



# First molecular analysis of wisteria vein mosaic virus in Italy: eight new variants reported in *Wisteria sinensis*

Athos Pedrelli<sup>1</sup> · Alessandra Panattoni<sup>1</sup> · Lorenzo Cotrozzi<sup>1</sup>

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## Abstract

Wisteria vein mosaic virus (WVMV) is a potyvirus infecting *Wisteria* spp. worldwide, making these largely used ornamental plants unattractive and even unsalable. In 2021, nine *Wisteria sinensis* plants in Sarzana (Liguria, Italy) showing WVMV symptoms like vein mosaic with irregular patterns, mottling, deformation and twisting margin on leaves were reported. This work describes research on symptomatic and asymptomatic leaves to confirm the identity of the virus infection. All plants tested resulted positive to WVMV. Sequencing of Nib/CP genomic region and comparison on GenBank revealed the presence of eight new genetic variants named Sar 5–12. The eight nucleotide sequences alignments revealed identity ranging between 87.13 and 99.85%. Negative selection ( $dN/dS < 1$ ) was detected suggesting well adaptation in the area here examined and stability in population. Through a phylogenetic tree, WVMV isolates were grouped in four clades with high bootstrap values, two of which included the eight Italian variants here identified. Since the late 1950 and 1960s, the present study represents the first additional report of WVMV in Italy, as well as its first molecular characterization ever. Further research is required to strengthen our understanding of the movement and transmission of WVMV. Examining how virus infected plants can disseminate this pathogen could help forestall potential risks that neighbouring species may face in their environments.

**Keywords** WVMV · Nib/CP region · Single nucleotide polymorphisms · Phylogenetic analysis

## Introduction

The genus *Wisteria* Nutt. (Fabaceae), characterized by deciduous woody perennial lianas with pinnately compound leaves and pendulous racemes with typical purple, violet or white papilionaceous corollas, includes species among the most beautiful ornamental flowering plants. For this reason, these plants are grown in many gardens and landscape settings worldwide (Wei and Pedley 2010). Native to China, Japan, Korea and Eastern United States (Li et al. 2013), *Wisteria* spp. were later introduced to United Kingdom and several other European countries (Compton 2015). Actually, non-native wisterias are now considered invasive species in some areas of the United States, especially the Southeast (Trusty et al. 2007). Other than ornamentals, wisterias are

also appreciated as edible and medicinal plants, and the fiber of their stems is also used for the production of paper (Mohamed et al. 2011).

*Wisteria* spp. can be affected by several viruses including wisteria vein mosaic virus (WVMV; family *Potyviridae*; genus *Potyvirus*) (Brierley and Lorentz 1957), subterranean clover stunt virus (SCSV; family *Nanoviridae*; genus *Nanovirus*) (Grylls and Butler 1959), cucumber mosaic virus (CMV; family *Bromoviridae*, genus *Cucumovirus*) (Milojević et al. 2016) and wisteria badnavirus 1 (WBV1; family *Caulimoviridae*; genus *Badnavirus*) (Li et al. 2017). While these diseases are notorious for causing damage to flora, it is important that research into their impacts continue in order to protect our local ecosystems from widespread destruction. WVMV is the subject matter of this work. The main WVMV symptoms consist of leaf chlorotic mottling and mosaic, distortions and twisting, which are commonly more severe on the leaflets closest to the petioles. Visible symptoms of WVMV can significantly mar the ornamental quality and commercial viability of infected plants, presenting a difficult challenge to growers. WVMV infection has

✉ Alessandra Panattoni  
alessandra.panattoni@unipi.it

<sup>1</sup> Department of Agriculture, Food and Environment,  
University of Pisa, Via del Borghetto 80, Pisa 56124, Italy

been reported in the more popular *Wisteria sinensis* (Chinese wisteria; De Beni 1964), *Wisteria floribunda* (Japanese wisteria; Brierley and Lorentz 1957), *Wisteria brachybotrys* (Silky wisteria; Clover et al. 2003), and its natural transmission is ascribed to aphids (*Aphis craccivora*, *Aphis fabae* and *Myzus persicae*; Conti and Lovisolo 1969; Valouzi et al. 2020).

This wisteria disease was likely first reported in the United States in the late 1950s (Brierley and Lorentz 1957), and later in Italy (Ciferri 1959; De Beni 1964; Conti and Lovisolo 1969) and the Netherlands (Bos 1970), but only with the serological studies by Conti and Lovisolo (1969) and Bos (1970) it was demonstrated the viral etiology and proposed the name WVMV. Later, an isolate of WVMV was obtained from *Wisteria sinensis* in Czech Republic (Brčák 1980), and ultrastructural analysis conducted to investigate its interaction with plant cell showed large complex inclusions previously reported for other potyviruses (Brčák and Králík 1981). First genetic characterization of WVMV was achieved on an Australian isolate by Clover et al. (2003), which confirmed the previously advised taxonomy. A few years later, Liang et al. (2006) performed a complete genome analysis on a Chinese isolate, showing that WVMV is a single strand, positive-sense RNA potyvirus of about 9,695 nucleotides excluding the poly (A) tail. Genome was characterized by one main ORF encoding for a polyprotein which cleavage generates ten mature proteins: P1, HCPro, P3, 6K1, CI, 6K2, VPg, NIapro, NIb, CP. Currently another protein is also known, namely P3N-PIPO, deriving from a short ORF positioned in the P3 coding region (Chung et al. 2008; Revers and García 2015). WVMV has had a far-reaching impact and it have been observed in seven countries across the globe in the last 18 years. Reports have emerged from China (Beijing municipality, Jiangxi and Jiangsu provinces; Liang et al. 2004; Ji et al. 2019; Zhu et al. 2019), Poland (Kaminska et al. 2006), United States (Naidu and Karthikeyan 2008), New Zealand (Ward et al. 2008), United Kingdom (Clover et al. 2015), and Iran (Al Jaber et al. 2018), further demonstrating the virus' spread through diverse geographical locations over time.

In Italy, the known records of wisteria viral infections by Ciferri (1959), De Beni in *Wisteria sinensis* (1964), and Conti and Lovisolo in *Wisteria floribunda* (1969), occurred in Northern regions only. No other reports came out in other Italian areas so far. In 2021, we actually reported some *Wisteria sinensis* plants in Sarzana district (Liguria, on the border with Central Italy) showing typical (and severe) WVMV symptoms on many leaves. This study confirms the WVMV infections and provide information on the biological properties, genetic diversity and evolutionary relationships of WVMV.

## Materials and methods

### Field surveys and sampling

In 2021, one *Wisteria sinensis* plant with some leaves showing WVMV-like symptoms (Supplementary Figure S1a) was reported in Sarzana district, Liguria, Italy (44°06'49"N, 9°57'36"E). Therefore, all *Wisteria sinensis* plants within a circular area of 0.5 km radius around the first symptomatic plant were surveyed, and further symptomatic ones were detected (i.e., showing WVMV-like symptoms). This surveying approach was repeated until no new symptomatic plants were reported in the monitored areas. In the end, nine plants were recovered in both public and private areas (e.g., streets, urban parks, gardens), all within a 1 km<sup>2</sup> region. Plants were around ten- to fifty-year-old [according to the indications by owners, confirmed by a plant aging using a Resistograph®, Rinntech, Heidelberg, Germany (Lukaszkiwicz et al. 2005)], and showed fully foliated canopies and no other diseases and infections.

In May (i.e., when symptoms were most evident on all nine plants) the foliar disease severity was classified and sorted according to a four-class pathometric scale based on the percentage of symptomatic area of leaflets: Class 0 = 0%; Class 1 = < 5%; Class 2 = 5–40%; Class 3 = > 40% (Supplementary Figure S1b). Symptomatic and asymptomatic leaves were collected from each of nine plants (i.e., two leaf samples for each plant; 18 in total) and kept refrigerated until quickly reaching the Plant Pathology Lab of the Department of Agriculture, Food and Environment, University of Pisa, where leaflets and petioles were detached and stored at -80 °C until RNA extraction.

### RNA isolation and cDNA synthesis

RNA was extracted from 500 mg of leaf tissue with cetyltrimethylammonium (CTAB) buffer, according to Li et al. (2008) with some modifications (Pedrelli et al. 2021, 2023a, b). Briefly, leaves were powdered in liquid nitrogen with 5 ml CTAB 2% buffer. After incubation at 65 °C for 15 min, one volume of chloroform:iso-amyl alcohol (24:1) was added and RNA precipitated with one volume of isopropanol. Pellet was then washed with 70% ethanol, air-dried and dissolved in 80 µL of RNase/DNase free water. cDNA synthesis was finally performed using M-MMLV reverse transcriptase (GeneSpin s.r.l., Milan, Italy), according to the manufacturers' instructions.

### Virus detection

An end point PCR was performed with WVMVF1/WVMVR1 specific primers (Clover et al. 2003) targeting

**Table 1** Symptom severity class, PCR assay, sequence variants, synonymous (dS) and non-synonymous (dN) mutations, accession number and identity (%) of isolates on GenBank database of WVMV identified in *Wisteria* plants in Sarzana district. (AY656816.1 = isolate Beijing; KP161267.1 = isolate JW\_2014). S = symptomatic sample; A = asymptomatic sample; + = Positive; - = Negative

| Plant | Sample type | Severity class | PCR assay | Sequence variant | dS | dN | Accession number | Identity (%)<br>NCBI |
|-------|-------------|----------------|-----------|------------------|----|----|------------------|----------------------|
| 1     | S           | 3              | +         | Sar 5            | 63 | 16 | OM417219.1       | 89.86<br>AY656816.1  |
|       | A           | 0              | -         |                  |    |    |                  |                      |
| 2     | S           | 2              | +         | Sar 6            | 2  | 0  | OM417220.1       | 99.55<br>KP161267.1  |
|       | A           | 0              | +         | Sar 6            | 2  | 0  |                  |                      |
| 3     | S           | 2              | +         | Sar 7            | 23 | 4  | OM417221.1       | 96.11<br>KP161267.1  |
|       | A           | 0              | +         | Sar 7            | 23 | 4  |                  |                      |
| 4     | S           | 1              | +         | Sar 8            | 1  | 0  | OM417222.1       | 99.70<br>KP161267.1  |
|       | A           | 0              | +         | Sar 8            | 1  | 0  |                  |                      |
| 5     | S           | 2              | +         | Sar 9            | 3  | 0  | OM417223.1       | 99.40<br>KP161267.1  |
|       | A           | 0              | +         | Sar 9            | 3  | 0  |                  |                      |
| 6     | S           | 3              | +         | Sar 5            | 63 | 16 | OM417219.1       | 89.86<br>AY656816.1  |
|       | A           | 0              | +         | Sar 10           | 1  | 0  | OM417224.1       | 99.85<br>KP161267.1  |
|       |             |                |           |                  |    |    |                  |                      |
| 7     | S           | 1              | +         | Sar 11           | 4  | 2  | OM417225.1       | 98.86<br>KP161267.1  |
|       | A           | 0              | +         | Sar 12           | 1  | 0  | OM417226.1       | 99.70<br>KP161267.1  |
| 8     | S           | 3              | +         | Sar 5            | 63 | 16 | OM417219.1       | 89.86<br>AY656816.1  |
|       | A           | 0              | +         | Sar 10           | 1  | 0  | OM417224.1       | 99.85<br>KP161267.1  |
|       |             |                |           |                  |    |    |                  |                      |
| 9     | S           | 3              | +         | Sar 5            | 63 | 16 | OM417219.1       | 89.86<br>AY656816.1  |
|       | A           | 0              | -         |                  |    |    |                  |                      |

genomic NIB/CP region (703 bp). PCR amplification was performed on a C1000 Touch® thermal cycler (Bio-Rad, Hercules, CA, USA) using PCR conditions as follows: 30 cycles of 95 °C/30 s, 55 °C/30 s, and 72 °C/45 s and final extension at 72 °C for 10 min. Amplified products were observed on 1.2% agarose gel electrophoresis. Infected and healthy control were used as references. All samples were analysed in a qPCR to determine the presence and quality of cDNA using 18 S rRNA as internal control (Osman and Rowhani 2008).

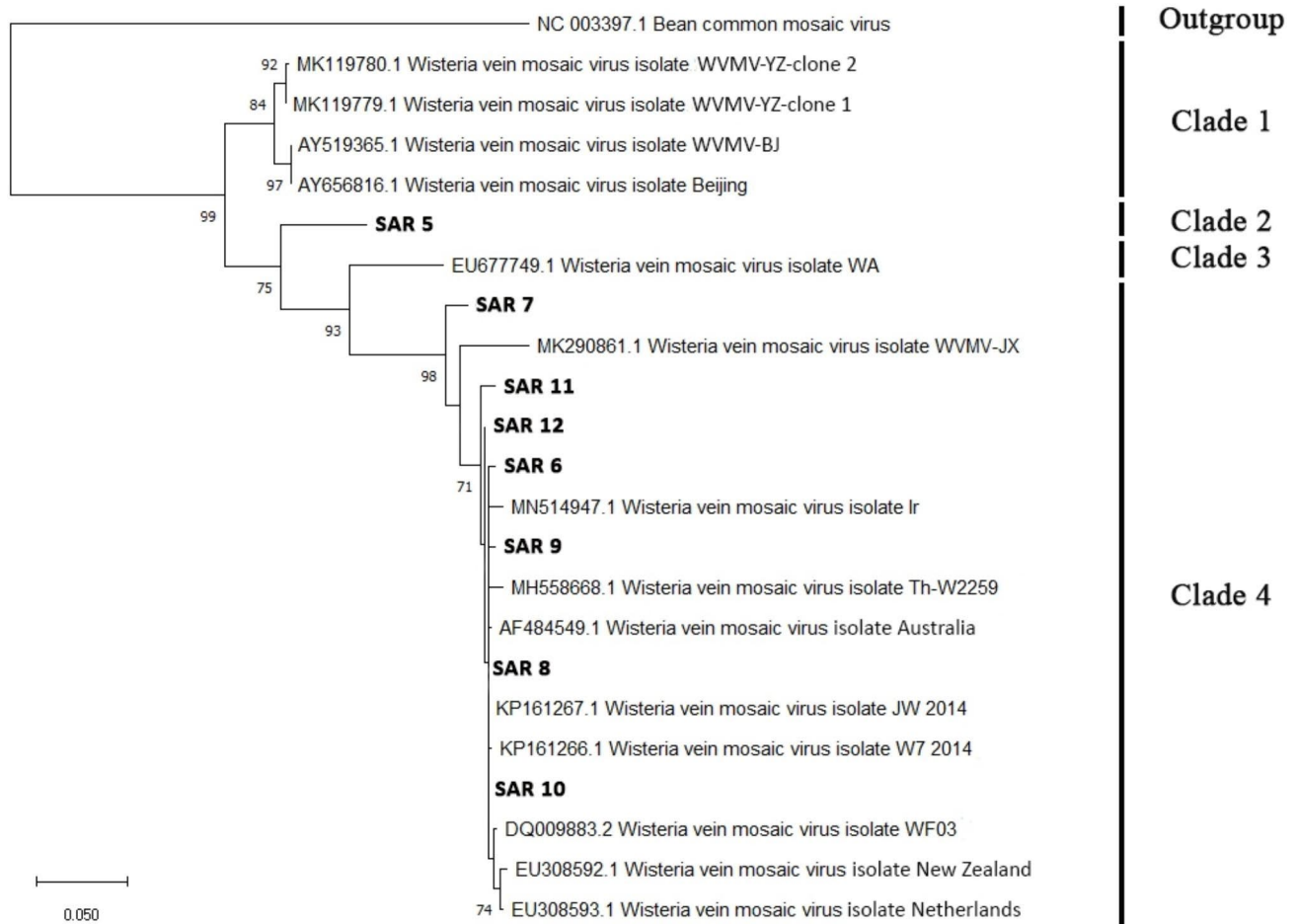
### Virus sequencing and *in silico* assays

The PCR products were cleaned up and directly sequenced by Sanger DNA method (Eurofins genomics, Ebersberg, Germany). The nucleotide and deduced amino acid sequences were *in silico* analysed using Bioedit (Hall 1999) and nucleotide sequences were also compared in BLASTn ([www.ncbi.nlm.nih.gov](http://www.ncbi.nlm.nih.gov)). The role of natural selection on population using Codon Z-test by the Nei-Gojobori method and the presence of non-synonymous (dN) and synonymous (dS) single nucleotide polymorphisms (SNPs) ratio by Felsenstein (1981) model was estimated using MEGA X

(Kumar et al. 2018). The recombinant events in the nucleotide sequences were evaluated by RDP4 program (v.4.101) using 3Seq, Bootscan, Chimaera, GENECONV, MaxChi, RDP, and SiScan algorithms (Martin et al. 2015) and they were accepted only if identified by at least 4 methods ( $p$  value of  $> 10^{-5}$ ). The nucleotide sequence alignment was used to construct phylogenetic tree by the Maximum Likelihood (ML) method (Jukes-Cantor model) with 1,000 bootstrap replicates on MEGA X (Kumar et al. 2018). WVMV sequences obtained from NCBI were used as ad hoc dataset (Supplementary Table S1). An isolate of bean common mosaic virus (NC\_003397.1) was used as the outgroup.

### Results and discussion

*Wisteria* spp. are ornamental plants largely used in public and private gardens in Italy (as well as in many other countries worldwide), and WVMV represents an important and spread viral agent of a detrimental disease that makes plants unattractive and even unsaleable (Clover et al. 2003). However, information about WVMV diffusion in Italy was lacking and restricted to the few and dated publications



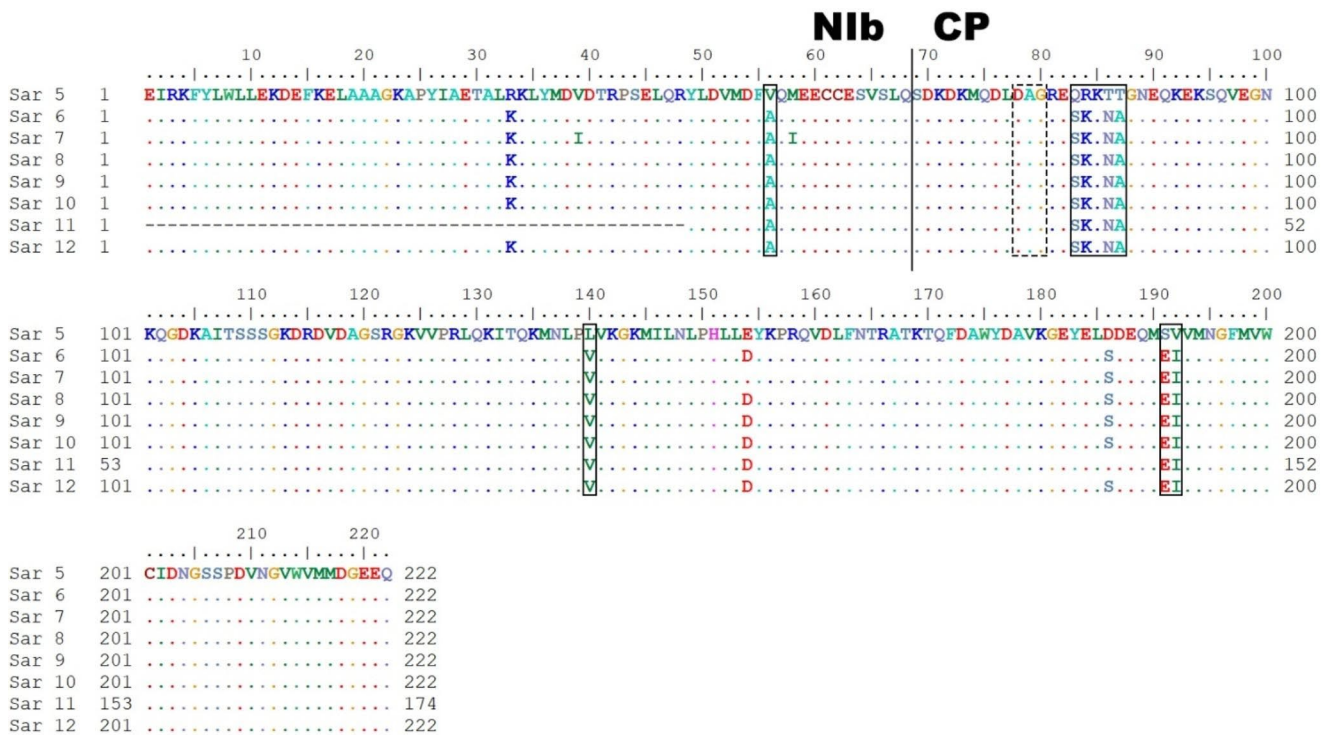
**Fig. 1** Phylogenetic tree of wisteria vein mosaic virus (WVMV) reconstructed from partial CP genomic region. The trees were generated by Maximum Likelihood (ML) using the Jukes-Cantor model of evolution for nucleotide. The significance of each branch was evaluated by constructing 1,000 trees in bootstrap analysis. Only bootstrap values > 70% are shown. The scale represents a distance of 0.05 substitutions per site. The isolates sequenced in this study are in bold. Bean common mosaic virus (NC\_003397.1) was used as the outgroup

by Ciferri (1959), De Beni (1964) and Conti and Lovisolo (1969), all of which referred to WVMV observations in Northern regions (Lombardy, Emilia-Romagna and Piedmont, respectively). To the best of our knowledge, after more than 50 years from these observations, the present work represents the first additional report of WVMV in Italy (Sarzana district, Liguria, on the border with Central Italy), as well as its first molecular characterization ever.

All the nine *Wisteria sinensis* plants identified in May 2021 showed typical leaf WVMV symptoms (Supplementary Figure S1a) scattered in the canopy (i.e., asymptomatic leaves were also present), which were especially noticeable in spring, whereas they became fade and masked in summer. Symptom severity assessment showed the presence of leaf samples in each class of the pathometric scale. Among symptomatic leaf samples, the harsher class 3 was mostly observed (44% of samples), followed by class 2 (34%) and 1 (22%; Table 1).

All nine tested plants resulted positive to WVMV (quality of cDNA samples was proved by 18 S rRNA gene amplification, and the Ct values of the internal control ranged from 10 to 13; data not shown). The electrophoretic analysis of WVMV amplicons identified the virus in 16 out of the 18 leaf samples (around 90%; Table 1). Specifically, nine symptomatic and seven asymptomatic leaf samples were positive, so only two asymptomatic leaves were negative (Supplementary figure S2). The presence of asymptomatic positive leaf samples was in accordance to the previous WVMV report in *Wisteria floribunda* by Kaminska et al. (2006), while the occurrence of the two negative leaf samples could be due to a very low infection rate in those tissues, resulting below the detection limit of the diagnostic technique (Rubio et al. 2020).

PCR amplicon sequencing identified 16 distinct sequences, eight of which were considered novel variants and deposited in GenBank as Sar 5–12 (Table 1). The most



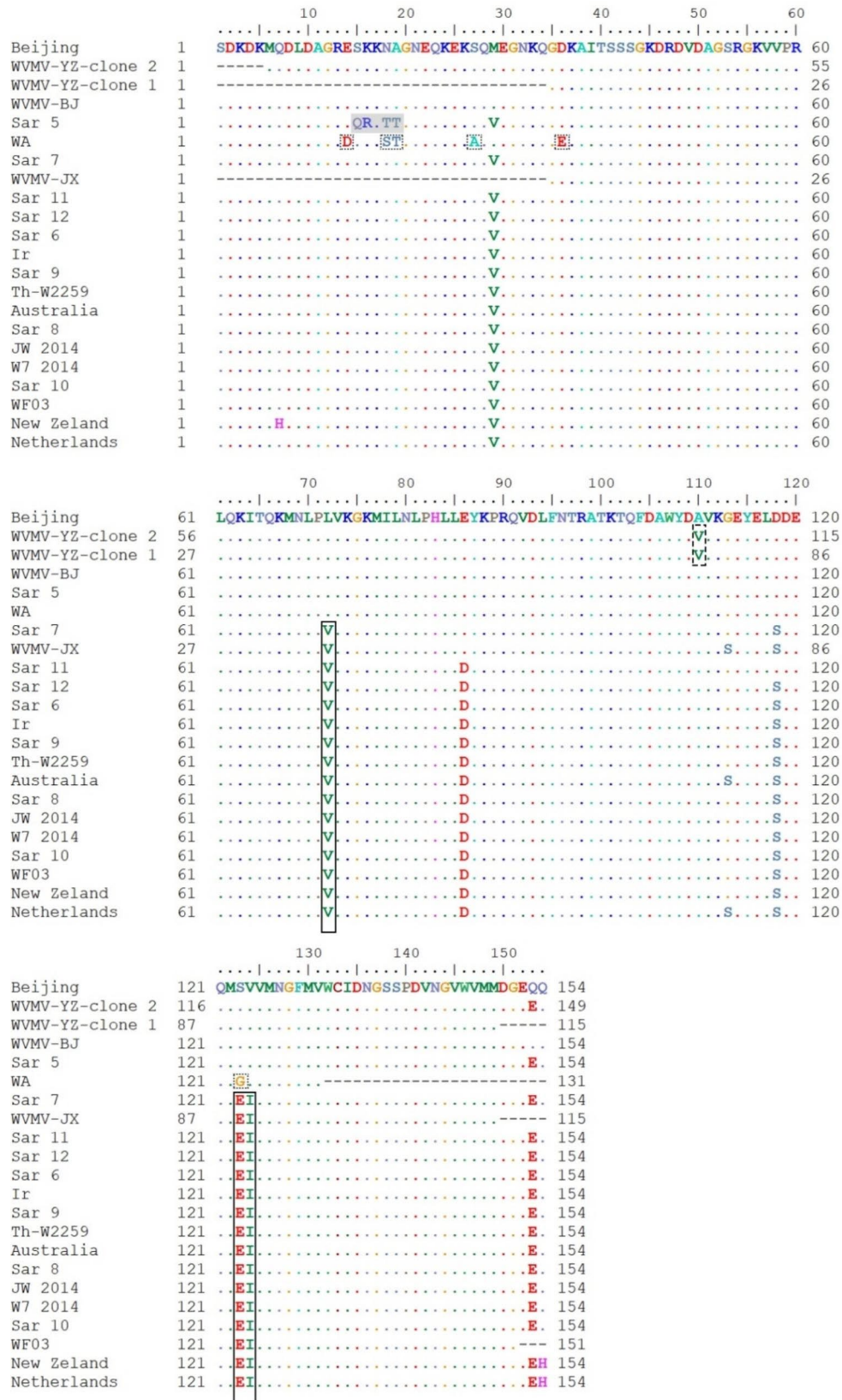
**Fig. 2** Alignment of the deduced amino acid sequences of wisteria vein mosaic virus isolates recovered in this study. The <sup>78</sup>DAG<sup>80</sup> motif are shown in dashed rectangles. Conserved amino acids and motif change are shown in black rectangles. Polyprotein processing site, i.e. Nib/ CP junction, is indicated with a black line

frequent variant was Sar 5 being identified in four leaf samples (25.0%), followed by Sar 6, 7, 8, 9 and 10 characterized in two leaf samples (12.5% each), and finally Sar 11 and 12 identified in only one leaf sample (6.3% each). All leaf samples infected by Sar 5 were symptomatic, whereas Sar 6, 7, 8 and 9 were found in both symptomatic and asymptomatic leaf samples of the same plant, Sar 10 only in asymptomatic leaf samples, and Sar 11 and 12 in the symptomatic and asymptomatic leaf samples of the only plant infected by these variants. Mixed strains infections included Sar 10, 11 and 12, while Sar 6, 7, 8 and 9 were present only as single infections, Sar 5 was detected in both conditions. Referring to the symptom severity, Sar 5 was always associated to the highest class (3); Sar 6, 7, 9 to the middle class (2), and Sar 8, 11 to the lowest class (1). Sar 10 and 12 were identified exclusively in class 0 (Table 1).

The nucleotide sequence alignments revealed similarity among variants ranging from 87.13 to 99.85%. In particular, Sar 5 showed the lowest similarity (87.13–87.60%) with all other variants, while Sar 10 displayed the highest one (95.69–99.85%), especially with Sar 8 and 12. The genetic variability ( $\pi$ ) was estimated in 0.045 ( $\pm$  0.005). Interesting, a viral population with higher genetic diversity is normally considered more ancient in an area (Wei et al. 2009). The RDP4 program using the 3Seq, Chimaera, MaxChi, and RDP algorithms detected a concordant result in the Sar 5 with the

242 nt beginning breakpoint and the 360 nt ending breakpoint and a mean  $p$ -value of  $2.073 \times 10^{-2}$ ,  $1.697 \times 10^{-2}$ ,  $1.516 \times 10^{-2}$ ,  $3.848 \times 10^{-2}$ , respectively. As major parent was identified the isolate Beijing (AY656816.1) with 90.3% similarity whereas minor parent was unknown. Although four algorithms found the same putative event, the result was rejected due to the probability not meeting the accepted criterion ( $p$  value  $> 10^{-5}$ ) for identifying a true recombinant variant. It is possible to hypothesize that a greater number of available sequences could confirm or deny the presence of this recombinant event, as reported in previous studies focused on other potyviruses (Santillan et al. 2018; Fuentes et al. 2021). The number of SNPs among the eight variants was 120. Maximum changes were recognized in Sar 5 (79 nt) with 16 dN, followed by Sar 7 (27 nt) with 4 dN, and Sar 11 (6 nt) with 2 dN (Table 1). The overall ratio between nonsynonymous and synonymous mutations ( $dN/dS$  ratio) was 0.05 ( $p < 0.000$ ) within population under study. These outcomes suggest a well-adaptation of WVMV in the small infected area here reported (i.e., around one km<sup>2</sup>), a phenomenon that likely promotes a high stability of the virus genetic structure (Escriu 2017).

Comparing the novel identified sequences with those already available in GenBank (Table 1), Sar 10 revealed the maximum similarity of 99.85% with the isolate JW\_2014 (KP161267.1) identified on *Wisteria floribunda* in England



**Fig. 3** Comparison of the partial coat protein amino acid sequences identified in this study and reported in the ad hoc WVMV database. Amino acid substitution in the Clade 1 is shown in dashed rectangles. Grey area underlines the distinctive motif of the Clade 2. Specific amino acids substitutions in the Clade 3 are highlighted by dotted rectangles. Conserved amino acids in Clade 4 are shown in black rectangles. The sequence of the Beijing isolate was used as the reference sequence and others were sorted as in the result of the phylogenetic analysis of the nucleotide sequences

(Clover et al. 2015), followed by Sar 6, 7, 8, 9, 11 and 12 with similar rates ranging from 96.11 to 99.85%. Sar 5 showed the higher similarity percentage (89.86%) with isolate Beijing (AY656816.1) previously found on *Wisteria sinensis* in China (Liang et al. 2006) (Table 1).

In the phylogenetic tree, WVMV isolates were grouped in four clades with high bootstrap values (i.e., > 70%), two of which included the eight Italian variants here identified (Fig. 1). Clade 1 indeed included only Chinese isolates (AY656816.1, AY519365.1, MK119779.1, MK119780.1), whereas clades 2, and 3 clustered only the Italian Sar 5 (OM417219.1) and a North American (EU677749.1), respectively. Differently, clade 4 included sequences with worldwide origin: Australia (AF484549.1), China (MK290861.1), the Netherlands (EU308593.1), New Zealand (EU308592.1), Poland (DQ009883.2), United Kingdom (KP161266.1, KP161267.1) and Iran (MN514947.1, MH558668.1), as well as the here recognized Italian Sar 6 (OM417220.1), 7 (OM417221.1), 8 (OM417222.1), 9 (OM417223.1), 10 (OM417219.1), 11 (OM417225.1) and 12 (OM417226.1). The deduced amino-acid sequences of our population revealed identity ranging from 94.59 to 100.00% with higher distance values identified between Sar 5 (94.59–95.05%) and other sequences. The alignment showed conserved amino acids in the population as A<sub>56</sub> in N1b and V<sub>140</sub>, E<sub>191</sub>, I<sub>192</sub> in CP, which were replaced by specific changes in the Sar 5, namely V<sub>56</sub> in N1b, L<sub>140</sub>, S<sub>191</sub> and V<sub>192</sub> in CP, respectively. Moreover, the <sup>83</sup>SKKNA<sup>87</sup> motif was changed in <sup>83</sup>QRKTT<sup>87</sup> motif in Sar 5. No variation in the <sup>78</sup>DAG<sup>80</sup> motif associated with the transmissibility of potyviruses by aphid vectors was instead observed (Kaminska et al. 2006; Fig. 2). A comparison of the partial CP amino acid sequences of our and ad hoc WVMV database isolates showed that the difference between isolates within Clade 1 is due to a V<sub>110</sub>, Clade 2 is characterized by the distinctive feature of <sup>13</sup>QRKTT<sup>19</sup> motif and Clade 3 displayed specific amino acids substitutions, namely D<sub>13</sub>, A<sub>27</sub>, E<sub>36</sub>, G<sub>123</sub>, and <sup>13</sup>SKKST<sup>19</sup> motif. Interestingly, Clade 4 showed high similarities between the isolates and V<sub>72</sub>, E<sub>123</sub>, I<sub>124</sub> as conserved amino acid sites. These results were in accordance with our previous analyses and with the observations on WVMV CP amino acids by Valouzi et al. (2020; Fig. 3).

The phylogenetic analysis and the observation of deduced amino acid sequences supported the division in four clades, each characterized by specific amino acids in the variable region of N-terminus of CP. Interesting, partial clustering based on country of origin was observed, while no one by host was identified. The Italian variants showed a different clustering with Sar 5 alone between the “Chinese” and the “American” clusters and all the other Italian variants with worldwide isolates in the same cluster. Overall, the analysis allowed to hypothesize a spread of the virus from the

original area in other worldwide areas, probably due to exchange of propagating material, as previously observed for other ornamental plants (Valverde et al. 2012). Moreover, the phylogenetic outcomes associated with the  $\pi$  and the selective pressure seems to suggest a long-term introduction of virus and a co-evolution between *W. sinensis* and WVMV in the studied area (LaTourrette and Garcia-Ruiz 2022). This interpretation is supported by the presence in Italy of several plants (e.g., Leonardo’s *W. sinensis* in Milan and Goethe’s *W. sinensis* in Tivoli, near Rome) which seem to have been attested before wisteria fortune as ornamental species starting from the early 1800s.

## Conclusions

Since the late 1950 and 1960 s, the present study represents the first additional report of WVMV in Italy, as well as its first molecular characterization ever. Here, eight new Italian variants with different performances were identified, with Sar 5 showing the highest distances from other variants. According to LaTourrette and Garcia-Ruiz (2022), data obtained could suppose a long-term introduction of virus and a co-evolution between *W. sinensis* and WVMV in the studied area. Moreover, the presence of asymptomatic-positive leaf samples enforces the essential role of monitoring plants and regular phytosanitary surveys to detect viral infections potentially dangerous for different species and cultivars. As *Wisteria sinensis* (as well as other wisterias) is a major woody perennial plant widely grown as ornamental in public and private gardens, more research should be carried out to improve our knowledge on WVMV diffusion (the collection and analysis of further samplings is highly encouraged), especially in Italian districts where the production and marketing of highly priced ornamental plants is crucial, as well as to elucidate the risk of growing virus infected plants that could represent a source for virus transmission to other potential neighbouring susceptible species.

**Supplementary Information** The online version contains supplementary material available at <https://doi.org/10.1007/s42161-023-01526-7>.

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## Declarations

**Competing interests** The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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