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Dynamics of *Scaphoideus titanus* population in southern South Tyrol (Italy) and detection of Grapevine Flavescence Dorée phytoplasma in the insect with a multiplex real-time PCR

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Summary

In South Tyrol the population of *Scaphoideus titanus*, the vector of Grapevine Flavescence dorée phytoplasma (FD), has been steadily increasing since 2010. The present work provides an overview of the monitoring activity of *Scaphoideus titanus* in South Tyrolean vineyards coupled with the description of a sensitive and reliable detection method of FD in the insect vector. We have developed an endogenous control of *Scaphoideus titanus* to be used in a multiplex real-time PCR, amplifying the map gene of FD phytoplasma. We present evidence on the performance of this new vector reference and its compatibility with FD detection.

Key words: Flavescence dorée; *Scaphoideus titanus*; vector; internal control.

Introduction

Flavescence dorée is the most important grapevine yellows disease in European vineyards. The disease is caused by the grapevine Flavescence dorée phytoplasma. The intracellular bacterium, restricted to phloem sieve tubes is highly pathogenic to several major grapevine cultivars and rapidly leads to the death of the vine (Chuche and Thiéry 2014, Oliveira *et al.* 2019).

The leafhopper *Scaphoideus titanus* Ball, a Cicadellidae of American origin, is monophagous for *Vitis vinifera*. By feeding on an infected grapevine and then subsequently feeding on noninfected grapevines, *Scaphoideus titanus* spreads the disease throughout the vineyard (Vidano 1966, Maixner 2010).

Scaphoideus titanus is native to the Nearctic ecoregion and was introduced in Europe during the fifties with the import of American rootstocks. The presence of *Scaphoideus titanus* was first reported in 1958 in France, (Bonfils and Schvester 1960) and shortly after in northwestern Italy (Vidano 1964) and southern Switzerland (Baggiolini *et al.* 1968).

Recent phylogenetic analyses suggest that the FD phytoplasma strain responsible for the FD disease originated in Europe (Arnaud *et al.* 2007, Malembic-Maher *et al.* 2011), but only after the introduction of *Scaphoideus titanus*, the main known and confirmed vector of FD phytoplasma, the FD disease was rapidly spreading over the European vineyards.

An occasional vector of FD is the European latern fly *Dictyophara europea*. This species transmits FD from clematis to grapevine (Filippin *et al.* 2009), but with lower efficacy and with a low rate of FD infection (and is therefore less epidemiologically significant).

Another emerging vector is the Mosaic leafhopper, *Orientus ishidae*, an Asian species now widespread in Europe that has been confirmed to transmit 16SrV phytoplasmas to grapevines. *Orientus ishidae* and *Scaphoideus titanus* have a similar life cycle and since *Orientus ishidae* is highly polyphagous and has a lower efficacy in FD transmission it can be considered a secondary vector of FD (Lessio *et al.* 2016 and 2019).

In Europe, FD was declared in 1983 as a quarantine disease (Boudon-Padieu 2002), but despite all the quarantine measures applied, it continued its geographical expansion. At the end of the nineties FD phytoplasma was reported in Spain, Portugal and Switzerland. Currently it is present also in Austria, France, Croatia, Hungary, Serbia, Slovenia and Italy (Papura *et al.* 2012; references therein and EPPO global database 2019).

At present, the control of the disease consists mainly of three parts: (1) Enhanced surveillance through monitoring programs, (2) strict removal of infected plants, and (3) chemical control of the vector.

In Italy, it was pronounced by ministerial decree (no. 32442, in year 2000), that viticultural areas where symptomatic plants and vectors are present must be officially declared as "infected zones". In those zones a chemical control of the vector is mandatory. Several regions such as Veneto, Emilia-Romagna, Liguria, and Toscana have shown a conspicuous presence of FD (Botti and Bertaccini 2007). FD is absent in most parts of southern Italy, only one outbreak was found in Campania region and is under eradication (ENPPO 2019).

In the Province of South Tyrol (Northern Italy, Fig. 1), the first report of FD in grapevines was in 2016 in Valle Isarco. In 2018 eight grapevines in the locality of Salorno (South Tyrolean Bassa Atesina) were found infected by FD phytoplasma. This area is located close to the neighbouring

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Fig. 1: Map of South Tyrol showing the sampling locations.

province of Trentino, an area highly infested with FD phytoplasma. In 2019, sixteen single plants, distributed over the Bassa Atesina region, were found infected with FD. Due to the increase of infected plants in South Tyrol, the monitoring of *Scaphoideus titanus* was intensified.

We wanted to evaluate the presence of FD phytoplasma in the vector. Since there was no report on a positive endogenous control for the vector, we modified one of the standard methods for FD detection (EPPO, 2016), the triplex realtime-PCR (Pelletier *et al.* 2009), by replacing the internal plant specific reference by a *Scaphoideus titanus* specific control.

Material and Methods

Sample collection: Individual *Scaphoideus titanus* leafhoppers were sampled in different vineyards throughout the southern Province of South Tyrol. Starting from 2011, every year from June to October two – three sticky yellow traps (Glutor - Biogard) were positioned in each monitored vineyard. Yellow traps were replaced every two weeks by new ones and brought to the laboratory. These were checked for the presence of *Scaphoideus titanus* individuals using a binocular microscope. *Scaphoideus titanus* individuals were transferred into reaction tubes and stored at -20 °C until analysis.

In the southernmost part of South Tyrol, in the locality of Salorno, every year starting from 2011, the same eleven vineyards were used for positioning the sticky yellow traps (three traps per vineyard) for monitoring purposes. In the other localities the monitoring sites were changed annually.

DNA isolation from insects: DNA from *Scaphoideus titanus* adults was extracted by homogenizing the insects in a 400 µL of CTAB buffer (CTAB 2.5 %, Tris pH 8 100 mM, NaCl 1.4 M, EDTA 50 mM pH 8, PVP-40 1%, Proteinase K 10 mg·mL^{-1}) in a reaction tube containing a 5 mm tungsten carbide bead (Qiagen, Hilden, Germany). Samples were crushed using a Mixer Mill MM 400 (Retsch, Germany) at 30 Hz for 3 min. The DNA was extracted using the DNeasy Plant Mini kit (Qiagen, Hilden, Germany) following the instructions of the provider. Two µL of the extracted DNA were used in real-time PCR assays.

Multiplex real-time PCR for the detection of FD in insects: A partial region of the 16S mtDNA of *Scaphoideus titanus* was amplified from total genomic DNA with primers 16SarL (5'CGCCTGTTTAACAAAAACAT3') and 16sbr-H (5'CCGGTTTGAACTCAGATCATGT3') (Palumbi 1996). The amplified 600 bp fragment was cloned into the pJET1.2 vector following the instructions of the provider (CloneJET PCR cloning Kit, Thermo Fisher Scientific/Thermo Scientific) and sequenced from both directions (LGC Genomics, Berlin, Germany).

Specific primers and probe (TaqMan® MGB™, Thermo Fisher Scientific/Life Technologies Italia) for the detection of *Scaphoideus titanus* partial mitochondrial 16S mtDNA gene were designed using the "Primer Express" software (Version 3.0; Applied Biosystems, Foster City, CA, USA). For the *Scaphoideus titanus* a 71 bp fragment was amplified with primers Stit16S_F (5'CTCCCCATTAAAATTACGCTGTT3') and Stit16S_R (5'CATGTATATTTGATCCTTTTGGAAGTAAG3') together with the Stit16S_P probe (5'NED-TCCCTAAGGTAATTTTG-MGB 3'). Primers and probe for the detection of the FD map gene of the 16SrV-C, D and E group phytoplasmas are described in Pelletier *et al.* (2009).

A Plasmid pFDmap was constructed by cloning the 71 bp fragment of the FD map amplicon into the pJET1.2 vector following the instructions of the provider.

Multiplex real-time PCR analysis was performed in a final volume of 20 µL containing onefold of QuantiTect Multiplex PCR buffer (Qiagen, Hilden, Germany), 200 nM final concentration of each primer and probe and 2 µL of insect DNA. Reactions were performed on a 7500 Fast PCR System (Thermo Fisher Scientific/Applied Biosystems) as follows: 15 min at 95 °C followed by 45 cycles of 60 s

denaturation at 94 °C and 90 s for annealing and elongation at 59 °C. Threshold cycles (CT) for each PCR reaction were calculated with Applied Biosystems 7500 Software (v 2.3).

Results and Discussion

Fig. 2 shows the *Scaphoideus titanus* population dynamics of adult individuals collected at eleven sites in the locality of Salorno, separated by year and time of flight. It was the first area in South Tyrol, where a notable population of *Scaphoideus titanus* was found. Those individuals were probably migrating from the neighbouring Province of Trentino in northern direction. Recently, the presence of *Scaphoideus titanus* was detected also in vineyards located in the more northern areas including Valle Isarco and Burgraviato.

The notable increase of captured *Scaphoideus titanus* individuals in 2013 led to the decision of the local extension service to recommend an insecticide treatment against *Scaphoideus titanus*. This could explain the lower numbers of adult leafhoppers present on the yellow traps in the years following 2013. The recommendation to treat against the vectors was kept up since that and according to the South Tyrolean Extension Service for Fruit- and Winegrowing, most of the farmers in Salorno followed these recommendations on an annual basis.

Monitoring control and sanitation measures of FD disease require sensitive and reliable diagnosis. Due to the lack of an internal quality control of the extracted insect DNA, we designed primers and probe targeting the 16S of *Scaphoideus titanus* to be adapted in the Multiplex real-time PCR for detection of FD in plants (Pelletier *et al.* 2009) at the place of the internal plant control gene.

In the real time PCR, the amplification and detection of the 16S mtDNA of *Scaphoideus titanus* produces a single curve which CT values were detected between 14 and 33 cycles, corresponding to a detection limit of 3 $pg \cdot mL^{-1}$ of insect DNA. No amplification curves were observed either with FD nor with 16S mtDNA *Scaphoideus titanus* primers in reactions containing any insect DNA template (Fig. 3A). To evaluate the reliability and efficacy of the method, we performed a control experiment using artificially infected specimens. A *Scaphoideus titanus* DNA extraction was used as a matrix to spike serial dilutions of a plasmid containing the map FD amplicon (pFD). In these artificially infected samples, it was possible to amplify the map FD amplicon in a range from 3 x 106 to 0.1 $copies \cdot \mu L^{-1}$ (Fig. 3B, blue lanes). The detection of 16S mtDNA *Scaphoideus titanus* (CT = 18) did not change at any tested concentration of plasmid pFD (Fig. 3B, orange lanes).

To demonstrate the suitability of this new vector reference, we tested fourteen naturally infected *Scaphoideus titanus* collected in the neighbouring province of Trentino. Fig. 3C shows that the amount of FD (blue lanes) range from 3 x 104 to 3 $copies \cdot \mu L^{-1}$ per individual and the concentration of DNA of *Scaphoideus titanus* range from 1.4 $\mu g \cdot \mu L^{-1}$ to 17 $\mu g \cdot \mu L^{-1}$.

Using this method, always with an artificial infected sample as a positive control of the PCR, we have analysed 2,206 *Scaphoideus titanus* individuals collected in South Tyrol from 2014 to 2018. No FD was detected in these individuals.

The diagnostic method described here allows for the analysis of a high number of samples which is advantageous when a big area of monitoring must be covered and that also allows the quantification of the amount of the phytoplasma in the insects.

In Trentino, studies were conducted to assess the relation between vineyards infected with FD and the presence of infected insect vectors. In highly infected areas, the percentage of infected *Scaphoideus titanus* is 16 % (Gelmetti *et al.* 2018). It is hence reasonable that the probability to find a FD infected insect drops down in vineyards were the inci-

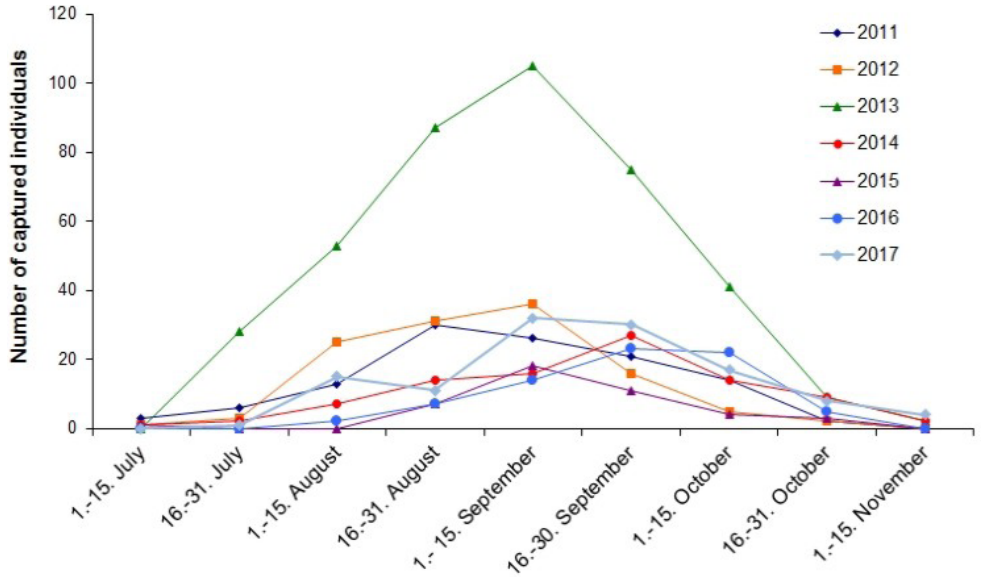

Fig. 2: Captured individuals of *Scaphoideus titanus* in the different years of monitoring. Each symbol represents the sum of all captured adult individuals per time period and corresponding year from 11 sites in the locality of Salorno (3 traps per site). Sites were the same in each year.

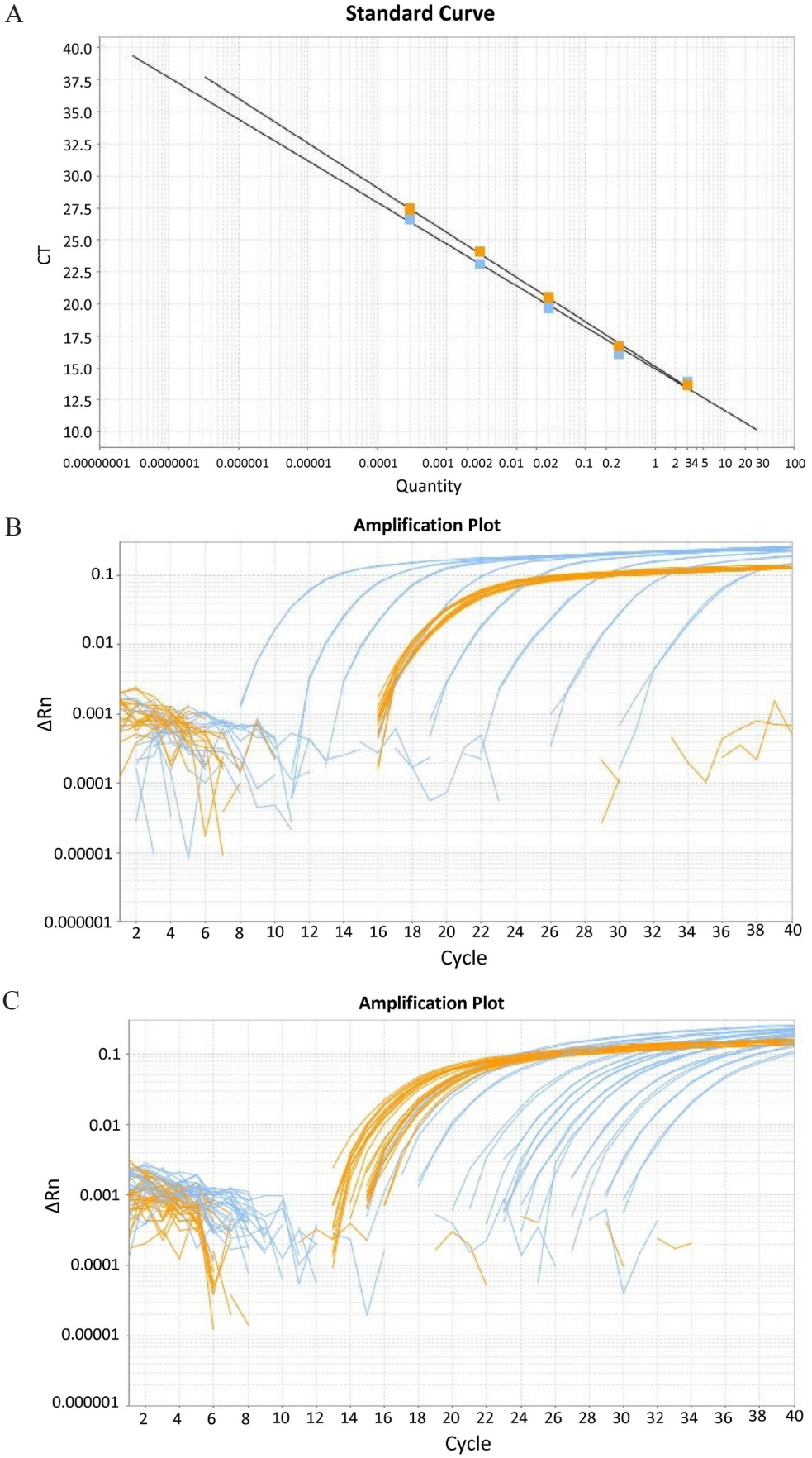

Fig. 3: (**A**) Range of a multiplex real-time PCR with primer/probe set Stit, curve with orange squares (slope = -3.483, R2 = 0.999) and FD map, curve with blue squares (slope = -3.245, R2 = 0.994). (**B**) Duplex Real-time Amplification of FD map in a DNA extraction of *Scaphoideus titanus*. Increasing tenfold dilutions of plasmid pFDmap (blue curves) were spiked into a DNA extraction of *Scaphoideus titanus* containing 66 ng·µL^{-1} of total genomic DNA (orange curves). (**C**) Duplex Real-time Amplification of FD map (blue curves) from DNA extracted from *Scaphoideus titanus* (orange curves) collected in Trentino.

dence of FD is almost zero. Currently, the Plant Protection Service in South Tyrol declare so called "infected zones" in proximity to the vineyards where the infected plants are detected. In these zones, plant protection measures against *Scaphoideus titanus* are mandatory and every symptomatic plant must be uprooted.

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