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Short communication

Redefining the medicago sativa alphapartitiviruses genome sequences

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ABSTRACT

In alfalfa samples analyzed by hightroughput sequencing, four *de novo* assembled contigs encoding gene products showing identities to alphapartitiviruses proteins were found based on BlastX analysis. The predicted amino acid (aa) sequences of two contigs presented 99–100% identity to the RNA-dependent RNA polymerase (RdRp) and the capsid protein (CP) of the recently reported medicago sativa alphapartitivirus 1 (MsAPV1). In addition, the remaining two contigs shared only 56% (CP) and 70% (RdRp) pairwise aa identity with the proteins of MsAPV1, suggesting that these samples presented also a novel *Alphapartitivirus* species. Further analyses based on complete genome segments termini and the presence/absence of alphapartitivirus RNA in several samples and public alfalfa RNA datasets corroborated the identification of two different alphapartitivirus members. Our results likely indicate that the reported MsAPV1 genome was previously reconstructed with genome segments of two different alphapartitiviruses. Overall, we not only revisited the MsAPV1 genome sequence but also report a new tentative alphapartitivirus species, which we propose the name medicago sativa alphapartitivirus 2. In addition, the RT-PCR detection of both MsAPV1 and MsAPV2 in several alfalfa cultivars suggests a broad distribution of both viruses.

In Argentina, alfalfa (*Medicago sativa* L.) is a primary forage crop and a major feed component in dairy and beef cattle production systems. In 2010, we observed alfalfa plants showing symptoms of shortened internodes (bushy appearance), leaf puckering and varying-sized vein enations on abaxial leaf surfaces (Bejerman et al., 2011). Deep sequencing of alfalfa samples collected in central region of Argentina revealed the presence of four RNA viruses: alfalfa mosaic virus (AMV), alfalfa dwarf virus (ADV), alfalfa enamovirus-1 (AEV-1) and bean leaf roll virus (BLRV) (Bejerman et al., 2015, 2016; Trucco et al., 2014, 2016) and one DNA virus: alfalfa leaf curl virus (ALCV) (Bejerman et al., 2018). Furthermore, four assembled sequences (contigs) analyzed by BlastX searches shared significant identity (E-value = 0), to the capsid and replication associated proteins encoded by alphapartitiviruses.

Partitiviruses particles are isometric and contain a double stranded RNA (dsRNA) bi-segmented genome; each segment contains a single open reading frame (ORF), which encodes a RNA dependent RNA polymerase (RdRP) or a coat protein (CP) (Nibert et al., 2014). Recently, the *Partitiviridae* family has been taxonomically reorganized; it comprises five genera, *Alphapartitivirus*, *Betapartitivirus*, *Gammapartitivirus*, *Deltapartitivirus,* and *Cryspovirus* (Nibert et al., 2014). Partitiviruses are usually reported in single infections; however they also have been described co-infecting a host (Lesker et al., 2013; Lyu et al., 2018; Ong et al., 2017; Osaki and Sasaki, 2018; Sabanadzovic and Valverde, 2011). In the latter case, it is a challenge to pair the two genomic segments to identify and annotate the genomes without mixing up genomic segments from different partitiviruses (Ong et al., 2017).

Alphapartitiviruses infect either plant or fungi. Plant-infecting alphapartitiviruses are associated with latent infections of their hosts and are transmitted with a high frequency via ovule and by pollen to the seed embryo, but they cannot be transmitted by grafting or mechanical inoculation (Nibert et al., 2014). A new alphapartitivirus member infecting alfalfa, named as medicago sativa alphapartitivirus 1 (MsAPV1), was recently reported by analyzing public transcriptome dataset (Kim et al., 2018).

In this work we report two medicago sativa alphapartitiviruses, redefining the reported MsAPV1 genome sequence by Kim et al. (2018), and describing the medicago sativa alphapartitivirus 2 (MsAPV2). Furthermore, we detected both viruses in mixed infections in several alfalfa cultivars samples from Argentina and public alfalfa RNA

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datasets, suggesting a high prevalence of them.

In 2011, five samples of symptomatic alfalfa plants showing dwarfism were collected from a field located in Manfredi (Córdoba, Argentina), and pooled, and total RNA was extracted using TRIzol Reagent (Life Technologies) according to manufacturer's instructions. Total RNA was DNaseI treated and sent to Fasteris Life Sciences SA (Plan-les-Ouates, Switzerland), where the bands located between 21 and 30 bp were excised, purified, processed and sequenced on an Illumina HiSeq 2000. The obtained 7,618,074 raw reads were filtered by quality and trimmed using the FASTX-Toolkit as implemented in http://hannonlab.cshl.edu/fastx_toolkit/index.html. The remaining 6,941,672 short reads were *de novo* assembled using Velvet 1.2.10 with the velvet optimiser parameter available at https://www.ebi.ac.uk/~zerbino/velvet/. The obtained contigs were subjected to bulk BlastX searches (E-value < 1e-5) against a local database of the non-redundant virus proteins. Four contigs ranging from 1,650-1,840 nt sharing high similarity (56–100%) with alphapartitivirus CP and RdRp genes were retrieved, further inspected and annotated as described in Debat and Bejerman (2019). The contigs were curated by mapping the filtered sequencing reads using the Geneious 8.1.9 (Biomatters, inc) mapper with low sensitivity parameters and assessing the consensus by hand. The curated contigs were initially designated as alphaA1 (1,872 nt, mean coverage 31.6X, supported by 2,782 reads, encoding an RdRP); alphaA2 (1,687 nt, mean coverage 11.5X, supported by 876 reads, encoding a CP); alphaB1 (1,845 nt, mean coverage 38.9X, supported by 3,357 reads, encoding an RdRP); and alphaB2 (1,714 nt, mean coverage 28.5X, supported by 2,314 reads, encoding a CP). AlphaA1 and alphaA2 shared 98.7% and 99.8% nt identity with the reported MsAPV1 dsRNA1 (MF443256) and dsRNA2 (MF443257), respectively, whereas alphaB1 and alpha B2 shared 68.9% and 55.6% nt identity with the reported MsAPV1 dsRNA1 (MF443256) and dsRNA2 (MF443257), respectively. Thus, we tentatively grouped the identified contigs into two putative viruses corresponding to MsAPV1 (alphaA1/alphaA2) and MsAPV2 (alphaB1/alphaB2). However, evidence presented below based on the complete genome segment sequences and RNA levels indicated that the segments could be paired differently. So, alphaA2 (almost identical to the reported MsAPV1 dsRNA2 (MF443257)) is in fact the RNA2 of MsAPV2, and thus alphaB2 should be paired with alphaA1.

To obtain the complete sequences of these putative alphapartitivirus genome segments, their terminal sequences were amplified by 5´and 3´rapid amplification of cDNA ends (RACE) that was carried out as described previously by Phelan and James (2016) which resulted in assembly of full genome sequences that were deposited in the GenBank database under accession numbers MK292286 (MsAPV1 dsRNA1), MK292287 (MsAPV1 dsRNA2), MK292288 (MsAPV2 dsRNA1) and MK292286 (MsAPV2 dsRNA2). Primers used to amplify the terminal regions are listed in Supplementary Table 1.

The complete sequences of the revisited MsAPV1 dsRNA1 (extended from the formerly designated alphaA1 contig) and dsRNA2 (formerly designated alphaB2 contig) were determined to be 1942- and 1806- nt long, respectively (Fig. 1A) with GC contents of 38.62% and 43.79%, respectively; whereas the complete sequences of the novel MsAPV2 dsRNA1 (alphaB1) and dsRNA2 (alphaA2) were determined to be 1939- and 1764- nt long, respectively (Fig. 1A) with GC contents of 43.73% and 44.72%, respectively. GC content is likely similar to those values reported for other partitiviruses (Phelan and James, 2016).

dsRNA was isolated according to Osaki et al. (1998), treated with DNase I and electrophoresed in a 5% polyacrylamide gel, which showed the dsRNA fragments of MsAPV1 and MsAPV 2 (Fig. S1).

Each dsRNA contains a single open reading frame (ORF) with 5´and 3´untranslated regions (UTRs). Interestingly, multiple alignments of 5´UTRs revealed that these sequences are highly conserved, being the first 9 nt at the 5´terminus of the dsRNA1 and dsRNA2 fragments of MsAPV1 identical, whereas the first 10 nt at the 5´terminus of the dsRNA1 and dsRNA2 fragments of MsAPV2 also are identical, but different from the 5′ termini of the MsAPV1 segments (Fig. 1B). Furthermore, the 5´UTR of MsAPV1 and MsAPV2 dsRNA1 and dsRNA2 were predicted to fold into stem-loop structures, when examined using Mfold software (http://mfold.rna.albany.edu) (Fig. 1C). Similar stem-loop structures, which are likely to play an important role in dsRNA replication and virion assembly, have been described in other partitiviruses (Guo et al., 2017; Lesker et al., 2013; Osaki and Sasaki, 2018).

Sequence analysis of MsAPV1 and MsAPV2 dsRNA1 revealed that it contained a single ORF encoding a putative 586-amino acid (aa) RdRp protein, where a conserved domain was found (cd01699, e-values = 3.73e-05 and 3.40e-07). Moreover, the multiple-protein alignment of the deduced aa sequences of the RdRps of MsAPV1, MsAPV2 and plant-infecting alphapartitiviruses confirmed that this protein includes the six conserved motifs (III to VIII) described for partitiviruses (Liu et al., 2015). Sequence analysis of MsAPV1 and 2 dsRNA2 revealed that it contained a single ORF encoding a putative 499- and 491-aa CP protein, respectively.

Pairwise comparison using Sequence Demarcation Tool (SDT v1.2) (Muhire et al., 2014) showed that at aa level the RdRp of the redefined MsAPV1 is 100% identical to that one encoded by the reported MsAPV1 (Fig. 2A), whereas its CP is just 56% identical to that one of the reported MsAPV1 (Fig. 2B). On the other hand, the RdRp of the MsAPV2 is just 70% identical to that one encoded by the redefined MsAPV1 and the reported MsAPV1 (Fig. 2A), whereas its CP is 99% identical to that one of the redefined MsAPV1 and the reported MsAPV1 (Fig. 2B), which likely suggest that genome segments of the reported MsAPV1 could be paired differently that previously described. Both the redefined MsAPV1, and MsAPV2 have a degree of identity below 90% and 80% for RdRp and CP protein, respectively, when compared with other partitiviruses, which is the species demarcation threshold recently recommended for alphapartitiviruses (Vainio et al., 2018).

To further assess our speculation that the reported MsAPV1 segments derived from two different viruses we analyzed the raw data which was the source of Kim et al (2018) study, which pooled eight libraries before de novo assembly. Raw reads corresponding to RNAseq NGS libraries (SRA: SRP017117), associated to NCBI Bioproject PRJNA179114 and additionally from an independent study oriented to elucidate the molecular genetic basis of herbivory between butterflies and their host plants, including alfalfa (Nallu et al., 2018, PRJNA437291, SRA: SRP134094), were analyzed according to Debat and Bejerman (2019). Briefly, the public datasets were downloaded and reads of each library including our NGS data were mapped against the assembled virus genomes sequences using Bowtie2 v2.3.4.3 available at http://bowtie-bio.sourceforge.net/bowtie2/ with default settings and the fast end-to-end preset. The obtained values were normalized as mapped reads per million (RPM) for each library. Interestingly, we observed a differential pattern of presence and absence of diverse alphapartitivirus segments in each library (Table 1). When we analyzed in detail the transcriptome data used by Kim et al. (2018) we observed that two of the eight pooled libraries presented virus RNA derived from both segments of the revisited MsAPV1 alphaA1 and alpha B2, and only one library harbored virus reads of segment 2 (alphaA2) of MsAPV2 (which was previously annotated as segment 2 of MsAPV1). Thus, we believe that the use of a pooled transcriptome dataset for virus discovery could misrepresent the virus landscape associated to each specific sample that led to a miss assignment of alphapartitiviruses genome segments. Moreover, when we explored 18 alfalfa RNAseq datasets from bioproject PRJNA437291 we observed that in three libraries only virus RNA from both genome segments of the redefined MsAPV1 (alphaA1 and alpha B2) were found, while in five additional runs we detected virus RNA from both segments of MsAPV1 (alphaA1 and alpha B2) and MsAPV2 (alphaA2 and alpha B1), suggesting mixed infections of alphapartitiviruses in those samples (Table 1). The detection of only both segments (alphaA1 and alpha B2) of the redefined MsAPV1 in several samples likely indicates that the former MsAPV1 genome could be revisited.

Following sequence annotation and the assessment of RNA levels,

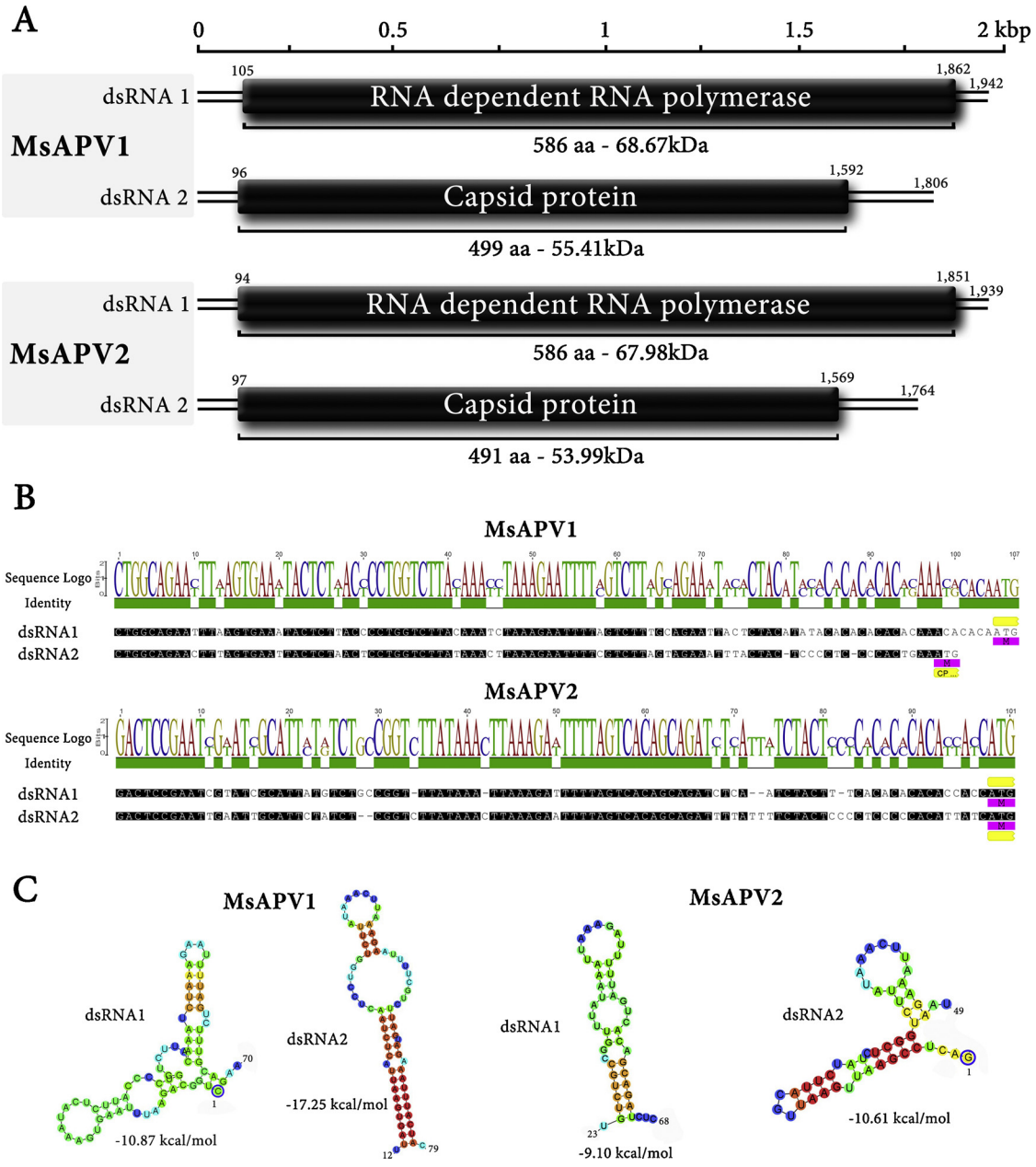

Fig. 1. A. Genomic organization of medicago sativa alphapartitivirus 1 and 2. The numbers above the solid lines indicate the nucleotide positions of the beginning and end of the 5′- and 3′-UTRs. B. Multiple alignments between 5´UTR of dsRNA1 and 2 of MsAPV1 and MsAPV2. C. Potential secondary structures formed by 5′-terminal sequences of positive strands of two segments of MsAPV1 and MsAPV2.

the redefined MsAPV1 and 2 predicted RdRp and CP aa sequences were aligned with the corresponding sequences of selected partitivir-usesusing MUSCLE (Edgar, 2004) as implemented in Mega X (Kumar et al., 2018). ML phylogenetic trees were inferred using Mega X with LG + G and LG + G + F aa substitution models, respectively, and 1000 bootstrap replicates. Phylogenetic analyses supported the pairwise comparison results showing that the redefined MsAPV1 RdRp grouped within alphapartitiviruses with the reported MsAPV1 whereas MsAPV2 RdRp clustered with those encoded by pyrus pyrifolia partitivirus 2 (PpPV2) and rose partitivirus (RoPV) (Fig. 2C). On the other hand, the revisited MsAPV1 CP formed a monophyletic cluster within alpha-partitiviruses whereas MsAPV2 CP grouped with those encoded by the reported MsAPV1, PpPV2 and RoPV (Fig. 2D). This suggest that the redefined MsAPV1 and MsAPV2 do not share a common immediate ancestor, so it is tempting to speculate that they originated from two distinct ancestral partitiviruses and adapted to the same host.

Recently, it was described the presence of the reported MsAPV1 in alfalfas from USA (Nemchinov et al., 2018). Given that the presence of alphapartitivirus RNA sequences in that study was assessed by RNAseq reads mapping using as reference the published sequences by Kim et al. (2018), it is tempting to suggest, based on our results, that these alfalfas could be infected with both MsAPV1 and MsAPV2.

It was reported that there is higher sequence identity between the corresponding 5′ UTRs of CP and RdRp segments belonging to the same alphapartitivirus than those belonging to different species (Ong et al., 2017). Therefore we verified if genomes were consistently annotated in this study by comparing the 5′ UTRs of segments of the redefined MsAPV1 and MsAPV2. The comparison revealed a 70.5% and 70.1% identity between the 5′ UTRs of CP and replicase segments of the re-defined MsAPV1 and MsAPV2, respectively. Whereas identities values when 5′ UTRs of CP and replicase segments of the redefined MsAPV1 were compared against those of MsAPV2 were lower than 60%. This

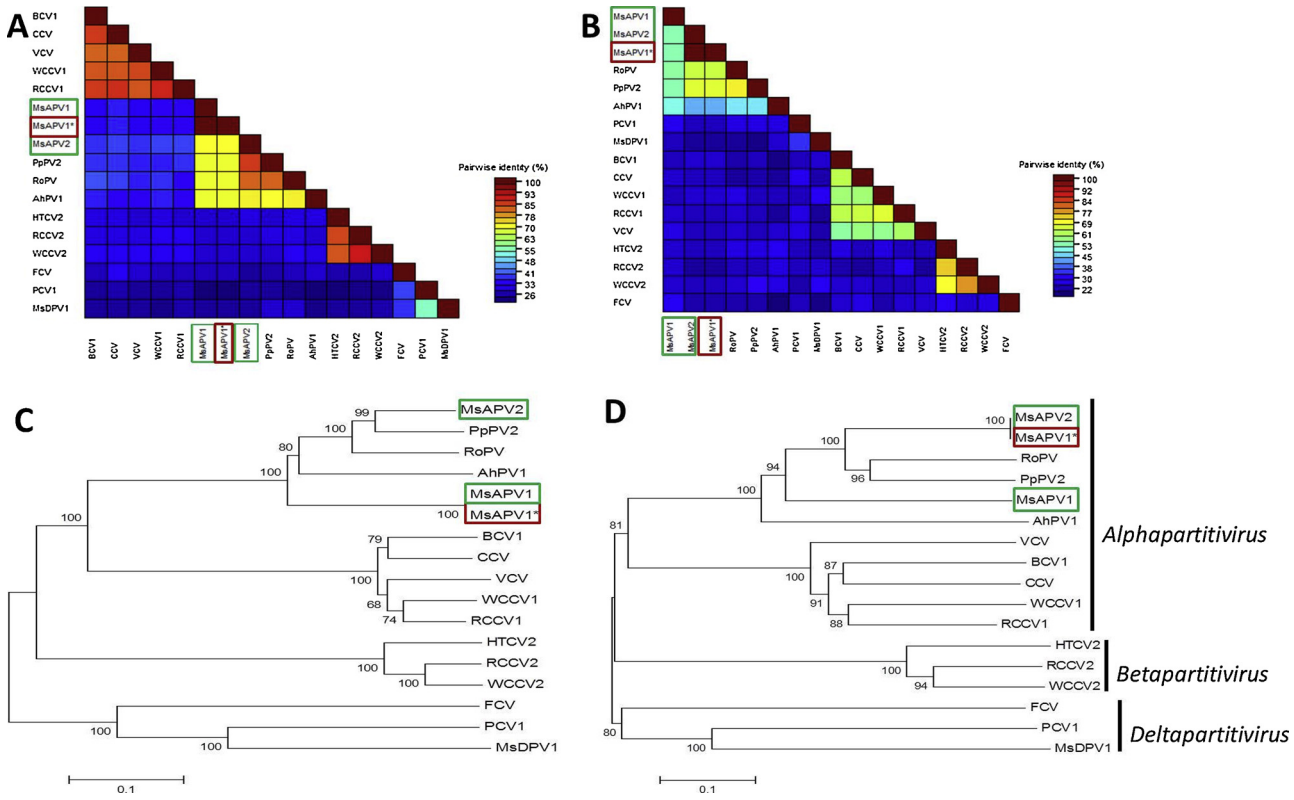

Fig. 2. A and B. Amino acid percentage pairwise identities between MsAPV1 and MsAPV2 RdRp (A) and CP (B) with those from selected partitiviruses members calculated using SDT v1.2 (Muhire et al., 2014). C. Maximum likelihood phylogenetic tree of MsAPV1 and 2 and selected partitiviruses members based on the RdRp amino acid sequences. D. Maximum likelihood phylogenetic tree of MsAPV1 and 2 and selected partitiviruses members based on the CP amino acid sequences. All trees were constructed using MegaX software. LG + G model was used to construct the phylogenetic tree based on RdRp amino acid sequences; whereas model LG + G + F was used to construct the phylogenetic tree based on CP amino acid sequences. Bootstrap values out of 1000 replicates are given at the nodes. The bar below each tree represents substitutions per site. The viruses used to construct the tree, and their accession numbers are as follows: arabidopsis halleri partitivirus 1 (AhPV1; LC151461/2), beet cryptic virus 1 (BCV1; EU489061/2), carrot cryptic virus (CCV; FJ550604/5), fig cryptic virus (FCV; FR687854/5), hop trefoil cryptic virus 2 (HTCV2; JX971980/1), * medicago sativa alphapartitivirus 1 (*MsAPV1; MF443256/7), medicago sativa delpartitivirus 1 (MsDPV1; MF443258/9), pepper cryptic virus 1 (PCV1; JN117276/7), pyrus pyrifolia partitivirus 2 (PpPV2; LC221826/7), rose partitivirus (RoPV; KU896858/9), red clover cryptic virus 1 (RCCV1; KF484724/5), red clover cryptic virus 2 (RCCV2; JX971978/9), vicia cryptic virus (VCV; AY751737/8), white clover cryptic virus 1 (WCCV1; AY705784/5), white clover cryptic virus 2 (WCCV2; JX971976/7).

supports our tentative pairing of the revisited MsAPV1 and MsAPV2 genomes in this study. Plant-infecting partitiviruses are efficiently pollen/seed transmitted, but are cryptic, so infected- plants are symptomless (Nibert et al., 2014), therefore they are not considered as quarantine pest and they are not tested when alfalfa seeds are traded. Consequently, both the redefined MsAPV1 and MsAPV2 could have been transmitted to many alfalfa cultivars inadvertently, resulting in their dissemination all around the world through the exchange of alfalfa seeds, which led to its detection in diverse contexts. In this regard, in order to assess a tentative prevalence landscape of alphapartitivirus in alfalfa, we studied the occurrence of the revisited MsAPV1 and MsAPV2 by RT-PCR assays in leaf tissue of nine alfalfa cultivars, using two set of virus-specific primers designed from dsRNA1 (Supplementary Table 1) in order to detect the redefined MsAPV1 and MsAPV2 in alfalfa leafs. Interestingly, we detected both dsRNA1 sequences in all samples tested, suggesting the presence of both viruses as mixed infections is common in alfalfa (Fig. 3).

In conclusion, this study describes the complete genome sequences of two tentative alphapartitivirus infecting alfalfa, revisiting the reported MsAPV1 genome sequence by Kim et al. (2018) and naming the second one as medicago sativa alphapartitivirus 2, which could be considered new members of the genus *Alphapartitivirus*. The revisited MsAPV1 was found in mixed and single infections in alfalfa samples from diverse regions of the world. On the other hand, our RNA evidence only supports MsAPV2 in mixed infections, given that the sample containing only MsAPV2 RNA (SRX203330) was found to have a significant number of dsRNA2 reads with no indication of dsRNA1. Thus, evidence of infection of that sample with MsAPV2 is weak. Future studies with larger sampling should unveil whether MsAPV2 might infect alfalfa in single infections. We also implemented here a simple and valuable tool based on RT-PCR detection to screen for the presence of both viruses in alfalfa cultivars. Our study provides new avenues to explore and confirm virus genome segments when partitiviruses (or any multi-segmented viruses) co-infect a sample, which appears to be genuinely common.

Conflict of interest

All authors declare that they have no conflict of interest

Ethical approval

This article does not contain any studies with human participants or animals performed by any of the authors.

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Table 1
Virus RNA levels corresponding to each genome segment of MsAPV1 & 2 in multiple public datasets. Values express virus derived reads per million of total reads (RPM) based on mapping to the assembled genome sequences. An arbitrary (and conservative) cut-off of 5 RPM was considered to indicate virus RNA presence, which is indicated in bold.

				Virus RPM			
BioProject	Run	BioSample	Experiment	MsAPV1 RNA1	MsAPV1 RNA2	MsAPV2 RNA1	MsAPV2 RNA2
PRJNA179114	SRR612164	SAMN01804647	SRX203401	3	1	0	0
PRJNA179114	SRR612163	SAMN01804646	SRX203400	0	0	0	0
PRJNA179114	SRR612116	SAMN01804612	SRX203369	**498**	**99**	0	0
PRJNA179114	SRR612115	SAMN01804610	SRX203368	0	0	0	0
PRJNA179114	SRR612114	SAMN01804609	SRX203367	0	0	0	0
PRJNA179114	SRR612113	SAMN01804577	SRX203355	0	0	0	0
PRJNA179114	SRR611885	SAMN01804552	SRX203330	0	0	0	**16**
PRJNA179114	SRR611809	SAMN01804510	SRX203284	0	0	0	0
PRJNA437291	SRR6814494	SAMN08647220	SRX3771495	0	0	0	0
PRJNA437291	SRR6814495	SAMN08647220	SRX3771494	**982**	**122**	**187**	**56**
PRJNA437291	SRR6814496	SAMN08647220	SRX3771493	**5202**	**625**	**837**	**284**
PRJNA437291	SRR6814497	SAMN08647220	SRX3771492	2	0	0	0
PRJNA437291	SRR6814498	SAMN08647220	SRX3771491	2	0	0	0
PRJNA437291	SRR6814499	SAMN08647220	SRX3771490	1	0	0	0
PRJNA437291	SRR6814500	SAMN08647220	SRX3771489	1	0	0	0
PRJNA437291	SRR6814510	SAMN08647220	SRX3771479	0	0	0	0
PRJNA437291	SRR6814527	SAMN08647220	SRX3771462	2	0	0	0
PRJNA437291	SRR6814528	SAMN08647220	SRX3771461	0	0	0	0
PRJNA437291	SRR6814531	SAMN08647220	SRX3771458	**7741**	**952**	0	0
PRJNA437291	SRR6814532	SAMN08647220	SRX3771457	**4123**	**865**	**1175**	**792**
PRJNA437291	SRR6814533	SAMN08647220	SRX3771456	**5719**	**758**	0	0
PRJNA437291	SRR6814534	SAMN08647220	SRX3771455	**4054**	**579**	0	0
PRJNA437291	SRR6814535	SAMN08647220	SRX3771454	1	0	0	0
PRJNA437291	SRR6814536	SAMN08647220	SRX3771453	1	0	0	0
PRJNA437291	SRR6814537	SAMN08647220	SRX3771452	**3423**	**705**	**930**	**663**
PRJNA437291	SRR6814538	SAMN08647220	SRX3771451	**5287**	**1108**	**1652**	**1182**

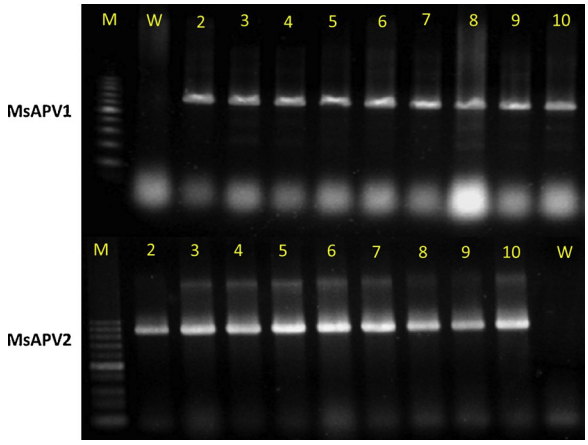

Fig. 3. Agarose gel electrophoresis showing the results of the RT-PCR detection of MsAPV1 and MsAPV2 in several alfalfa cultivars. The order of alfalfa samples is: cvs "Monarca" (lane 2), Antares (lane 3), SARDI 10 (lane 4), Sardi 7 (lane 5), Sardi Grazer (lane 6), Pegasis (lane 7), Soraya (lane8), Genesis II (lane 9), Traful (lane 10). Water control (W) is in lane 1 and 100 pb DNA ladder (Biodynamics) is in lane M.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.virusres.2019.03.021.

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