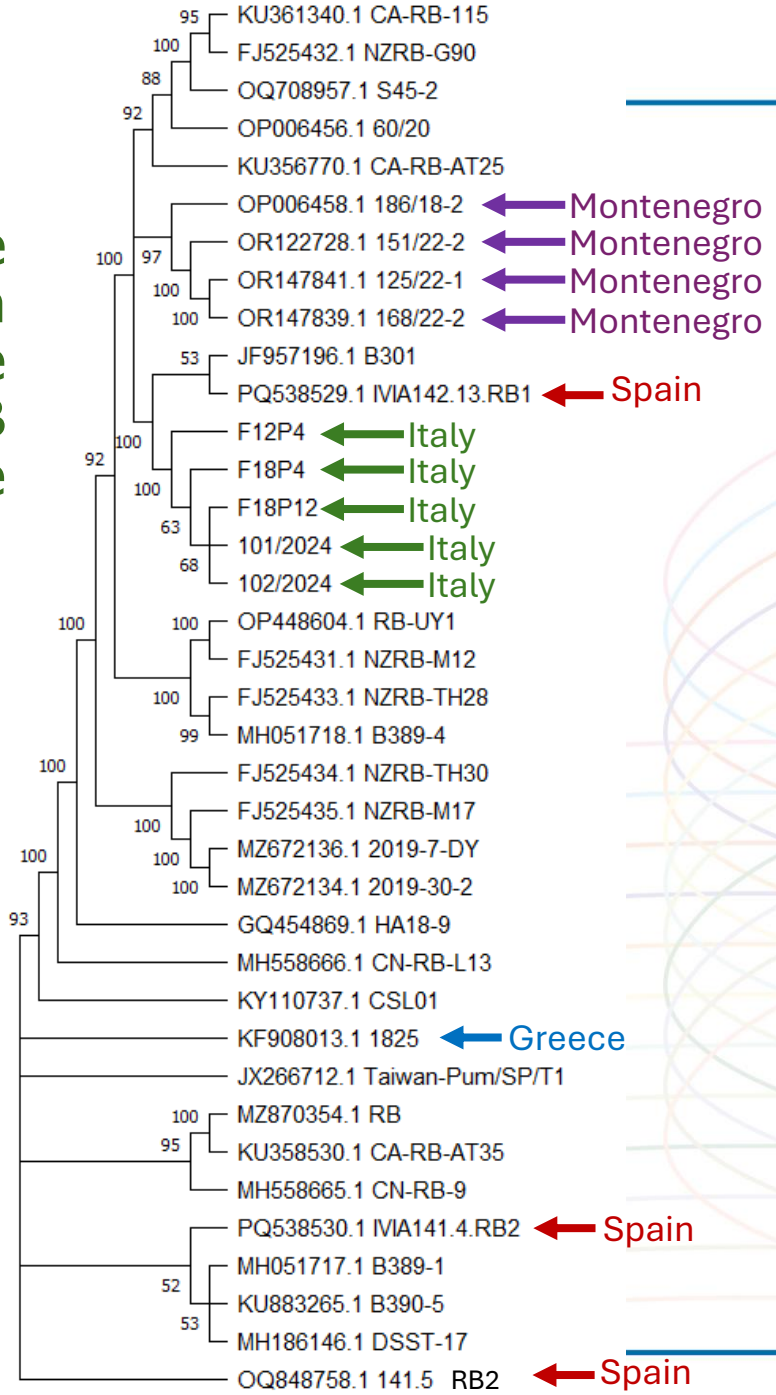
Phylogenetic analysis

- Phylogenetic tree including complete genome sequences from **Bester *et al.* (2021)** and **Zindović *et al.* (2024)**



Analyses were conducted using the Maximum Composite Likelihood model [1]. The rate variation among sites was modeled with a gamma distribution (shape parameter = 1). This analysis involved 102 nucleotide sequences.

- Phylogenetic tree generated with complete genome sequences of **RB isolates** available in GenBank



Conclusions and considerations

- The presence of CTV-RB isolates, genetically similar among themselves and to a Spanish isolates, was confirmed in *C. reticulata* plants cv Valley Gold® in 2 of the 3 citrus groves planted with this variety in Sardinia
- No symptoms resembling those of CTV were never observed in the field regardless the trees were infected by RB or VT/T3 isolates
- To date, no proofs are available on a possible introduction of RB isolates throughout the plant propagation material of cv Valley Gold®: when the nursery in Policoro (Basilicata region) was inspected by the Regional Phytosanitary Service plant propagation material of this variety was not present
- However, the fact that no CTV infected plants were found in the citrus groves located away from the outbreak area, planted with propagation material of the same type and origin makes it possible to hypothesize an in-field infection

Conclusions and considerations

Should we still consider RB isolates as non-EU quarantine pest?

- They have been detected in several countries of the European territory, to date (Greece, Italy, Montenegro, Spain)
- Although timely phytosanitary measures must be taken when detected (including uprooting of infected plants), there are evidences that a spread in the field is already occurring in some citrus growing areas
- They are generally reported as biologically mild isolates for which not noticeable symptoms are currently described in the field (symptoms in trees infected by RB isolates are often due to co-infections with severe CTV isolates)

All this considered, could it be more realistic to categorize these isolates as RNQP?

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Thank you for your attention!