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Research Notes

Virome analysis reveals apple mosaic virus in the monumental tree *Castagno dei cento cavalli* in Sicily

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Summary. The *Castagno dei cento cavalli* (One hundred horse chestnut) is a monumental *Castanea sativa* (sweet chestnut) located on the eastern slope of Mount Etna in Sicily (Italy), and is the oldest (>2000 years) known sweet chestnut in the world. In the spring of 2022, symptoms indicating virus infection were observed on this tree. Attempts to visualize virus particles from extracts using transmission electron microscopy were unsuccessful, so high-throughput RNA sequencing was carried out. This identified a tripartite genome (RNA1, RNA2, and RNA3) corresponding to apple mosaic virus (species *Ilarvirus ApMV*). Serological analyses using polyclonal antiserum confirmed infection in symptomatic but not in asymptomatic leaf samples. This is the first report of ApMV infecting *C. sativa*, and this virus possibly threatens monumental trees elsewhere internationally. Although efficient ApMV vectors are lacking, limiting the risk of spread, this knowledge highlights the need for monitoring monumental trees as potential reservoirs of novel host-virus associations, and the importance of careful management of this Sicilian botanical monument.

Keywords. HTS-based virus detection, *Castanea sativa*, chlorotic mottling, ApMV host range.

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INTRODUCTION

The One Hundred Horse Chestnut (*Castagno dei Cento Cavalli*) is a monumental *Castanea sativa* Mill. located in Sant'Alfio (Catania Province, Sicily, Italy; 37°45'00.66" N, 15°07'49.34" E), at 710 m a.s.l., about 10 km from the Etna crater (Figure 1). Estimated to be 2,000 to 3,000 years old, this tree is regarded as the world's oldest *C. sativa* specimen, and has survived multiple Etna eruptions and extreme climatic events (Schicchi and Raimondo, 2007). Recognized by UNESCO as a "World Heritage messenger of peace," the tree holds European records for size and longevity. Molecular analyses have shown that the tree, once believed to be three individuals, is a single

plant, with three trunks sharing a common root system probably resulting from radial fragmentation of an ancient trunk (Pereira-Lorenzo et al., 2019; Mattioni et al., 2020; Nunziata et al., 2022). Radial growth estimates indicate that the tree is at least 2,276 years old (Pereira-Lorenzo et al., 2019).

The apple mosaic virus (ApMV, species *Ilarvirus ApMV*) belongs to *Ilarvirus* (*Bromoviridae*). Early classification of this virus relied on serology, but was later refined through genomic data into four molecular subgroups (Pallas *et al*., 2013). The term "ilar-" derives from "**i**sometric **la**bile **r**ingspot." These thermolabile viruses often mask symptoms at high temperatures (Aramburu and Rovira, 1998; Dal Zotto et al., 1999). ApMV particles are quasi-spherical and contain a tripartite, positive-sense RNA genome and RNA3, and produce a subgenomic RNA that carries the open reading frame (ORF) encoding the coat protein (Roossinck *et al*., 2005). All three RNA components are required for host plant infection (Grimová et al., 2016). The virus occurs widely in woody and herbaceous hosts, mainly *Malus domestica* and *Rubus idaeus* (Manzoor *et al*., 2023), but has been detected in more than 65 plant species (Grimová *et al*., 2016; EPPO, 2024).

This paper reports the results of virome analysis carried out on the monumental sweet chestnut tree using high-throughput sequencing, and documents the first detection of ApMV in *C. sativa*.

MATERIAL AND METHODS

Following a report from the Regional Plant Protection Service, a first survey of *Castagno dei Cento Cavalli* was conducted in June 2022, the symptoms of which indicated viral infection. Foliar symptoms of chlorotic ring mottling, spotting, and oak-leaf patterns were observed on suckers and apical branches of one of the three main trunks of the tree (Figure 2), while the other two trunks had no symptoms. Leaf samples were collected from all three trunks. Additional surveys in spring and autumn 2023 and 2024 confirmed persistence and localization of symptoms on the tree. Field observations were also extended to the surrounding area to record comparable symptoms in other plant species, including hazelnut (*Corylus avellana*), an abandoned apple orchard, and another monumental chestnut (*Castagno della Nave*) located about 600 m away from *Castagno dei Cento Cavalli*.

Attempts to visualize virus particles using transmission electron microscopy (TEM) were unsuccessful, probably due to virion instability. For virome analy-

Figure 1. The *Castagno dei Cento Cavalli* (One hundred horse chestnut) *Castanea sativa* tree in Sant'Alfio (Catania), Sicily, Italy.

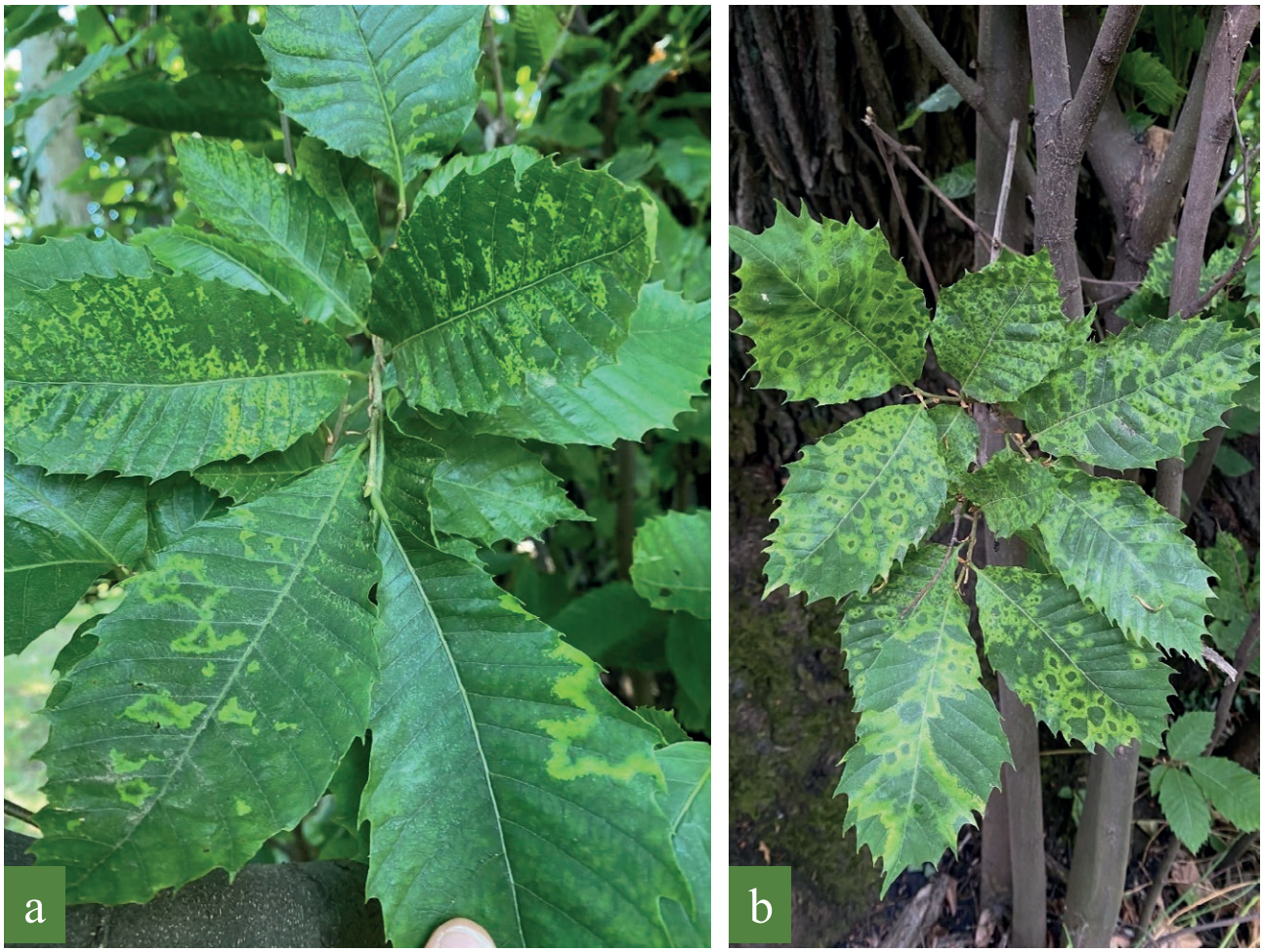

Figure 2. Symptoms of leaf chlorotic ring mottling, oak-like leaf, and chlorotic spotting on suckers, in initial (a) and advanced (b) stages of symptom development on the monumental chestnut tree *Castagno dei Cento Cavalli*.

sis, leaf samples were freeze-dried and stored at –20°C. Total RNA was extracted using the Spectrum™ Plant Total RNA Kit (Merck). RNA integrity was checked by 1% agarose gel electrophoresis, and RNA was quantified using a NanoDrop spectrophotometer (Thermo Fisher Scientific). Total RNA was processed by Macrogen Inc. (Seoul, Republic of Korea), for rRNA depletion (TruSeq Stranded Total RNA with Ribo-Zero Plant, Illumina), library preparation, and high-throughput sequencing on an Illumina NovaSeq 6000 platform. Resulting 150 bp paired-end reads were quality-filtered using the Joint Genome Institute (JGI) pipeline (https://doi.org/10.17504/protocols.io.gydbxs6), and were assembled *de novo* with Trinity v2.3.0. Viral contigs were identified through BLAST search against the NCBI nr database (e-value $1e^{-10}$), as described by Forgia *et al.* (2022). Filtering with the *dplyr* package (R software) and manual check yielded three contigs blasting against the protein coded by RNA1, RNA2 and RNA3 of ApMV. Read mapping was performed with Bowtie2 (Langmead and Salzberg, 2012), and alignments visualized with Tablet. ApMV detection was validated by RT-qPCR on cDNA synthesized from the same RNA used for HTS, using primers specifically designed on RNA1 by using Primer3 (Untergasser *et al.*, 2012) (amplicon 70–120 bp, 60°C annealing) (Table 1). cDNA synthesis employed the High-Capacity cDNA Reverse Transcription Kit (Thermo Fisher Scientific), and RT-qPCR was run in 10 µL reactions with three technical replicates using iTaq Universal SYBR Green Supermix (Bio-Rad) on a CFX Connect System (Bio-Rad) (Picarelli *et al.*, 2019). Open reading frames (ORFs) prediction and identification of domains in the putative proteins were carried out using the NCBI ORF Finder tool. The RNA-dependent RNA polymerase (RdRp) of the ApMV isolate MT3 was aligned with RdRp sequences of recognized *Ilarvirus*

species (ICTV database). Multiple sequence alignment was performed using MAFFT (Katoh and Standley, 2013), and a maximum-likelihood tree was constructed with IQ-TREE (Trifinopoulos *et al*., 2016) under automatic model selection and 1,000 ultrafast bootstraps. The resulting tree was edited using MEGA version X.

To confirm presence of the ApMV, and its association with observed symptoms, four asymptomatic and four symptomatic leaf samples were collected during each spring and autumn of 2023 and 2024 (total of 16 asymptomatic and 16 symptomatic samples). The samples were assessed using DAS-ELISA. Serological analyses were carried out using a polyclonal antiserum raised against the ApMV-hop isolate (LOEWE), following the manufacturer's instructions, using 1 g of leaf tissue homogenized in extraction buffer. To validate the three RNA sequences obtained by HTS and to verify possible insertions, deletions, or SNPs in comparison with genomes available in GenBank, specific primers were designed and used for RT-PCR on total RNA from four symptomatic samples collected in spring 2024 (Supplementary Table S1). Two asymptomatic samples served as negative controls. Amplicons were sequenced in both directions by Sanger sequencing.

RESULTS AND DISCUSSION

Illumina sequencing produced 102,025,671 raw reads, of which 91,999,781 high-quality reads were retained, and these were assembled *de novo* with Trinity, yielding 409,219 contigs. All raw sequencing data generated in this study have been deposited in the NCBI Sequence Read Archive (SRA) under accession number SRR36266065. The HTS library was established through the collection of diverse plant specimens suspected of containing virus infections. Each of the identified viruses (prunus necrotic ringspot virus, prune dwarf virus, eggplant mottled dwarf nucleorhabdovirus) were checked in the chestnut samples through qRT-PCR (protocol described above).

Virus screening exclusively identified three viral contigs corresponding to the complete coding sequences of a novel ApMV isolate, designated MT3. These sequences were deposited in GenBank, under accession numbers PQ137752 (RNA2), PQ137753 (RNA1), and PQ137751 (RNA3). Read mapping using Bowtie2 confirmed coverage of all genomic segments (40,882 reads for RNA1, 21,078 for RNA2, and 76,316 for RNA3). RT-qPCR assays with primers targeting RNA1 (Supplementary Table S1) confirmed ApMV presence in RNA extracted from symptomatic chestnut leaves collected in spring 2023. BLAST analyses of each RNA sequence (July 2025 NCBI release) revealed that RNA1 and RNA2 of ApMV MT3 were most similar to the Iranian apple isolate Alborz-A2 (92.7% similarity for RNA1, 89.5% for RNA2). In contrast, RNA3 of ApMV MT3 exhibited greater divergence, with 87.9% similarity with its closest match, a German ApMV isolate from *Rubus* sp. (DSMZ PV-0742). The predicted proteins shared 88.8 to 94.8% similarity with ApMV homologs (Table 1). Phylogenetic analysis of the RdRp amino acid sequence positioned ApMV MT3 within the established ApMV clade (Figure. 3). Mutations in regions containing indels or

Table 1. BLAST analysis results of the nucleotide and protein sequences of ApMV MT3.

Genomic segment	Isolate	Blastn best hit						
		Query cover	E value	Percent similarity	Acc. Len	Accession	Host	Origin
RNA1	Alborz-A2	98%	0.0	92.67%	3440	OR537857.1	*Malus domestica*	Iran
RNA2	Alborz-A2	98%	0.0	89.45%	2979	OR537858.1	*Malus domestica*	Iran
RNA3	DSMZ PV-0742	100%	0.0	87.88%	2104	OR477282.1	*Rubus* sp.	Germany

Genomic Segment	Protein	Isolate	Blastp best hit						
			Query cover	E value	Percent similarity	Acc. Len	Accession	Host	Origin
RNA1	Replicase	Alborz-A2	100%	0.0	94.84%	1047	XCO66467.1	*Malus domestica*	Iran
RNA2	RNA-dependent RNA polymerase	DSMZ PV-0742	100%	0.0	91.45%	877	WNS50456.1	*Rubus* sp.	Germany
RNA3	Movement protein	ApMV_RNA3_hop_pool_2021	100%	0.0	88.77%	285	XQU58012.1	*Humulus lupulus*	Germany
RNA3	Coat protein	India3	100%	0.0	89.24%	223	ACJ44917.1	*Malus domestica*	India

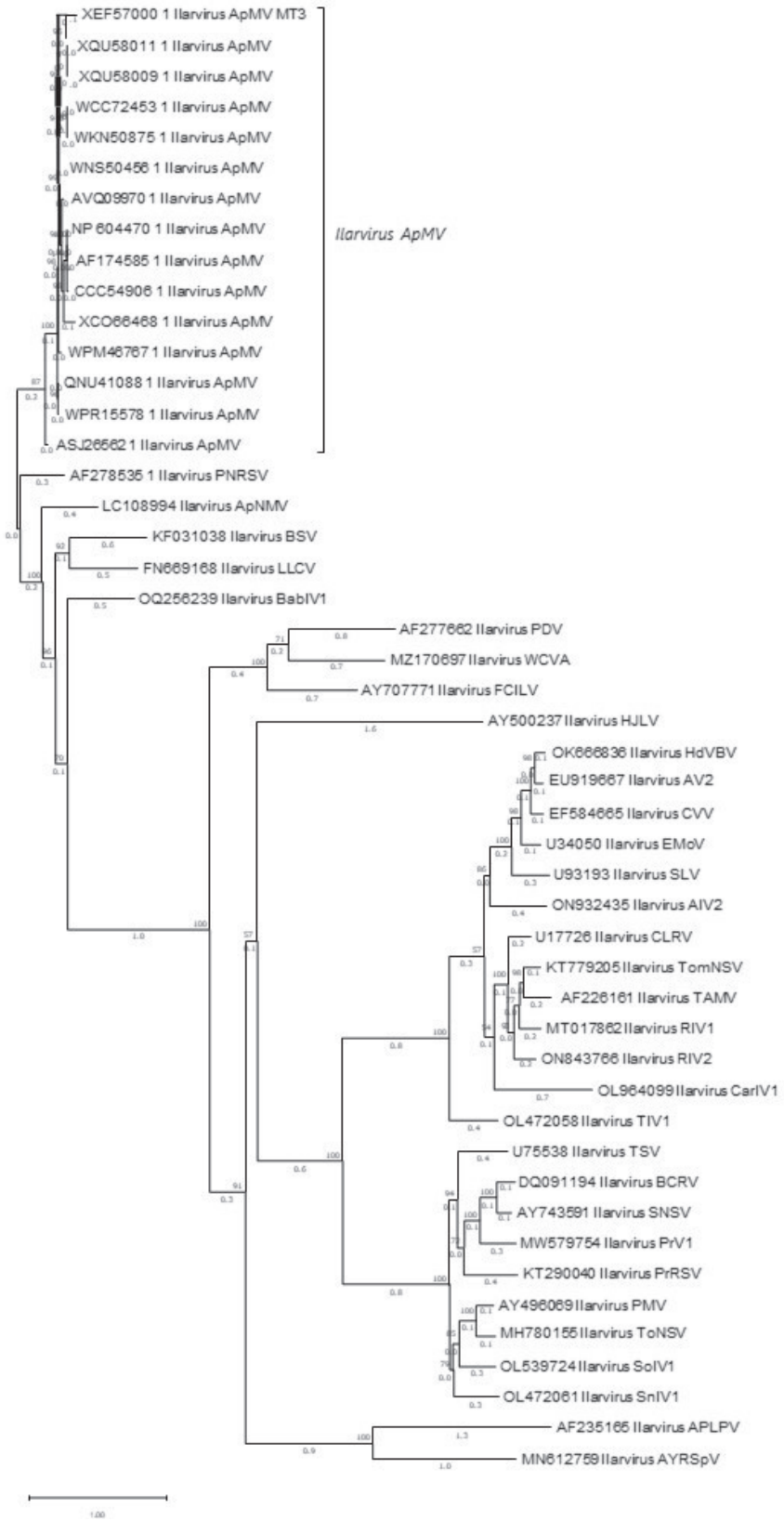

Figure 3. Maximum likelihood phylogenetic tree of RdRp sequences from all viruses accepted in *Ilarvirus*.

SNPs, initially identified in the original sample, were confirmed by RT-PCR with specific primers and Sanger sequencing, using RNA extracts from samples collected in autumn 2024.

DAS-ELISA consistently detected ApMV only in the symptomatic trunk of the monumental chestnut tree, while the other two trunks remained virus-free. Visual surveys conducted during 2023 to 2024 did not reveal virus- or ApMV-like symptoms in nearby hazelnut or apple trees, nor in the *Castagno della Nave* tree (600 m away from *Castagno dei Cento Cavalli*). While latent or asymptomatic infections cannot be ruled out without molecular analyses, no symptomatic hosts were detected in the surrounding vegetation. The restriction of ApMV detection to a single trunk may reflect limited systemic movement of the virus within the monumental chestnut, potentially constrained by the age and structural complexity of the host.

This study highlights the sensitivity and specificity of HTS-based virus detection. *Castagno dei Cento Cavalli* lacked symptom-based indicators associated with specific viruses in *C. sativa*, and this combined with inconclusive electron microscopy results, made conventional diagnostic approaches ineffective. High throughput sequencing was the only method that enabled the detection of ApMV in this newly reported host. The present paper is the first to report virome characterization of a monumental tree, and the first detection of ApMV in *C. sativa* and within *Fagaceae*. Although sequence divergence exists, nucleotide and protein identities, along with phylogenetic placement and serological reactivity, confirm the classification of ApMV MT3 as a member of apple mosaic virus. These results expand the known host range of ApMV, confirming that sweet chestnut can be naturally infected. For the monumental *Castagno dei Cento Cavalli*, this detection can inform future pruning or management interventions for the tree, helping to prevent potential virus spread within this historically important tree.

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