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Transcriptomic analyses reveal highly conserved plant amalgavirus genomes in different species of Allium

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Summary. – This work aims to study amalgavirus diversity in different species of allium collected around the world. Transcriptomic data of 19 Sequence Read Archive runs available at GenBank, as well as RNA-seq data generated from onion tissue from fields in Brazil were used to assemble nine allium cepa amalgavirus 1 (AcAV-1) and nine allium cepa amalgavirus 2 (AcAV-2) genomes from different species of allium worldwide. Sequence demarcation tool analyses of RdRp amino acid sequences revealed identities above 99% within each species, except for an isolate of AcAV-1 from *Allium escalonicum* from China. This work contributes to the understanding of the genetic diversity of amalgaviruses that infect the genus *Allium*.

Keywords: amalgaviruses; Allium transcriptomic datasets; *Allium* sp.

Introduction

The genus *Allium* belongs to the *Amaryllidaceae* botanical family and is one of the largest plant genera in the world with more than 900 species (*The Plant List*, March, 2021). Several species of allium are cultivated worldwide due to their therapeutic properties, aroma and flavor and are used as a spice in many cuisines. Central and northwest of China are considered as the center of origin of different species of allium and Asia accounts for more than half of the world production with over 43 million tons (FAO, 2019). In Brazil, among the horticultural species of importance, *Allium cepa* L. is considered the third crop in economic terms, second only to tomatoes and potatoes (Nunes *et al*., 2014).

In the last few years, several studies have used RNA-seq data to discover and assemble complete plant viral genomes (Goh *et al*., 2018; Kim e*t al*., 2018; Lee *et al*., 2019; Park *et al*., 2018). Transcriptome or metatranscriptome data often contain sequence reads derived from viral genomic RNAs. In this study, we analyzed data from RNA-seq from four different species of allium available at the GenBank, as well as a sequenced sample of our work of *Allium cepa* to understand the diversity of amalgavirus genomes. In 2013, the ICTV recognized the family *Amalgaviridae*, whose name comes from amalga, which means mixture, since amalgaviruses have characteristics of species belonging to both genera *Partitivirus* and *Totivirus*, indicating a probable genetic relationship with these two families (Liu and Chen, 2009; Martin *et al*., 2011; Sabanadzovic *et al*., 2009). However, phylogenetic analyses have shown that the RdRp sequences of amalgaviruses are more closely related to partitiviruses than totiviruses and the coat proteins are homologous to the nucleocapsid pro-

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Abbreviations: AcAV-1 = allium cepa amalgavirus 1; AcAV-2 = allium cepa amalgavirus 2; HTS = High-Throughput Sequencing; NCBI = National Center for Biotechnology Information; ORF(s) = open reading frame(s); RdRp = RNA dependent RNA polymerase; RNA-seq = RNA sequencing; SDT = sequence demarcation tool; SRA = Sequence Read Archive; TSA = transcriptome shotgun assembly.

Table 1. Lengths of nucleotides of AcAV-1 and AcAV-2 genomes

AcAV-1							
TSA accessions prefix*	**5'UTR**	**ORF1**	**ORF1+ORF2**	**ORF1 regulatory (UUUCGU)**	**ORF2**	**3'UTR**	**Total length**
NC_036580	148	1176	3174	6	2349	130	3453
GEOY	145	1176	3174	6	2349	130	3450
GFAK	122	1176	3174	6	2349	130	3427
GFAJ	126	1176	3174	6	2349	89	3390
GHMJ	138	1176	3174	6	2349	115	3428
GBJZ	126	1176	3174	6	2349	111	3412
GBGJ	148	1176	3174	6	2349	110	3433
GBRN	148	1176	3174	6	2349	91	3414
GBRQ	60	1176	3174	6	2349	130	3365
GBRO	60	1176	3174	6	2349	95	3330
AcAV-2							
TSA accessions prefix*	**5'UTR**	**ORF1**	**ORF1+ORF2**	**ORF1 regulatory (UUUCGU)**	**ORF2**	**3'UTR**	**Total length**
NC_036581	148	1173	3198	6	2208	106	3453
MZ063690	131	1173	3198	6	2208	58	3388
GFAJ	131	1173	3198	6	2208	58	3388
GEOY	131	1173	3198	6	2208	79	3409
GHMM	131	1173	3198	6	2208	58	3388
GHMJ	135	1173	3198	6	2208	75	3409
GHMP	131	1173	3198	6	2208	58	3388
GBRN	128	1173	3198	6	2208	48	3375
GETF	131	1173	3198	6	2208	58	3388
GBRQ	131	1173	3198	6	2208	58	3388

5' UTR and 3' UTR regions were not verified with Rapid amplification of cDNA ends (RACE). *TSA and SRR files used to assemble amalgavirus genomes are described in Supplementary Table S1.

teins of negative-stranded RNA viruses of the genera *Phlebovirus* and *Tenuivirus* (*Phenuiviridae*) (Krupovic *et al*., 2015; Martin *et al*., 2011; Sabanadzovic *et al*., 2009).

Currently, the *Amalgaviridae* family consists of two genera: the genus *Amalgavirus*, which currently contains nine species (*Allium cepa amalgavirus* 1, *Allium cepa amalgavirus* 2, *Blueberry latent virus, Rhododendron virus A, Southern tomato virus, Spinach amalgavirus* 1, *Vicia cryptic virus M, Zoostera marina amalgavirus* 1, *Zoostera marina amalgavirus* 2), found in plants, and the second genus *Zybavirus* that infects fungi and consists of only one species, the *Zygosaccharomyces bailii virus Z* (Adams *et al*., 2014). Their genomes are monopartite consisting of double-stranded RNA of about 3.5 kilobases (kb) in length (Sabanadzovic *et al*., 2009, 2010). Two partially overlapping open reading frames (ORFs) are predicted to be expressed from the genome: a 5'-proximal ORF (ORF1) that encodes a putative nucleoprotein that forms the replication factory matrix like protein and a second ORF (ORF2) encoding the RNA-dependent RNA polymerase (RdRp). The RdRp-containing protein is expressed as an ORF1+ORF2 fusion and is produced by a +1 ribosomal frame displacement mechanism (PRF) (Depierreux *et al*., 2016; Sabanadzovic *et al*., 2009).

Interestingly, amalgaviruses have shown beneficial effects in their host. Recently, Fukuhara *et al*. (2020) showed that southern tomato virus (STV) interacts with tomato plants in a mutualistic mode. Infected tomato plants were taller and produced more fruits. Authors showed from transcriptomic analysis that STV infection affected the expression of plant genes involved in ethylene biosynthesis, and that selection of superior traits may be involved with virus infection.

This work aims to use transcriptomic data available at the GenBank as well as RNA-seq data generated for this work to assemble amalgavirus genomes of different species of allium collected worldwide. The study of plant virus genetic diversity is important to understand epidemiological and evolutionary processes and provides relevant information on amalgaviruses.

Materials and Methods

Sample collection and transcriptomic datasets. Symptomatic onion leaf materials (*Allium cepa* L.) were collected from producing fields in the city of Petrolândia, Pernambuco, Brazil. Leaves were immediately dried at 4°C and stored in silica to avoid degradation of the genetic material. Total RNA was extracted using the Quick-RNA™ Plant miniprep kit (Zymo Research, USA) according to the manufacturer's instructions and sent for HTS sequencing by Macrogen (Seoul, South Korea). In addition, Sequence Read Archive (SRA) and contigs datasets were downloaded from the National Center for Biotechnology Information (NCBI) and employed to assemble the amalgavirus genomes (Supplementary Table S1). The SRAs datasets SRR3144579, SRR3144593, SRR3144570, SRR9077155, SRR1293377, SRR1051882, SRR1056448, SRR1312067, and SRR1312066 were used to assemble nine genomes of allium cepa amalgavirus 1 (AcAV-1) and SRR3144570, SRR3144579, SRR9077160, SRR9077155, SRR9077156, SRR1312067, SRR2814815, SRR2814816, SRR2814817, SRR2814818, SRR2814819, SRR2814820, SRR2814821, SRR2814822, and SRR1312066 to assemble nine genomes of allium cepa amalgavirus 2 (AcAV-2). The AcAV-2 genome assembled from the Macrogen HTS sequencing of collected samples of *A. cepa* from Brazil received the following accession number MZ063690 (Table 1).

HTS bioinformatic pipeline. The bioinformatic pipeline for *de novo* assembling of viral genomes was performed according to Blawid *et al*. (2017). Briefly, the quality of the transcriptomic data and the one generated in this work were monitored by FastQC v.011.8 (Andrews, 2010). TRIMMOMATIC v.039 (Bolger *et al*., 2014) was used for clipping leftovers of adapters and for quality trimming. Contigs were assembled with SPAdes v.3. 15. 2 (Bankevich *et al*., 2012), using k-mers of 21, 33, 55, 77. When necessary, the available platform GALAXY (www.usegalaxy.eu) was used for assembling the viral genomes. The generated contigs were analyzed with tBLASTX against a database of viral genome sequences implemented manually in the Geneious v 11.0 (R11) software. Mapping and extension steps were carried also in Geneious until the complete genomes of amalgaviruses were assembled. In addition, a strategy using the allium cepa amalgavirus 1 and allium cepa amalgavirus 2 genomes (NC_036580 and NC_036581) as reference were employed to obtain complete genomes from SRA/TSA data originating from other allium species. Gaps were closed using the original SRA datasets.

Phylogenetic and sequence analyses. Assembled ORF2 and RdRp amino acid sequences were used in multiple sequence alignments generated with MAFFT v.7 (Katoh and Standley, 2013). The statistical selection of best-fit models of nucleotide substitutions was assessed with ProtTest 3. 4. 2 (Darriba *et al*., 2011). Based on the AIC and BIC criteria, the most appropriate model was RtREV + I + G + F. Phylogenetic trees were constructed using PhyML (Guindon *et al*., 2010). The starting tree was obtained by BioNJ and optimized by branch length and tree topology. The type of tree improvement was according to the best of nearest neighbor interchange and subtree pruning and regrafting. Branch support values (%) were estimated by the approximate likelihood ratio test (aLRT) with SH-like criteria. Phylogenetic trees were visualized and edited with iTOL v4 (https://itol.embl.de) (Letunic and Bork, 2019). The Sequence Demarcation Tool (SDTv.1.2) (Muhire *et al*., 2014) was used for multiple nucleotide-level pairwise analyzes of different regions of the amalgavirus and partitivirus genomes with other sequences retrieved from GenBank.

Results

Genome assemblies and sequence identities of amalgaviruses

In the present work, a total of 19 SRA files were analyzed for the search of new amalgavirus sequences. Of the 19 SRA samples, a total of nine viral genomes of AcAV-1 and nine of AcAV-2 were assembled (Table 1). Eight AcAV-1 sequences were derived from *Allium cepa* datasets and one from *A. ascalonicum*, while six AcAV-2 sequences were assembled from *A. cepa* and one from *A. ascalonicum*, one from *A. fistulosum,* and one from *A. angulosum* datasets (Supplementary Table S1).

All assembled sequences of AcAV-1 showed the same ORFs size length of the published AcAV-1 available at NCBI (NC_036580). The ORF1 of AcAV-1 genomes that putatively encode the replication factory matrix-like protein consists of 1176 bp while the ORF2 (encoding the RdRp) is 2349 bp long and the ORF1+ORF2 consists of 3174 bp. Blast searches of ORF1 amino acid sequences revealed an identity of 100% with ORF1p of AcAV-1 (acc. YP_009447920), followed by identities of around 40% with sequences from the ORF1 of AcAV-2. SDT analyses (Supplementary material) using ORF1 amino acid sequences revealed identities of 100% between the AcAV-1 sequences, except for the AcAV-1 isolated from *A. ascalonicum* from China (acc. GFAJ01023991), where identities were of 83.9%. The total length of assembled genomic sequences of AcAV-1 varied from 3330 to 3453 bp. In general, it was possible to assemble longer 5' UTRs for AcAV-1 genomes than the 3' UTR. The conserved regulatory sequence (UUUCGU), involved in the +1 programmed ribosomal frameshift mechanism was found in all assembled sequences of AcAV-1 and AcAV-2 isolated from *A. ascalonicum, A. angulosum, A. cepa*, and *A. fistulosum*. SDT analyzes using RdRp amino acid sequences revealed percentages of identities ranging from 89.1 to 100% between RdRp sequences of AcAV-1, while AcAV-2 sequences showed identities varying from 99 to 100%. When comparing the two RdRp amino acids sequences from the two amalgavirus species, identities varied from 66.9 to 67.8%.

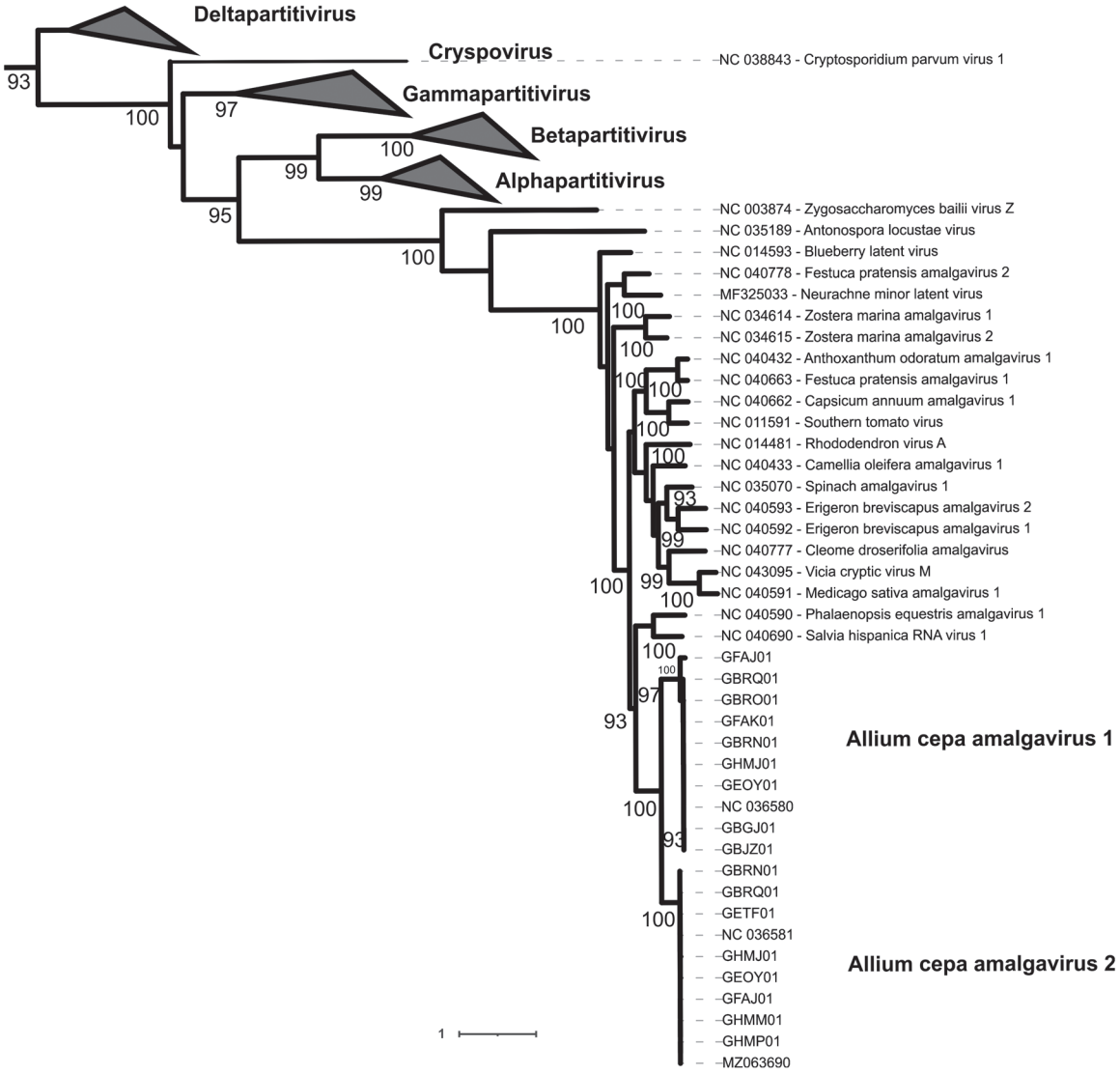

Fig. 1

Phylogenetic relationship between members of the family *Partitiviridae* and *Amalgaviridae* based on the amino acid sequences of the RdRp region

Phylogenetic trees were generated by the Maximum-Likelihood method. Numbers at nodes represent bootstraps values (displayed above 80).

Like AcAV-1, all assembled AcAV-2 sequences showed ORFs of the same length as the genomic sequence available in the NCBI (NC_036581). The ORF1 consisted of 1173 bp, the ORF1+ORF2 of 3198 bp and the ORF2 of 2208 bp. Thus, the ORF1 from the Brazilian AcAV-2 genomic sequence has also 1173 bp and ORF2 is smaller than the ORF2 from AcAV-1, with 2208 bp. The ORF1 amino acid sequences of AcAV-2 are highly conserved sharing identities varying from 99 to 100% with sequences isolated from *A. cepa*, *A. angulosum*, *A. ascalonicum*, and *A. fistulosum* plants. Contrary to AcAv-1, the ORF2 of all AcAV-2 sequences starts after the regulatory sequence. SDT analyses also showed that the ORF1+ORF2 amino acid sequences are highly conserved in both AcAV1-1 and AcAV-2 sequences, except for AcAV-1 isolated from *A. ascalonicum* (GFAJ01023991), as mentioned before (59.4–59.7%). Comparisons between species showed identities from 19.9 to 77.7% in this region. The 5' UTR sequences also showed to be longer than the 3' UTR sequences.

The AcAV-2 genomic sequence assembled from onion samples from the state of Pernambuco has a total length of 3388 bp with ORF1 (nt position 132–1304) and ORF2 (nt position 1123-3330) partially overlapping. The regulatory ORF is located at position 963 to 968. The assembled

5' UTR is 131 bp long, and the 3' UTR is only 58 bp long (3331–3388). The 5' UTR and 3' UTR of the AcAV-2 sequence are rich in AU (61.0% and 65.6%), as the AcAV-1 5' UTR and 3' UTR sequences.

In general, multiple alignments using amino acid sequences of RdRp of amalgaviruses and partitiviruses generated levels of identities below 27.0% (data not shown), showing the high divergence between the genomic sequences of amalgavirus and the partitiviruses characterized so far. Comparisons between RdRps aminoacid sequences between amalgavirus species revealed identities varying from 24.1 to 80,8%. Several structural motifs (A-G) (Bruenn, 1993; Jia and Gong, 2019) of RdRp were found in the amalgavirus proteins (Supplementary Table S2 shows 11 motifs in addition to motifs A-G). The motif C (GDD) is followed by an asparagine (N) amino acid in all amalgavirus RdRps, except for Antonospora locustae virus 1 (NC_035189) and Zygosaccharomyces bailii virus Z (NC_003874). In addition to the catalytic motifs of RdRp, eleven regions of conserved amino acid sequences were observed by multiple alignments (Supplementary Table S2). Motif 1 was not found in the RdRp amino acid sequence of Antonospora locustae virus 1 (NC_035189) and motif 11 was not found in the Zygosaccharomyces bailii virus Z sequence (NC_003874).

Phylogenetic analyses

Phylogenetic analyses were performed using the RdRp amino acid sequences of all 20 *Allium cepa* amalgaviruses as well as of classified and unclassified partitiviruses. As a result, eight main clusters were observed from the generated tree (Fig. 1).

The AcAV isolates were divided into two clusters. The AcAV-1 isolate from China of *A. ascalonicum* (GFAJ01023991), and the isolates of *A. cepa* from China, Czech Republic, India, New Zealand, Republic of Korea and USA formed a single group. The AcAV-2 isolates formed a second cluster with several isolates from samples of A. *cepa, A. ascalonicum, A. fistulosum,* and *A. angulosum* from different parts of the world (Brazil, China, Czech Republic, Republic of Korea and USA). The closest relationship with *Allium cepa* amalgaviruses was with the phalaenopsis equestris amalgavirus 1 and salvia hispanica RNA virus 1.

Discussion

The type species of the *Amalgaviridae* family is the Southern tomato virus (STV, 3,437 bp), which was first described in 2009 (isolated from tomatoes from the United States and Mexico), with suspicion of association with a new tomato disorder (Sabanadzovic *et al*., 2009). Attempts with no success were made to mechanically and graft transmit amalgaviruses (Sabanadzovic *et al*., 2010; Zhan *et al*., 2019). Experiments with seeds collected from STV infected tomatoes confirmed high rates of vertical transmission (70 - 90%) (Sabanadzovic *et al*., 2009). Therefore, viruses belonging to the genus *Amalgavirus* are transmitted vertically through seeds, although it is not known whether the transmission is by pollen or ovary or both (Sabanadzovic *et al*., 2010, Martin *et al*., 2011). Amalgaviruses have been found infecting different crops in fields from France (Candresse *et al*., 2013), Spain (Verbeek *et al*., 2015), China (Padmanabhan *et al*., 2015), Bangladesh (Padmanabhan *et al*., 2015) and Italy (Iacono *et al*., 2015). In all cases, co-infections with other viruses were verified, making it difficult to recognize the association between virus infection and symptomatology (Sabanadzovic *et al*., 2010). Recently, Elvira-González *et al*. (2020) showed that geographically distant isolates of STV showed very low genetic diversity in the CP gene. Indeed, amalgavirus CP homologs have been detected in the genomes of *Populus trichocarpa*, *Medicago truncatula* and *Theobroma cacao* and spurious integration of amalgavirus sequences into host DNA has been suggested (Krupovic *et al*., 2015).

Aiming to understand the relationship between amalgaviruses and hosts, we analyzed all available allium transcriptomic data from GenBank in addition to the one generated for this work and assembled complete AcAV-1 and AcAV-2 genomic sequences. So far, most of the identified amalgavirus sequences have been discovered by analyzing available transcriptome datasets. In this work, nine complete genomic sequences of allium amalgavirus 1 and 2, respectively, were assembled from transcriptome data of *Allium cepa* collected around the world (Brazil, China, Czech Republic, India, New Zealand, Republic of Korea, and USA). Multiple pairwise analyses showed that allium amalgavirus complete nucleotide sequences share identities higher than 97% from the same species from different hosts, with exception of the AcAV-1 isolated from *A. ascalonicum* from China. In addition, it was possible to observe from the analyzed transcriptomic data that mixed infections of AcAv-1 and AcAV-2 occurred in *A. cepa*. As suggested for fig mosaic virus (Valia *et al*., 2014), the close genetic relationship to geographically distant isolates might suggest long-distance migration and it would be interesting to know if amalgaviruses contribute to broad adaptability to a wide range of climatic conditions. Moreover, the low genetic variability observed in the amalgaviruses genomic sequences might be the result of integration events into the allium DNA genome, which probably provides ideal settings for long-term evolution. Therefore, exciting experimental new approaches in the area of plant virology will help to understand the

function of highly conserved amalgavirus sequences across different host species around the world. Finally, computer analysis predicted five conserved motives (A-G) known for several plant and animal viruses as well as eleven additional motives conserved with RdRps from amalgaviruses. Specific functions of these motives are yet to be determined.

Taken together, this work shows that *Allium cepa* amalgavirus genomes are highly conserved among different allium species around the world indicating long-term virus-host coevolution.

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Supplementary information is available in the online version of paper.

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