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Virology

Long-Term Monitoring of Aphid-Transmitted Viruses in Melon and Zucchini Crops: Genetic Diversity and Population Structure of Cucurbit Aphid-Borne Yellows Virus and Watermelon Mosaic Virus

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Abstract

Understanding the emergence and prevalence of viral diseases in crops requires the systematic epidemiological monitoring of viruses, as well as the analysis of how ecological and evolutionary processes combine to shape viral population dynamics. Here, we extensively monitored the occurrence of six aphid-transmitted viruses in melon and zucchini crops in Spain for 10 consecutive cropping seasons between 2011 and 2020. The most prevalent viruses were cucurbit aphid-borne yellows virus (CABYV) and watermelon mosaic virus (WMV), found in 31 and 26% of samples with yellowing and mosaic symptoms. Other viruses, such as zucchini yellow mosaic virus, cucumber mosaic virus, Moroccan watermelon mosaic virus, and papaya ring spot virus, were detected less frequently (<3%) and mostly in mixed infections. Notably, our statistical analysis showed a significant association between CABYV and WMV in melon and zucchini hosts, suggesting that mixed infections might be influencing the evolutionary epidemiology of these viral diseases. We then carried out a comprehensive genetic characterization of the full-length genome sequences from CABYV and WMV isolates by using the Pacific Biosciences single-molecule real-time (PacBio) high-throughput technology to assess the genetic variation and structure of their populations. Our results showed that the CABYV population displayed seven codons under positive selection, and although most isolates clustered in the Mediterranean clade, a subsequent analysis of molecular variance revealed a significant, fine-scale temporal structure, which was in part explained by the level of the variance between isolates from single and mixed infections. In contrast, the WMV population genetic analysis showed that most of the isolates grouped into the Emergent clade, with no genetic differentiation and under purifying selection. These results underlie the epidemiological relevance of mixed infections for CABYV and provide a link between genetic diversity and CABYV dynamics at the whole-genome level.

Keywords: aphid-transmitted plant virus, CABYV, cucurbit crops, evolutionary epidemiology, genetic variability, mixed infections, WMV

Epidemiological monitoring of plant viruses at the molecular level is essential for increasing our understanding of their disease risk across space and time (García-Arenal et al. 2000; Jeger et al. 2006; Jones 2014; Madden et al. 2017; Parnell et al. 2017). Additionally, it can help with the development of accurate and rapid detection procedures for emerging viral diseases, facilitating the implementation of disease management strategies (Rubio et al. 2020), as well as understanding the evolutionary dynamics of their populations (Elena et al. 2014; García-Arenal et al. 2001, 2003; Lefeuvre et al. 2019). However, current molecular epidemiology studies on plant viruses primarily rely on partial genome sequencing analysis and only a few short-term studies of full-length viral genomes, lacking a thorough examination of the impact of various agro-ecological factors on the viral genetic diversity in the long term at the complete entity level. Thus, the combination of long-term monitoring of viral diseases with a plant survey and full-length genome characterization is valuable for comprehensively exploiting temporal information to better understand the evolutionary dynamics of the viral populations in crops.

Cucurbits are economically important crops worldwide, and their production can be affected by several plant viral diseases (Keinath et al. 2017; Lecoq and Desbiez 2012; Lecoq and Katis 2014; Radouane et al. 2021). Among them, 28 viruses are currently known to cause important economic losses in the Mediterranean region (Lecoq and Katis 2014; Radouane et al. 2021). These viruses mainly belong to the families *Geminiviridae*, *Closteroviridae*, *Potyviridae*, *Bromoviridae*, and *Solemoviridae* and prevalently infect cucurbits (Lecoq and Desbiez 2012). Thus, viruses belonging to the *Potyvirus* genus, such as watermelon mosaic virus (WMV), zucchini yellow mosaic virus (ZYMV), Moroccan watermelon mosaic virus (MWMV), and papaya ringspot virus (PRSV), in addition to cucumber mosaic virus (CMV, genus *Cucumovirus*), are commonly reported worldwide and in most Mediterranean countries (Bertin et al. 2020; De Moya-Ruiz et al. 2021; Desbiez et al. 2007; Harth et al. 2018; Juarez et al. 2013; Lecoq et al. 2009). Similarly, cucurbit aphid-borne yellows virus (CABYV, genus *Polerovirus*) is one of the most prevalent viruses. In fact, CABYV is becoming epidemiologically relevant in Europe; it is distributed in France, Italy, and Spain (Desbiez et al. 2020; Rabadán et al. 2021), and recent outbreaks have been reported in Germany (Menzel et al. 2020), Slovenia (Mehle et al. 2019), Poland (Minicka et al. 2020), and Bulgaria (Radeva-Ivanova et al. 2022). Thus, there seems to be an increasing incidence of viruses transmitted by aphid vectors in cucurbit crops, possibly as a result of the lack of accurate detection and/or ineffective countermeasures, in combination with the potential of climatic changes and agroecological factors (Anderson et al.

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2004; Cleaveland et al. 2007; Jones 2009; Lefeuvre et al. 2019; Ristaino et al. 2021).

In particular, CABYV and WMV have been reported to affect all major cultivated cucurbit species in Spain (Luis Alonso-Prados et al. 2003; De Moya-Ruiz et al. 2021; Juarez et al. 2013; Kassem et al. 2013; Maachi et al. 2022; Rabadán et al. 2021). The CABYV genome consists of a single-stranded positive-sense RNA molecule of about 5.7 Kb. It is limited to phloem tissues in infected plants and transmitted in a persistent (circulative and non-propagative) manner by the aphid species *Aphis gossypii* Glover, *Myzus persicae* Sulzer, and *M. euphorbiae* Thomas (Gray and Gildow 2003). CABYV is best known for its infection of cucurbit species such as cucumber, melon, watermelon, zucchini, pumpkin, and bitter cucumber, in addition to other cultivated species such as fodder beet *(Beta vulgaris*) and lettuce (*Lactuca sativa*) and a variety of wild plant species, which could serve as reservoirs and/or sources of inoculum (Lecoq and Desbiez 2012; Maachi et al. 2022). Symptom expression can vary according to the plant species, the age of the plant at the time of infection, and environmental conditions. In general, infected plants show yellowing, with necrotic interveinal sectors on the older leaves that develop close to the major side veins, along with a significant yield reduction as a consequence of flower abortion. In fact, a novel CABYV variant has been recently described as being responsible for the exacerbation of watermelon plant yellowing, which is accompanied by a drastic yield reduction because of the high rates of fruit abortion, with its concurrent presence suggesting that CABYV disease is becoming a problem in cucurbits (Rabadán et al. 2021). The sequencing of different CABYV isolates has identified two main groups: Asian or Chinese (C) and Mediterranean (M) groups, with phylogenetic analysis further distinguishing a Recombinant (R) group of isolates (Knierim et al. 2010; Shang et al. 2009). Nevertheless, the recent characterization of CABYV isolates from passion fruit plants in Brazil and their comparison with other CABYV sequences have unveiled a complex of different species within the CABYV group (Vidal et al. 2023). To date, In Spain, the CABYV isolates sequenced from a local survey in 2003 to 2005, and more recently in 2020, were associated with the Mediterranean group (Kassem et al. 2013; Maachi et al. 2022). In the case of WMV, its genome consists of a single-stranded (ss) RNA molecule of 10 kb, which encodes for a polyprotein that is processed proteolytically to about 10 smaller mature proteins (Desbiez and Lecoq 2008). WMV has a wide host range and is non-persistently transmitted by at least 30 aphid species (Lecoq and Desbiez 2012). The WMV population is mainly represented by two groups: Classic (CL) and Emergent (EM) (Desbiez et al. 2009), although several Asian isolates seem to cluster between both groups (Bertin et al. 2020; Desbiez and Lecoq 2008; Desbiez et al. 2009; Glasa et al. 2011; Moreno et al. 2004). The WMV Spanish population was initially described to belong to the Classic group (Luis-Arteaga et al. 1998) until its apparent displacement by the Emergent group from the study of partial sequences of Spanish isolates from a survey in 2005 to 2006 (Juarez et al. 2013).

Despite the importance of these viruses in cucurbit crops in Spain, only limited local and temporal information is available regarding the distribution and genetic diversity of their viral populations. Furthermore, the recurrent co-detection of CABYV and WMV in the same plant is comparatively common in these crops (Juarez et al. 2013; Kassem et al. 2007; Rabadán et al. 2021), and virus-virus interactions within the same plant could invariably impose a selection pressure on viral populations as opposed to single infections, shaping the evolutionary dynamics of viral populations (Alcaide et al. 2020a, b; Gómez et al. 2010; Susi et al. 2015; Tollenaere et al. 2016; Zhang et al. 2001). To increase our knowledge about the evolutionary epidemiology of aphid-transmitted viral diseases in cucurbit crops, and to evaluate whether mixed infections may act as an ecological driver of the evolutionary dynamics of their populations, we examined the distribution of cucurbit aphid-transmitted viruses through extensive and systematic monitoring of symptomatic samples of melon and zucchini crops in three major producing areas in Spain for 10 consecutive (2011 to 2020) growing seasons. We also carried out a comprehensive genetic characterization of CABYV and WMV isolates by using the Pacific Biosciences single-molecule real-time (SMRT, PacBio) high-throughput sequencing technology. This technology generates highly accurate long reads that are derived from single genomes (Rhoads and Au 2015). This approach allowed us to generate high-quality, full-length genome sequences from a single virus in each sample and to examine the genetic variability and population structure within and between different producing areas, growing seasons, cultivated hosts, and type of infection (single and mixed infections), along with evolutionary forces that shape the evolutionary dynamics of the viral populations.

Materials and Methods

Sample collection

The phytosanitary inspection of melon (*Cucumis melo* L.) and zucchini (*Cucurbita pepo* L.) crops was carried out seasonally from April to September, with peak surveys conducted in July and August in several field plots located in the three main producing areas of Spain: Murcia (37°45′50.9″N, 1°02′55.4″W), Alicante (38°07′06.9″N, 0°47′58.2″W), and Castilla-La Mancha (39°11′37.8″N, 3°12′56.7″W). The coordinates given for each area represent the central point around which most of the plots were located within a radius of approximately 35 km. A total of 1,608 samples were collected from both crops, covering the growing seasons from 2011 to 2020 in Murcia, 2013 to 2020 in Alicante, and 2018 to 2020 in Castilla-La Mancha. In particular, 1,276 melon samples were collected from 245 field plots in Murcia, 82 from Alicante, and 46 from Castilla-La Mancha. For zucchini crops, 332 samples were collected from 87 field plots in Murcia and 21 from Alicante. Note that there were no zucchini samples from Castilla-La Mancha because this crop is not extensively grown in this region. During each field plot survey, 10 apical leaf samples displaying yellowing and mosaic virus-like symptoms were collected. Assuming that the large number of surveyed field plots is well representative of each production area, between two and four samples per plot were processed in the laboratory for total RNA extraction. All RNA extraction and duplicated plant samples were stored frozen at −80°C.

Cucurbit virus detection

Total RNA from plant samples was extracted using Tri-reagent (Sigma-Aldrich, U.S.A.) and used for virus detection through dot-blot hybridization. RNA from each sample was placed on two positively charged nylon membranes, and the RNA was fixed with an ultraviolet crosslinker. Dot-blot molecular hybridization was carried out using specific RNA probes for CABYV, WMV, CMV, MWMV, PRSV, and ZYMV detection (De Moya-Ruiz et al. 2021; Kassem et al. 2007). The membranes were incubated overnight at 68°C with the specific DIG-labeled probes. After the hybridization, a series of washes were carried out, followed by an incubation with the anti-digoxigenin antibody conjugated to phosphatase alkaline (Anti-Digoxigenin-AP, Roche Diagnostics, Germany) and the chemiluminescent substrate CDP-Star (GE Healthcare UK Ltd., England) (Gómez-Aix et al. 2019). The membrane analyses were performed using a chemiluminescent detector, Amersham Imager 600 (GE Healthcare Bio-Sciences AB, Sweden). RT-PCR was also performed on a subset of samples for confirmation of the dot-blot hybridization result, such as described in Rabadán et al. (2021). PCR was performed with the NZYTaq II 2× Green Master Mix (Nzytech), with the following cycling conditions: 95°C for 5 min; 30 cycles of 94°C for 10 s, 50 to 60°C for 30 s, and 72°C for 90 s; with a final extension at 72°C for 10 min before cooling to 4°C.

Full-length CABYV and WMV genome amplification

The genetic characterization of the populations was carried out with the analysis of the full-length CABYV and WMV genome sequences that were obtained from the SMRT-PacBio high-throughput technology. We randomly selected CABYV and WMV isolates from each subset of positive samples, considering the hosts (melon and zucchini), location (Murcia, Alicante, and Castilla-La Mancha), growing season (2011 to 2020), and type of infection (single and mixed). The cDNA synthesis of the entire CABYV and WMV genomes was performed by using a reverse transcriptase (Roche) with the specific primers CE-2513 (5,650 nt) 5′-ACACCGAAACGCCAGGGGGAATC-3′ for the CABYV isolate under accession number MW051362.1 and CE-2620 (10,014 nt) 5′-TTTTTTTTTTTTTAGGACAACAAACATTACCG-3′ for the WMV isolate under accession number MW147356.1. Then, full-length CABYV genomes of 24 isolates were amplified with the Expand high-fidelity system (Roche) according to the following cycling conditions: 94°C for 2 min; 10 cycles of 94°C for 15 s, 62°C for 30 s, and 72°C for 4 min; 15 cycles of 94°C for 15 s, 62°C for 30 s, and 72°C for 4 min; with a final extension of 72°C for 7 min before cooling to 4°C, and using the specific PacBio primers (in italic), along with the specific viral sequence (with capital letters): CE-2634 (1 nt) Fw 5′-5AmMC6*gcagtcgaacatgtagctgactcaggtcac*ACAAAAGATACGAGCGGGTGATG-3′ and CE-2635 (5,672 nt) Rv 5′-5*AmMC6tggatcacttgtgcaagcatcacatcgtag*ACACCGAAACGCCAGGGGG-3′. Similarly, full-length WMV genomes of 22 isolates were amplified with VeriFi Mix Red (PCR-Biosystem, U.K.) following the manufacturer's recommendations: 95°C for 2 min; 25 cycles of 95°C for 15 s, 60°C for 15 s, and 72°C for 5 min, and using the PacBio primers (in italic), along with the specific viral sequence (with capital letters): CE-2638 (1 nt) Fw 5′-*5AmMC6gcagtcgaacatgtagctgactcaggtcac*AAATTAAAACAACTCATAAAG-3′ and CE-2790 (10,031 nt) Rv 5′-5*AmMC6tggatcacttgtgcaagcatcacatcgtag*GACAACAAACATTACCGTACCTCG-3′.

Full-length genome sequencing by PacBio

After the first PCR round, the PCR fragments from each viral sample were submitted to the Centre for Genomic Research (CGR, University of Liverpool, U.K.) for PacBio amplicon libraries. Briefly, samples were cleaned up with AMPure BD, and the specific amplified fragment (CABYV: 5.6 kb and WMV: 10 kb) was size-selected by the BluePippin system through a 0.75% agarose gel. Then, a second round of PCR was performed with Phusion Hot Start II High-Fidelity to add a unique PacBio-validated barcode, according to the PacBio recommendations. Then, PCR products were purified with AMPure PB magnetic beads, and around 1 to 5 μg of DNA was used to prepare the SMRTBell library by ligating the corresponding adapters, according to PacBio protocols. This SMRTBell library reduces sequencing bias, whereas each SMRTBell template generates one pass on each single molecule sequenced. An exonuclease reaction was used to remove any unligated DNA. Finally, three rounds of DNA purification using AMPure BD magnetic beads were performed to guarantee DNA quality input, obtaining two libraries with concentrations ranging between 30 and 50 ng/μl (5,600 bp) and 50 ng/μl (10,000 bp). Primer annealing and P4 polymerase binding to the SMRTBell libraries were performed, followed by SMRT sequencing on the PacBio Sequel/RSII platform, using 20-h movies. After sequencing, the sequences were demultiplexed using the previously added PacBio barcodes. High-quality sequences (QV $>$ 20, 400,000 to 250,000 reads) were then selected by ranking the high-quality reads based on length and similarity using cd-hit-est (https://github.com/weizhongli/cdhit/wiki/#CDHITEST).

Assembling and multiple sequence alignment of the full-length CABYV and WMV genomes

The assembly of long-read sequences of the CABYV and WMV genomes was performed with the LoReTTa (Long Read Template-Targeted Assembler) tool, which was specifically developed for assembling viral genomes from long PacBio reads (Qaffas et al. 2021). It allows us to obtain a highly accurate and uniform coverage of the full-length genome sequences by minimizing the effect of long-read sequencing errors through the use of the longest read of each dataset as a reference to align the shortest reads and by correcting errors by calling consensus of the alignments (Wick and Holt 2019). LoReTTa was used under the Linux operating system, according to the command version with default cutoff values. The accessions MW051362.1 (CABYV) and MW147356.1 (WMV) were provided as the genome references, obtaining a consensus sequence for each viral sample. Consensus sequences were examined, and the alignments were carried out with ClustalW algorithm in MEGA7.

Phylogenetic analysis of the full-length CABYV and WMV genomes

The phylogenetic relationships between CABYV isolates were inferred from the 24 full-length isolates from this study, combined with 33 full-length CABYV sequences from the GenBank database (accessions indicated in Supplementary Table S1), including the sequence of melon aphid-borne yellows virus as the outgroup rooting and using MEGA7 software (Kumar et al. 2016). Similarly, for WMV, a phylogenetic analysis was inferred from the 22 full-length isolates of this study and 36 WMV accessions sequences from the GenBank database (accessions indicated in Supplementary Table S1). CABYV and WMV sequences were aligned with the ClustalW algorithm in MEGA7, and thereafter, we analyzed the evolution analysis to find the best model to study the evolutionary and phylogenetic history in MEGA7. It was analyzed for the following models: GTR: General Time Reversible; HKY: Hasegawa-Kishino-Yano; TN93: Tamura-Nei; T92: Tamura 3-parameter; K2: Kimura 2-parameter; and JC: Jukes-Cantor. We considered General Time Reversible the model with the lowest Bayesian information criterion scores to describe the substitution pattern. The evolutionary history and phylogenetic tree were inferred with the maximum likelihood method based on the General Time Reversible model and with 1,000 bootstrap replicates.

Reconstruction of time-scaled phylogenies of CABYV and WMV populations

To assess the temporal structure, we first used TempEst v1.5.3 (http://tree.bio.ed.ac.uk/software/tempest/) by submitting each iqtree file obtained from the multiple viral alignment upload in the IQ-Tree server. Then, the sample collection dates were imported into the TempEst program, selecting the best-fitting root to perform the best linear regression, according to the sequence dataset compatibility for a strict or relaxed clock evolutionary model (Rambaut et al. 2016). Additionally, the arrangement of phylogenetic trees was confirmed using only the sequences in which no recombination events were detected. Also, we performed Bayesian phylogenetic analyses in BEAST v1.10.4. (Suchard et al. 2018) to infer the evolution rate and timescales of CABYV and WMV populations. After comparing strict and relaxed molecular clocks, the tree was created with the strict molecular clock, and the distributions of parameters were estimated by the Markov chain Monte Carlo (MCMC) iterations, with the first 10% of samples discarded and the rest drawn every 10^4 MCMC steps. The MCMC process was examined using the program Tracer v1.7.2 (http://tree.bio.ed.ac.uk/software/tracer/) to ensure adequate sampling. Tree files were generated, and Bayesian maximum-clade credibility trees were visualized using FigTree v1.4.4 (http://tree.bio.ed.ac.uk/software/figtree/).

CABYV and WMV recombination analysis

The analysis of recombination events was carried out with the RDP4 software using all CABYV and WMV sequences from this study, as well as the accessions (indicated above) from the NCBI GenBank. RDP4 utilizes different analytical methods (RDP, GENECONV, BootScan, MaxChi, Chimera, and SiScan), which are implemented with different assumptions, and here, we used them

with default settings and a Bonferroni corrected *P* value of 0.05 (Martin et al. 2015). Only those recombination events detected by four or more methods were considered significant and were further verified by Simplot 3.5.1 software, according to the consistency between the potential recombination isolate and its major and minor parents.

Genetic diversity analysis of the viral populations

Genetic diversity (π), number of haplotypes (h), Tajima's D (D), gene flow index (Fst), and a nucleotide sequence-based statistic (Kst) were estimated with DnaSP6 software (Rozas et al. 2017) among the CABYV and WMV subpopulations. Briefly, a positive value on the Tajima's D neutral test means a relative abundance of polymorphic alleles, whereas negative values indicate the presence of rare alleles. The Fst values can range from 0 to 1, meaning no or complete population differentiation, as a result of the migration flow between populations. Kst can range from 0 to 1, meaning no or complete genetic differentiation. The Shannon index (H) was calculated with the poppr package, displaying species diversity into a population, with values that can range from 0.5 to 5, where <2 means low diversity and >3 high diversity. Additionally, to ascertain the direction and strength of selection operating on the CABYV and WMV populations, the ratio between the number of nonsynonymous substitutions per nonsynonymous site (dN) and the number of synonymous substitutions per synonymous site (dS) was calculated using the Pamilo-Banchi-Li method in MEGA7 (Pamilo and Bianchi 1993). The dN/dS ratio (≈ 1 indicates neutral evolution, <1 negative or purifying selection, and >1 positive or adaptive selection) was calculated with MEGA7. To identify individual codon positions evolving under natural selection, four different codon-based maximum-likelihood algorithms, single likelihood ancestor counting (SLAC), fixed effects likelihood (FEL), internal fixed effects likelihood (IFEL), and mixed effects model of evolution (MEME), were used within the HyPhy software package as implemented in the DataMonkey server (https://www.datamonkey.org) (Weaver et al. 2018), with a significance level (*P* value) less than 0.05.

Spatio-temporal differentiation analysis

Population subdivisions were examined using a model-free discriminant analysis of principal components (DAPC), implemented in the R package adegenet (Jombart et al. 2010). DAPC first transforms the data using a principal component analysis (PCA) and then assigns individuals to clusters by DA, maximizing variation between groups while minimizing variations within groups (J. Wang et al. 2017). Genetic distances were calculated with the poppr package (diss.dist) using a dissimilarity matrix utilized by the index of association. The distances between haplotypes were visualized by a minimum-spanning network (MSN), using the function poppr.msn implemented in the R poppr package (Kamvar et al. 2014). Additionally, an analysis of molecular variance (AMOVA) was carried out in R with the Poppr package to estimate the portion of molecular variation into potential population subdivisions due to variation within and between subpopulations portioned by host, growing season, and/or type of infection. This multivariate analysis was calculated with the Euclidean distance between populations with multiple pairwise tests and a significance level (*P* value) less than 0.05. The population subdivision based on the type of infection was represented using a PCA implemented in the R package ade4 (Dray and Dufour 2007).

Statistical analyses

The proportion of each type of infection (single or mixed) was estimated as the number of positive samples for each virus (CABYV and WMV either alone or in combination) according to the total samples analyzed from different years (2011 to 2020), host (melon and zucchini), and location (Murcia, Alicante, and Castilla-La Mancha). For comparison, the frequency of each virus was averaged through time and analyzed with a generalized linear model with the detection outcome (presence/absence) as an independent variable and assuming a binomial regression with logit function. Virus species, plant hosts, and locations were fitted as treatments, with their interactions as appropriate. The analysis of potential associations between viruses in multiple infections was carried out using a chi-square distribution based on a binary (2×2) contingency table approach. This analysis assessed the dependence of mixed infections with the presence or absence of CABYV and WMV in single infections. For this, the observed and expected ratios of single and mixed infections were compared ($df = 1$, $P < 0.05$), including the number of samples that were negative. Additionally, the potential association between CABYV and WMV was further analyzed by the calculation of the association index (Malpica et al. 2006), considering the distribution of CABYV and WMV in melon and zucchini crops, and where no association between viruses was indicated by values close to 0. Thus, the prevalence homogeneity for both viruses in melon or zucchini was calculated with the index of selectivity of the host (Malpica et al. 2006). All analyses were carried out using the package stats in R studio.

Nucleotide sequence accession numbers

The CABYV sequences were deposited in GenBank under accession numbers OM948834 to OM948857, and WMV sequences were deposited in GenBank under accession numbers OM948812 to OM948833. See Supplementary Table S1 for further information about each accession reference.

Results

Distribution of cucurbit aphid-transmitted viruses in melon and zucchini crops

A total of 1,276 melon samples exhibiting yellowing and mosaic symptoms were collected from the three main cucurbit-producing areas of Spain through consecutive growing seasons, Region of Murcia (2011 to 2020), Alicante (2013 to 2020), and Castilla-La Mancha (2018 to 2020), along with 332 zucchini samples from Murcia (2011 to 2020) and Alicante (2013 to 2020). Each sample was analyzed for CABYV, WMV, MWMV, ZYMV, CMV, and PRSV detection by dot-blot molecular hybridization. In general, our analysis revealed that the occurrence of virus species varied among melon and zucchini crops (interaction between virus species and plant host; $\chi^2 = 72.63$, $df = 5$; $P < 0.001$), with a relatively higher ratio of CABYV and WMV than the rest of the viruses. This variation was comparable for each location ($\chi^2 = 19.67$, $df = 2$; $P < 0.001$), with scarce differences across years (Fig. 1). In particular, in melon crops, the percentage of CABYV was similar in all three areas: Murcia (28.7%), Alicante (24.6%), and Castilla-La Mancha (30.4%), between all growing seasons ($\chi^2 = 2.38$, $df = 2$; $P = 0.30$). WMV was detected in higher numbers in samples from Alicante (34.3%) and Castilla-La Mancha (31.5%) than in those from Murcia (24.8%) ($\chi^2 = 10.66$, $df = 2$; $P = 0.004$). CMV was detected in Alicante (2.4%), Castilla-La Mancha (2%), and Murcia (1.7%) at similar ratios ($\chi^2 = 0.55$, $df = 2$; $P = 0.75$), whereas MWMV and ZYMV were only detected in Murcia in very low percentages (0.2 to 0.1%). We did not detect PRSV in melons and single infection in any location. Furthermore, some of these viruses were co-detected in the same melon sample (mixed infections), with the combination of CABYV and WMV being the most common (10%) (Fig. 1). CABYV and WMV were co-detected at similar ratios in Alicante (9%), Murcia (10%), and Castilla-La Mancha (13%) in melon samples ($\chi^2 = 4.81$, $df = 2$; $P = 0.09$). Similarly, the CABYV + CMV combination was also detected in Murcia (1%) and Castilla-La Mancha (3%), WMV + MWMV was detected in Alicante (2%) and Murcia (0.1%), and CABYV + ZYMV was only detected in melon in Murcia (0.1%). Triple infections were also recorded in melon, but at a very low ratio. For example, the CABYV + WMV + CMV combination was detected in Murcia (1%). CABYV + ZYMV + WMV

and CABYV + PRSV + WMV were observed at similar ratios in Alicante and Murcia (0.3 to 0.1%), and CABYV + WMV + MWMV was only detected in Alicante (0.3%). Moreover, the occurrence of different virus species was higher in melons from 2011 to 2018, whereas CABYV and WMV became highly prevalent in the last growing seasons. There were also some relative differences over the growing seasons and locations. In Alicante, WMV was found in a high ratio of the infected samples (24%) from 2019, and CABYV was present in only 2% of samples. In Murcia, CABYV was the most common virus found every year in the infected samples, with one major peak (76%) in 2016.

In zucchini crops, 334 samples were analyzed from Murcia and Alicante, and CABYV was detected in a higher ratio than in melon samples ($\chi^2 = 15.03$, df = 5; $P < 0.001$), with an average of 36% in Alicante and 25% in Murcia, whereas WMV was detected at a lower ratio in Alicante (9%) and Murcia (4%). CMV was only detected in Murcia (2%), and the rest of the viruses, MWMV, ZYMV, and PRSV, were not detected in any zucchini sample under single infections (Fig. 1). In the case of zucchini crops and mixed infections, CABYV + WMV was detected at a higher ratio in Alicante (25%) than in Murcia (8%) ($\chi^2 = 18.49$, df = 1; $P < 0.001$). Likewise, CABYV + ZYMV was also detected in Alicante (4%) and Murcia (1%). CABYV + PRSV was only detected in Murcia (1%), and CABYV + CMV was found in Alicante (1%). The combinations of CABYV + CMV, WMV + ZYMV, CABYV + MWMV, and WMV + CMV were all detected in zucchini samples from Murcia at a similar ratio (0.4%). Finally, we observed triple infections of CABYV + ZYMV + WMV, CABYV + MWMV + WMV, and CABYV + ZYMV + MWMV in Murcia, ranging from 0.4 to 1%, whereas WMV + PRSV + ZYMV was detected in Alicante (1%). Moreover, the occurrence of mixed infections was higher in zucchini than in melon, with major peaks in 2020. Also, the occurrence of CABYV was found to peak at >50% in 2015, 2016, 2018, and 2019 in Murcia and Alicante. In Murcia, PRSV and MWMV were found in mixed infections in 2018.

Furthermore, whereas a high number of co-occurrences between CABYV and WMV was observed, we also sought to assess the extent to which these mixed infections could be related to their frequencies in single infections. Thus, according to the observed viral frequencies in single and mixed infections in all growing seasons and locations for each crop, we analyzed if there were any associations between CABYV and WMV sharing the same host. This analysis was performed by a binary chi-square contingency table approach, as well as by estimating the indices and using a test of selectivity. The contingency analysis indicated that the co-occurrence of CABYV and WMV was significantly independent of the single infections in either melon ($\chi^2 = 43.59$, $P < 0.001$) or zucchini ($\chi^2 = 27.31$, $P < 0.001$) samples. Similarly, the analysis of the association index (AI) between CABYV and WMV indicated

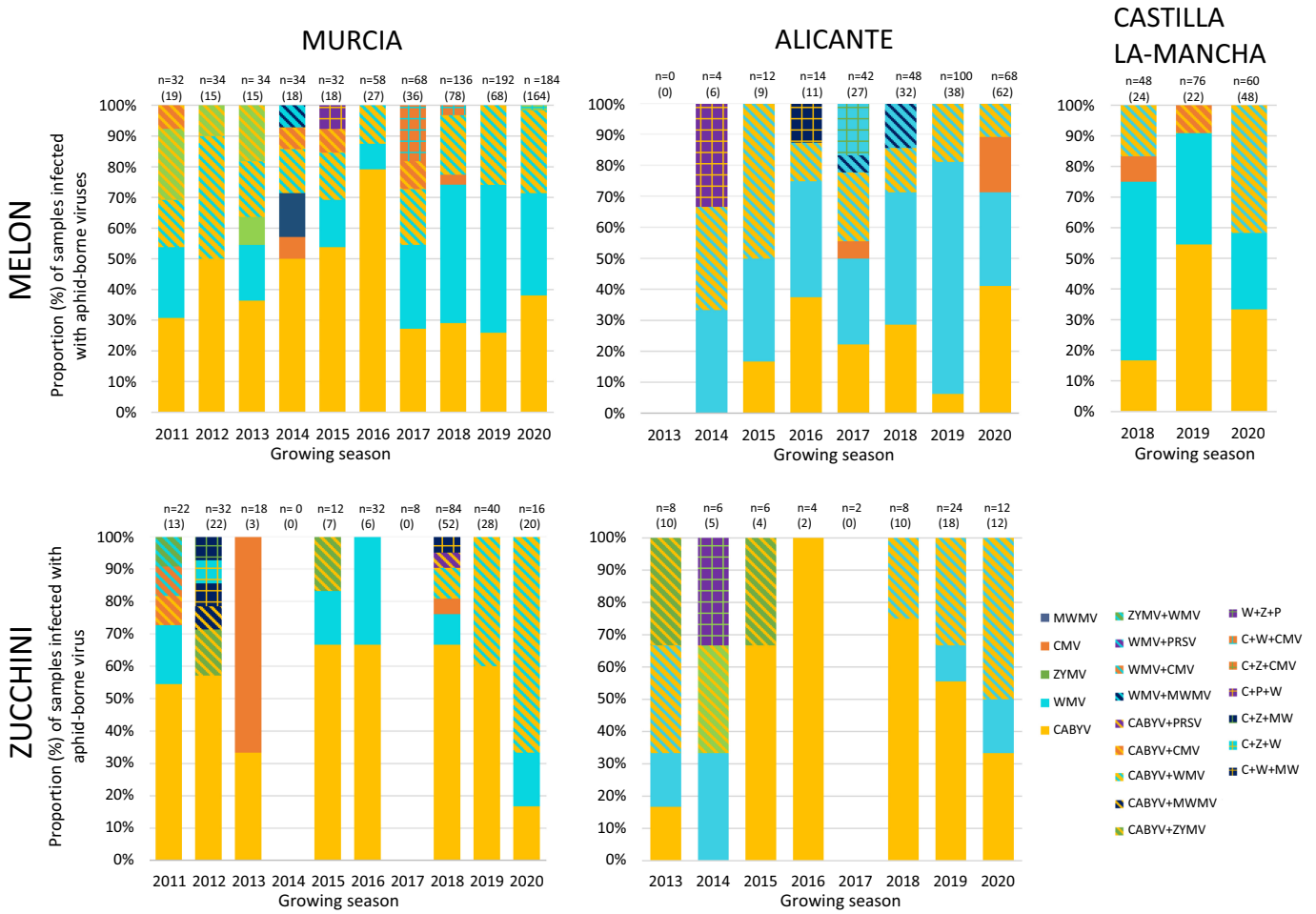

Fig. 1. Temporal and geographical distribution of aphid-transmitted virus in melon and zucchini crops across three Spanish regions. Each panel displays the occurrence of each virus in samples showing virus-like symptoms over the growing seasons in Murcia, Alicante, and Castilla-La Mancha. Bars represent the proportion (%) of virus detected within the infected samples, with solid bars indicating single infections and stripped bars for mixed infections. Viral species and coloring are indicated in the legend. The number of infected samples (x) out of the total number of samples (n) is shown at the top of each bar.

their association in melon (CABYV, AI = 0.27; WMV, AI = 0.24) and zucchini (CABYV, AI = 0.28; WMV, AI = 0.45). Additionally, the analysis of the index of selectivity of the host (IHS) showed a moderate selectivity in both cases (melon, IHS = 0.57; zucchini, IHS = 0.46), suggesting that the occurrence of both viruses in both crops, and potential interactions between CABYV and WMV in mixed infections, may play a role in the evolutionary epidemiology of their populations.

Phylogenetic relationship among CABYV and WMV isolates

We inferred the phylogenetic relationship and examined the genetic differentiation of the most common aphid-transmitted viruses (CABYV and WMV) in melon and zucchini crops from 44 isolates that were randomly collected, considering the host, location, growing season, and type of infection (single and mixed infection). For this, we characterized the complete nucleotide sequences of each viral genome via the PacBio Sequel/RSII sequencing approach. In particular, 24 CABYV isolates were sequenced, and their phylogenetic analysis, including another 34 isolates from NCBI GenBank, indicated that Spanish CABYV isolates were mainly grouped within the Mediterranean cluster, except for one recombinant isolate (19.1Zu/A/Sg/2019) (Fig. 2A). Indeed, the RDP4 analysis suggested a breakpoint in the P3 to P5 region (4,218 to 4,706 nucleotides) of this isolate, identifying an isolate (9.1Me/M/Sg/2014) from a sample of Melon 2014 as the minor parent, and another isolate (9.1Me/M/Mx/2019) from Melon 2019 as the major parent. In the WMV population, 22 isolates were sequenced and analyzed together with another 36 isolates from NCBI GenBank, showing that Spanish WMV isolates were mainly grouped within the Emergent cluster, as well as two isolates (2.1Me/Mu/Mx/2011 and 64.2Me/CLM/Mx/2018) that were grouped in the Classic group, in addition to one isolate (8.1Me/M/Mx/2016) that was further identified as a putative recombinant, with a breakpoint in the P1 region (22 to 822 nucleotides), suggesting isolate MG194419.1 as the minor parent and KT992078.1 as the major parent (Fig. 2B). Note that phylogenetic trees for CABYV and WMV were also reconstructed

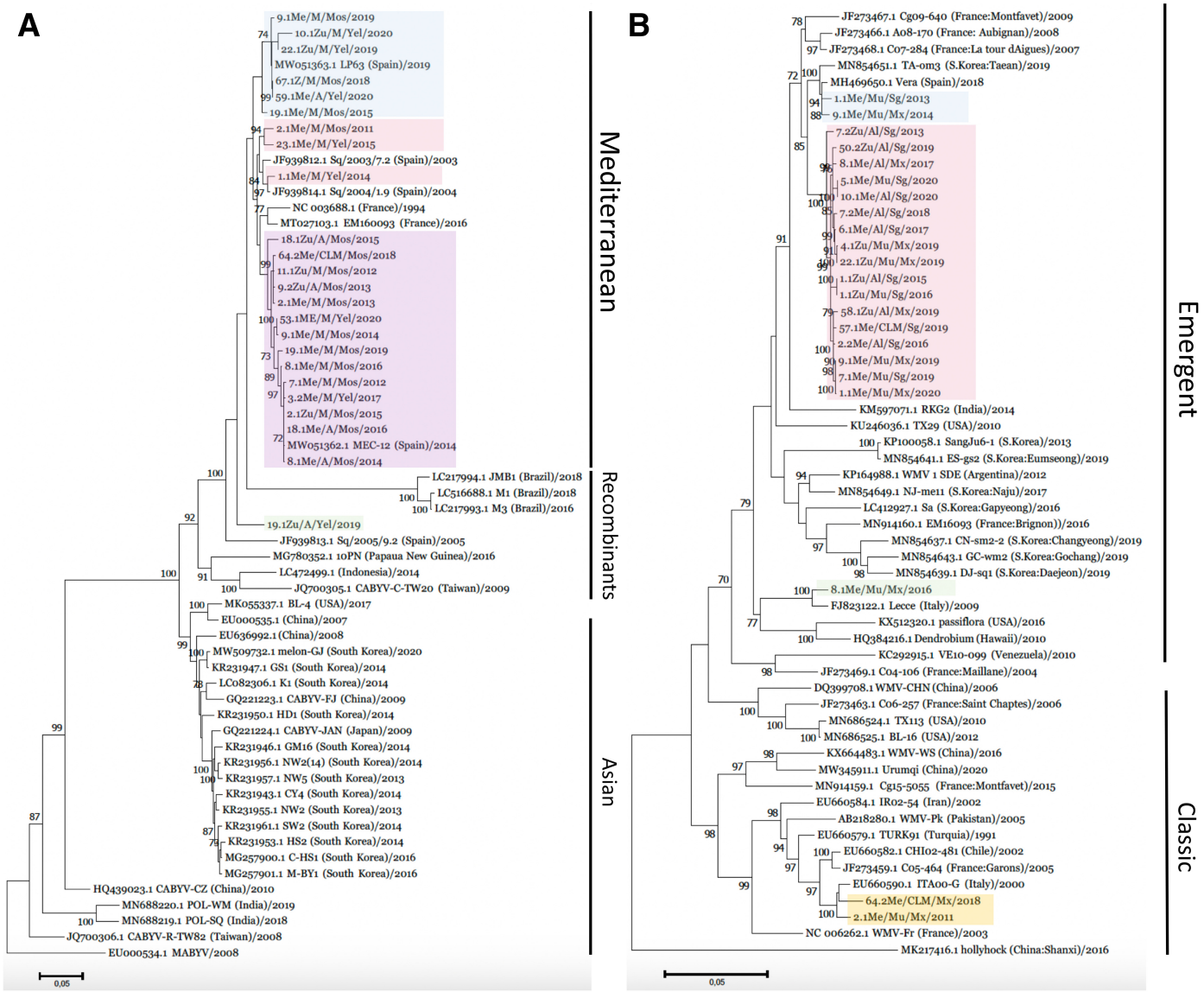

Fig. 2. Full-length phylogenetic relationships among **A,** cucurbit aphid-borne yellows virus (CABYV) and **B,** watermelon mosaic virus (WMV) isolates. Phylogenetic relationships between Spanish isolates from 2011 to 2020 together with isolates from GenBank database (represented by accession number, country, and year). The phylogenetic tree for each virus was built by the maximum likelihood method with 1,000 bootstrap replications and using the General Time Reversible model in MEGA. Only branches with bootstrap values >70% are shown. The lengths of the branches represent the genetic distances that are also represented by the scale bars. Our isolates are colored according to the branch group where they are clustered. Me: Melon; Zu: Zucchini; Mu: Murcia; Al: Alicante; CLM: Castilla La-Mancha; Sg: Single; Mx: Mixed.

without the recombinant isolates, and the tree shape was robust for each of them.

Genetic diversity and population structure of CABYV and WMV populations

To estimate the genetic diversity of the CABYV and WMV populations, we used the nucleotide sequences from either the full-length sequence or each gene separately. The analysis showed an average low nucleotide diversity in the CABYV ($\pi = 0.029$) and WMV ($\pi = 0.027$) populations at the genome level, with the smallest π values in the WMV populations from zucchini, Alicante, and single infection (Table 1). This low genetic diversity was also in accordance with the analysis of nucleotide diversity by gene, with the highest values: $\pi = 0.034$ in ORF0 of CABYV and $\pi = 0.038$ in ORF1 of WMV. This indicates that the genetic variabilities of CABYV and WMV at the genome level were not evenly distributed on the viral genome. Likewise, the number of segregation sites (S) differed among WMV subpopulations. The full-length genome sequences of WMV isolates from melon, Murcia, and mixed infection displayed the largest number of segregating sites (1,439, 1,375, and 1,164, respectively). In turn, S was similar among CABYV subpopulations, ranging from 440 to 548. Thus, the pairwise comparisons of the genetic parameters of Kst and Fst values between subpopulations were all close to 0, indicating no differentiation in either CABYV or WMV subpopulations according to host, location, and type of infection. Furthermore, to ascertain the direction and strength of selection operating in the CABYV and WMV populations, we evaluated average dS and dN values across all (concatenated) genes, as well as each gene separately. In CABYV, the genetic diversity for each gene in synonymous sites was similar to nonsynonymous sites by host, location, and infection type subpopulations (data not shown), showing overall dN/dS values of 0.08 to 0.3. However, for ORF4, the dN/dS value was 2.12, most certainly due to overlap with ORF3. Nevertheless, the dN/dS ratio was 0.8 to 0.9 considering the entire CABYV genome (Table 1). In WMV, the genetic diversity in synonymous sites was typically higher than in nonsynonymous sites for each gene in all subpopulations (data not shown), with the overall dN/dS ratio ranging from 0.01 to 0.3, thus corroborating the negative (purifying) selection acting on the full-length sequence of WMV. Next, we performed codon-based tests and found 90 codons under negative selection and seven codons ($P < 0.05$), ORF0 (position 146), ORF1 (positions 461 and 539), ORF2 (positions 36, 46, and 90), and ORF5 (position 478), under positive selection in several isolates of the CABYV population (Supplementary Table S2). Conversely, in the WMV population, 35 codons were under negative selection, and no codon was identified under positive selection. As such, the results of the Tajima's D neutrality tests indicated that the CABYV and WMV subpopulations had similar demographic or selective histories, suggesting that both viral populations are currently undergoing a population size expansion through a low frequency of rare mutations. All the D values of the WMV subpopulations were negative, whereas for the CABYV populations, these values ranged from negative to positive, and the positive values were not significantly different from negative values in zucchini, Murcia, or mixed infections ($P > 0.10$) (Table 1). It is likely that the temporal dynamics of these virus populations are contributing toward the fluctuation in nucleotide diversity values across growing seasons, ruling out the possibility of an evolutionary equilibrium in these virus populations.

Temporal dynamics on CABYV and WMV populations

To contextualize the nucleotide variability across the growing seasons, we next examined the genetic diversity of CABYV and WMV populations by performing a time-based DAPC. This DAPC revealed that the CABYV population was discriminated into different subpopulations per year, with contemporary CABYV isolates (2019 to 2020) differentiated from all the other temporally preceding isolates (Supplementary Fig. S1A). This temporal differentiation was also observed in the MSN analysis, showing the close genetic relatedness between contemporary CABYV isolates (Supplementary Fig. S1B). However, the analysis of the WMV population showed no temporal differentiation along the component axis, and it appeared to be relatively grouped (Supplementary Fig. S2A). In fact, the samples with a distant placement (2011 and 2018) belonged to the isolates 2.1Me/Mu/Mx/2011 and 64.2Me/CLM/Mx/2018, which were grouped in the phylogenetic tree within the Classic cluster. This genetic homogeneity of the WMV population was also validated by the MSN analysis (Supplementary Fig. S2B). Additionally, this temporal ascertainment was tested using a statistical clock analysis of the collection date of the CABYV and WMV isolates, according to the root-to-tip regression implemented in TempEst. Indeed, a moderate signal of sequence divergence was observed in the Spanish CABYV isolates ($n = 26, R^2 = 0.20$) during the sampling time, whereas there was no significant temporal structure in the WMV population ($n = 22, R^2 = 0.004$). Furthermore, the corresponding BEAST analysis revealed that the Spanish CABYV

TABLE 1. Genetic diversity parameters and neutrality tests estimated for cucurbit aphid-borne yellows virus (CABYV) and watermelon mosaic virus (WMV) in Spanish populations[z]

										Host				Location				Type infection	
										Melon		Zucchini		Murcia		Alicante		Single	
Virus		S (n)	π	dS	dN	dN/dS	H	D	Hd	Fst	Kst	Fst	Kst	Fst	Kst	Fst	Kst	Fst	Kst
CABYV																			
Host	Melon	477 (15)	0.025	0.027 ± 0.0023	0.023 ± 0.0015	0.851	2.83	−0.02	1	–	–								
	Zucchini	486 (8)	0.034	0.039 ± 0.0035	0.032 ± 0.002	0.82	2.08	0.17	1	0.07	0.03	–	–						
Location	Murcia	526 (17)	0.028	0.032 ± 0.0028	0.026 ± 0.0018	0.81	2.94	0.07	1	−0.05	−0.02	0.007	0.003	–	–				
	Alicante	440 (6)	0.033	0.037 ± 0.0035	0.031 ± 0.0021	0.83	1.79	−0.11	1	−0.005	−0.002	−0.12303	−0.061	−0.027	−0.01	–	–		
Type of infection	Single	548 (12)	0.03	0.032 ± 0.0029	0.029 ± 0.0019	0.9	2.46	−0.2	0.97	−0.048	−0.024	−0.025	−0.012	−0.049	−0.024	−0.07927	−0.034	–	–
	Mixed	451 (11)	0.029	0.030 ± 0.027	0.025 ± 0.0016	0.83	2.56	0.27	1	−0.04	−0.02	−0.026	−0.013	−0.06	−0.026	−0.06049	−0.028	−0.0356	−0.018
WMV																			
Host	Melon	1,623 (16)	0.038	0.116 ± 0.0038	0.011 ± 0.0007	0.094	2.69	−1.05	0.991	–	–								
	Zucchini	171 (7)	0.006	0.015 ± 0.0015	0.002 ± 0.0003	0.133	1.95	−0.68	1	0.086	0.031	–	–						
Location	Murcia	1,564 (12)	0.038	0.114 ± 0.0036	0.010 ± 0.00075	0.087	2,485	−1.34	1	−0.069	−0.034	0.068	0.029	–	–				
	Alicante	213 (9)	0.005	0.0142 ± 0.0012	0.0019 ± 0.00033	0.135	2,197	−1.49	1	0.08	0.033	−0.078	−0.039	0.075	0.036	–	–		
Type of infection	Single	382 (11)	0.008	0.023 ± 0.0015	0.0026 ± 0.0003	0.113	2.4	−1.52	0.981	0.046	0.021	−0.044	−0.021	0.031	0.016	−0.05321	−0.026	–	–
	Mixed	1,424 (12)	0.042	0.141 ± 0.004	0.013 ± 0.00093	0.092	2.48	−0.534	1	−0.068	−0.033	0.09	0.041	−0.064	−0.032	0.093	0.047	0.072	0.039

[z] CABYV and WMV populations were divided according to the sample host, localization, and type of infection. S = segregating sites, n = haplotypes, π = nucleotide diversity, dS = the number of synonymous substitutions per synonymous site, dN = the number of nonsynonymous substitutions per nonsynonymous site, H = Shannon-Weiner diversity index, D = Tajima's test, Hd = haplotype diversity, Fst = gene flow index, and Kst = nucleotide sequence-based statistic. Values for dS and dN are presented as the mean ± SD.

population diverged shortly after the introduction of CABYV in Spain in 2003, resulting in a temporal structure of its population (Supplementary Fig. S3).

To assess whether this temporal inference was correlated to the host, location, or type of infection, we performed a hierarchical AMOVA to probe for any differentiation within and between populations. Given that location may bias any temporal correlation due to the long-term surveillance in Murcia, as compared with Alicante and Castilla-La Mancha, this analysis was only assigned at this local level, estimating the haplotype diversity by host and type of infection. The AMOVA showed no differentiation between melon and zucchini CABYV populations ($P = 0.15$). However, there was a moderate differentiation among the types of infection within each growing season (phi $= 0.25$; $P = 0.0009$), explaining 28.4% of the variation (Supplementary Table S2). In contrast, the WMV population was differentiated between neither hosts nor type of infection ($P > 0.05$). This nucleotide variation at the complete viral genome level was further examined with a single-nucleotide polymorphism (SNP)-based PCA, which showed the haplotypic diversity of the CABYV and WMV population (Fig. 3). We observed that the variation between single and mixed infections within each growing season was more diverse in the CABYV population (Fig. 3A) than in the WMV one (Fig. 3B). The first and second principal components clearly separated isolates coming from single and mixed infection samples with CABYV, whereas WMV was not differentiated by these components. Together, these results indicate that some within-host interference in the CABYV population may occur by the presence of WMV in mixed infections but that genetic variability was limited from selection.

Discussion

This study describes the occurrence and distribution of six aphid-transmitted viral diseases affecting symptomatic melon and zucchini plants over long-term monitoring in Spain, coupled with phylogenetic and genetic variability analysis of the CABYV and WMV populations at the complete genome level. Although the survey of samples showing symptoms could introduce a bias for virus detection due to the fact that some viral infections can exacerbate symptom expression, our results show, first, that CABYV and WMV are predominantly responsible for the yellowing and mosaics symptoms in cucurbit crops, respectively, and these are widely distributed. Second, there is a high occurrence of mixed infections, with unexpected associations between CABYV and WMV in both hosts. Third, the CABYV populations are mainly grouped into the Mediterranean clade, with a fine-scale temporal structure that was in partly explained by WMV presence in mixed infections over the growing seasons. Fourth, there is a genetically uniform WMV population mostly composed of isolates from the Emergent genotype, with no differentiation pattern between plant hosts, location, season, or type of infection. These findings could be particularly relevant for the integration of viral disease management programs in cucurbit crops given that they update our knowledge about the phylogenetic and geographical distribution of the aphid-borne plant viruses. However, it is also worth noting that the majority of molecular epidemiological studies have focused on short-term contexts, whereas this study presents an evolutionary epidemiological history of CABYV and WMV to understand their evolutionary dynamics and to determine which ecological and evolutionary forces may shape the genetic diversity of virus populations.

Occurrence of aphid-transmitted viruses in cucurbits

Spain is among the largest producer of cucurbits in the Mediterranean basin, with a total of 67,500 ha cultivated in the central and southeastern areas and over 3 million tons harvested a year (MAPAMA 2021). Even without a reliable estimation of the viral disease incidence on these crops in Spain, it appears to have increased in the last few years, possibly due to rising aphid abundance in the early crop stages associated with the production of organically grown vegetables. The transmission and spread of most plant viruses depend on insect vectors, and more than 70% of them are transmitted by hemipterans (Fereres and Raccah 2015). Among

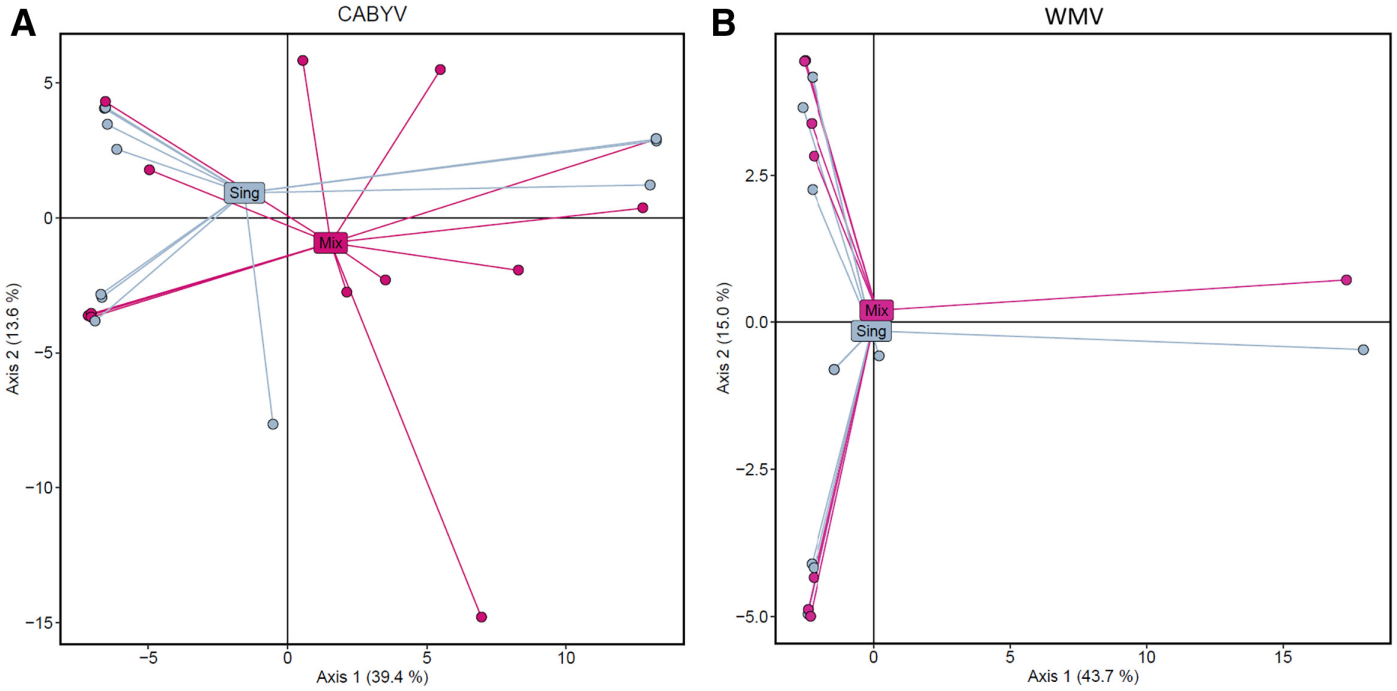

Fig. 3. Principal component analysis (PCA) of **A,** cucurbit aphid-borne yellows virus (CABYV) and **B,** watermelon mosaic virus (WMV) populations. CABYV and WMV populations are represented by infection type, mixed infection (pink), and single infection (blue). PCA was performed based on the single-nucleotide polymorphism difference among population single and mixed infection in R studio with the ade4 package. PCA showed the population differentiation among mixed infection and single infection in CABYV and WMV populations.

these, aphids transmit nearly 30% of all plant virus species described to date (Brault et al. 2010). Our results show that an average of 61% of the samples were positive for viral infection. CABYV and WMV were on average more readily detected in melon (28 and 27%, respectively) and zucchini (42 and 18%, respectively) symptomatic samples, as compared with CMV, MWMV, ZYMV, and PRSV, which ranged between 4 and 0.1%. Despite this variation between melon and zucchini crops, the frequency distribution of these aphid-transmitted viruses was relatively comparable in the three producing areas, CABYV (31%) and WMV (26%), with small differences fluctuating over the growing seasons (Fig. 1). This virus occurrence appeared to be at a low proportion, but only samples showing virus-like symptoms were collected. However, it must be considered that cultivated species, growing conditions, or nutritional deficiency could frustrate our visual survey, introducing a bias for virus detection. In addition, other virus species transmitted by other insect vectors could also be responsible for the yellowing symptoms, but this would require further research on these cucurbit samples. Nevertheless, our results clearly indicate that CABYV and WMV are the most prevalent aphid-transmitted viruses causing yellowing and mosaics in cucurbit crops in Spain. Whereas similar results were obtained in our recent epidemiological study on watermelon and squash crops (Rabadán et al. 2021), they also seem to be consistent with previous studies in cucurbit crops in closely related producing areas of Spain 18 years ago (Juarez et al. 2013; Kassem et al. 2007). Additionally, the spread and recent identification of CABYV in the Mediterranean basin and Europe is noteworthy (Abou-Jawdah et al. 2000; Desbiez et al. 2020; Minicka et al. 2020; Mnari-Hattab et al. 2009), where it is becoming a threat to cucurbit crops.

The prevalence of plant viruses affecting cucurbits could be associated with the commercial exchanges of seeds, plants, or fruits, in addition to the presence of aphids, alternative cultivations, and wild plant host species. Many aspects could account for the differential virus distribution observed in this study, for example, the aphid vector abundance and transmission efficiency of these viruses, the behavior of the aphid vector, and even a differential CABYV and WMV titer with the rest viruses. According to the type of virus−vector relationship, field experiments have shown that CABYV epidemics greatly correlate with *A. gossypii* abundance during the first 2 weeks after planting (Schoeny et al. 2020), and CABYV distribution could be explained because aphids remain viruliferous for weeks before reaching the plant. CABYV is transmitted in a circulative non-propagative manner by *Aphis gossypii*, *Myzus persicae*, and *M. euphorbiae* (Carmo-Sousa et al. 2016; Dogimont et al. 1996; Kassem et al. 2013). However, WMV is non-persistently transmitted by these aphid species and also has a wider distribution. We speculate that the potential WMV transmission by several other aphid species and a broad host range, as compared with CABYV (Lecoq and Desbiez 2012), may likely be responsible for this WMV prevalence (Luis-Arteaga et al. 1998; Sacristan et al. 2004). In contrast, CMV also has a broad host range and is aphid-transmitted in a non-persistent manner (Palukaitis et al. 1992; Sacristan et al. 2004), although its distribution was lower (2%) than that of WMV. It is likely that the virus−plant−aphid interactions could impact aphid probing and feeding behavior, and, along with the role of mixed infections and host ecology by different cultivated plant species, could be determining the prevalence and evolutionary dynamics of these virus populations in cucurbit crops. However, further studies on host ecology and mixed infections, including aphid feeding behavior, should be considered. Additionally, several studies have reported how host preference and vector behavior influence the transmission and spread of plant viruses (Fereres and Moreno 2009; Mauck et al. 2018). In particular, aphids have also shown a high preference for CABYV-infected plants (Carmo-Sousa et al. 2016). Additionally, there is evidence that potyviruses (such as PRSV) may encourage long-term feeding and alter aphid behavior through host plant nutrient enrichment (Gadhave et al. 2019). Given the potential effects of polerovirus and potyvirus on aphid performance, it could be thought that CABYV and WMV may play a beneficial role in their distribution by their influence on aphid behavior. Furthermore, the CABYV viral load can increase in the presence of ZYMV, without an increase in aphid transmission (Bourdin and Lecoq 1994). Thus, the co-occurrence of CABYV and WMV viruses in the same plant could also affect viral accumulation of both or one of them, which may consequently alter virus transmission rates (Alcaide et al. 2020a; Moreno and López-Moya 2020; Wintermantel et al. 2008). In this sense, it has been shown that WMV accumulation can be reduced in the presence of either ZYMV or cucurbit yellow stunting disorder virus (CYSDV), and despite the low accumulation, WMV was efficiently transmitted by the ability of ZYMV or CYSDV to increase aphid attraction (Domingo-Calap et al. 2020; Salvaudon et al. 2013). Thus, it is possible that a differential CABYV and WMV accumulation in the same plant, combined with aphid transmission efficiency, may also play a role in their occurrence and distribution in cucurbit crops, and further research is required.

Genetic diversity of CABYV populations

To our knowledge, this study provides the first genetic diversity analysis of a plant virus using the PacBio SMRT approach, with long-term surveillance. The advantage of this technology is the highly accurate long reads (thousands of sequences for each sample) that are produced using circular consensus sequencing, derived from single genomes, unambiguously warranting the detection of mutations and natural recombinants. Our CABYV phylogenetic analyses confirmed previous reports that most isolates clustered into the Mediterranean group (Juarez et al. 2013; Kassem et al. 2007, 2013) (Fig. 2). Some other phylogenetic studies suggest a geographical differentiation, providing distinct subgroups of CABYV that depend on the ORF analyzed (Asad et al. 2022; Kassem et al. 2013; Maachi et al. 2022; Minicka et al. 2020; Mnari-Hattab et al. 2009). However, based on the full-length CABYV genome, there was no differentiation according to host or geographical location in our Spanish isolates. Moreover, this phylogenetic approach revealed one CABYV recombinant isolate (19.1Zu/Al/Sg/2019), which showed a recombination event in the P3 to P5 read-through protein that was reported as a hotspot of CABYV (Kassem et al. 2013; Kwak et al. 2018), suggesting that recombination may drive the genetic diversification of the CABYV population. The occurrence of CABYV recombinant isolates has been reported from several locations (Asad et al. 2022; Kassem et al. 2013; Knierim et al. 2010; Kwak et al. 2018; Vafaei and Mahmoodi 2017), as the polerovirus genome contains sites with a variable frequency of mutations (Latourrette et al. 2021) and frequent intraspecific and interspecific recombination events (Pagán and Holmes 2010). Furthermore, we also observed a low nucleotide diversity in the CABYV population and under purifying selection (Table 1), despite different codons identified under positive selection at ORF0 (position 146), ORF1 (positions 461 and 539), ORF2 (positions 36, 46, and 90), and ORF5 (position 478). Given that aphids could reduce the size of the population bottleneck during transmission of CABYV, one potential explanation might be that a selective sweep provides CABYV haplotypes with a fitness gain. Indeed, our contemporary isolates grouped close to the recently described novel CABYV variant, which is characterized by an increase in severe CABYV symptoms in watermelon, and a relative fitness over cultivated cucurbit species (Rabadán et al. 2021). Additionally, our time-based DAPC, together with the MSN analysis, revealed that CABYV populations were discriminated into different subpopulations by growing season, with contemporary isolates differentiated from previous growing seasons. This temporal divergence in the CABYV population in Spain (Supplementary Fig. S3), could have possibly originated from separate different introductions, as, according to the neutrality tests by Tajima's D statistics, the CABYV population is under demographic expansion. Altogether, these results suggest that the CABYV population structure is still uncertain,

as most genetic variability studies come from partial genome sequences, and contemporary CABYV isolates appear to be a threat to cucurbit crop production.

Genetic diversity of WMV populations

Our WMV phylogenetic analyses showed that although two isolates (2.1Me/Mu/Mx/2011 and 64.2Me/CLM/Mx/2018) clustered into the Classic group, and one isolate (8.1Me/M/Mx/2016) displayed a recombination event in the P1 protein, most isolates clustered into the Emergent group, confirming that the WMV population in Spain is dominated by this Emergent group (De Moya-Ruiz et al. 2021) (Fig. 2; Juarez et al. 2013). These analyses also revealed a lack of genetic differentiation within isolates from different hosts, locations, growing seasons, and types of infection. Thus, WMV populations were under purifying selection, unlike other studies on WMV (Bertin et al. 2020; Desbiez and Lecoq 2008; Desbiez et al. 2007; Glasa et al. 2011). This is likely due to the recent introduction of these Emergent WMV isolates in Spain. In fact, Tajima's D values were negative for the WMV population, similar to another study (Nematollahi et al. 2021), suggesting that the WMV population is still under demographic expansion. Nevertheless, note that the P1 protein displayed a higher diversity than the other coding regions, which is consistent with recent WMV studies in India, Iran, Italy, China, and France (Bertin et al. 2020; Desbiez et al. 2020; Nematollahi et al. 2021; Verma et al. 2020; D. Wang et al. 2017). This P1 protein has been recently described to modulate replication and host defense and to act as an RNA silencing suppressor of the P25 protein of CYSDV when co-infecting *Nicotiana benthamiana* (Desbiez et al. 2009; Domingo-Calap et al. 2021; Pasin et al. 2014), and it may possibly shape the evolutionary dynamics of WMV populations. Nevertheless, several plant virus studies on population structure are often oversimplified, and further spatiotemporal variations, along with environmental heterogeneity, have been shown to be relevant in the evolutionary dynamics of WMV populations (Peláez et al. 2020; Valverde et al. 2020). The lowest nucleotide diversity in our WMV population may be due to a host association as reported for other plant RNA viruses (Peláez et al. 2020; Valverde et al. 2020).

Differential impact of mixed infections on CABYV and WMV populations

The implications of mixed infections on the evolution of pathogens has yet to be investigated. With the recent diagnosis improvements, the co-detection of viruses in diseased plants is increasingly being recognized as an integrated biotic factor in crop epidemics that can affect the ecology and evolution of the disease (Alcaide et al. 2020b; Mascia and Gallitelli 2016; Moreno and López-Moya 2020; Syller 2012). Our long-term monitoring strategy allowed us to confirm previously reported viral infection patterns in these cucurbit crops (Juarez et al. 2013; Kassem et al. 2007; Rabadán et al. 2021). Mixed infections are frequent, and 10% of all positive samples derived from a combination of CABYV and WMV, with positive associations between them regardless of the host. Many factors may influence the recurrent co-detection of viruses in plants. For example, both viral and plant intrinsic factors (e.g., generalist viruses, cultivar, plant age, and nutritional status), as well as external factors (e.g., mode of virus transmission, polyphagous vectors, environmental conditions, growing season overlap, intensification and expansion of agricultural production, and proximity of alternative hosts), are thought to occur in agricultural contexts and likely combine, leading to a range of ecological interactions between plants and viruses that might be favorable the prevalence of mixed infections. However, what is less clear is the importance of mixed infections to viral distribution and genetic diversity of their populations in cultivated and wild plant species, relative to purely random effects. To date, most studies have focused on pairwise plant-virus interactions, and although very informative in particular contexts, recent studies have attempted to determine the relative impact of mixed infections versus single infection on driving evolutionary dynamics (Alcaide et al. 2020b, 2021; Ali and Roossinck 2017). In this framework, we further examined how SNP variation was distributed among the CABYV and WMV populations through an AMOVA, showing a fine-scale temporal genetic differentiation in CABYV populations (28.44%) that could be partially explained by the level of variance between single and mixed infections with WMV (84.14%). However, the WMV population remained undifferentiated between type of infections within growing seasons (Supplementary Table S3; Fig. 3). We speculate that WMV could be influencing CABYV population diversity and underline the importance of considering the interplay of virus-virus interactions on their evolutionary dynamics and viral disease ecology. Collectively, our findings indicate that mixed infections deserve attention, and further studies involving pathogenicity and transmission mode, combined with ecological and evolutionary analysis, need to be considered to understand to what extent abiotic and biotic factors may shape viral population dynamics.

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