1 **Exploring the tymovirids landscape through metatranscriptomics data**

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11

12 Abstract

13 *Tymovirales* is an order of viruses with positive-sense, single-stranded RNA genomes that mostly infect
14 plants, but also fungi and insects. The number of tymovirid sequences has been growing in the last few
15 years with the extensive use of high-throughput sequencing platforms. Here we report the discovery of 31
16 novel tymovirid genomes associated with 27 different host plant species, which were hidden in public
17 databases. These viral sequences were identified through a homology searches in more than 3,000 plant
18 transcriptomes from the NCBI Sequence Read Archive (SRA) using known tymovirids sequences as
19 query. Identification, assembly and curation of raw SRA reads resulted in 29 viral genome sequences with
20 full-length coding regions, and two partial genomes. Highlights of the obtained sequences include viruses
21 with unique and novel genome organizations among known tymovirids. Phylogenetic analysis showed
22 that six of the novel viruses were related to alphaflexiviruses, seventeen to betaflexiviruses, two to
23 deltaflexiviruses and six to tymoviruses. These findings resulted in the most complete phylogeny of
24 tymovirids to date and shed new light on the phylogenetic relationships and evolutionary landscape of this
25 group of viruses. Furthermore, this study illustrates the complexity and diversity of tymovirids genomes
26 and demonstrates that analyzing SRA public data provides an invaluable tool to accelerate virus discovery
27 and refine virus taxonomy.

28

29 Introduction

30 The application of high-throughput sequencing (HTS) is becoming a fundamental tool in biomedical
31 research, resulting in a steep increase in the number of genome and transcriptome sequencing projects
32 that is leading to a massive number of nucleotides deposited in the Sequence Read Archive (SRA) of the
33 National Center for Biotechnology Information (NCBI). Over 16,000 petabases (10^{15} bases) have been
34 deposited in the SRA, with over 6,000 petabases available as open-access data [1], and this number is
35 increasing significantly each year. This huge amount of data provides a significant challenge derived in
36 the high costs of data storage and management. Given the nature, objectives and magnitude of the
37 sequencing projects most of the generated data remains largely unexplored.

38 In the last years the application of metagenomics approaches resulted in the discovery of an abundant
39 number of novel viruses. Most of them likely do not induce any apparent symptoms in their host or their
40 host is unknown. In addition, the detection of multiple viruses in single samples suggest that mixed
41 infections are common and represent an important constrain to an unbiased assessment of the
42 contribution of each virus in a tentative pathosystem. Importantly, detections in metagenomics datasets
43 usually correspond to novel and unreported viruses. This illustrates how limited is our knowledge about
44 the richness of the plant virosphere, that seems to be exceedingly diverse in every host assessed so far [2-
45 4]. Even though the application of HTS has resulted in the discovery of a great number of viruses, the
46 pace of discovery suggest that it is still a minuscule portion of the whole virosphere, which has led to
47 initiatives to accommodate this massive diversity in a proposal for a first comprehensive megataxonomy
48 of the virus world [5].

49 The submitters of transcriptome datasets often limit their scientific interest to their specific field of study,
50 leaving behind a large amount of unused data which is potentially valuable [6]. Several studies in the last
51 few years have been focusing in the analysis of such transcriptome datasets, where viral sequences could

be hidden in plain sight, which resulted in the discovery of novel viral sequences [7-13]. Simmonds and colleagues [14] consensually stated that viruses that are known only from metagenomic data can, should, and have been incorporated into the official classification scheme overseen by the International Committee on Taxonomy of Viruses (ICTV). Therefore, analyzing public sequence databases is becoming a great source for mining novel plant viruses, that results in the identification of novel viruses in hosts with no previous record of virus infections [7]. The analysis of publically available databases does not require the acquisition of samples and subsequent sequencing, resulting in an inexpensive and sustainable approach for virus discovery. Proper attribution to the generators of the original data is essential to promote the good practices of open and transparent research, which enable the production of new results with existing data. Thus, the secondary analysis of publically available transcriptomic data to address novel research question and objectives is likely a wide-ranging, efficient and cost-effective approach for virus discovery due to the millions of datasets from a vast variety of potential host species available at the NCBI-SRA [11].

Tymovirales is an order of viruses which encompass viruses with a positive-sense, single-stranded RNA genome that mostly infect plants, but some are associated to fungi, while others are associated with insects. This order is taxonomically classified into five families: *Alphaflexiviridae*, *Betaflexiviridae*, *Deltaflexiviridae*, *Gammapflexiviridae*, whose members have flexuous filamentous virions, and *Tymoviridae*, whose members have isometric virions [15].

The family *Alphaflexiviridae* contains seven genera: *Allexivirus*, *Lolavirus*, *Mandarivirus*, *Platypuvirus* and *Potexvirus* genera comprised by plant-associated viruses, while *Botrexvirus* and *Sclerodarnavirus* genera correspond to fungi-associated viruses [16]. However, a recent proposal suggested to abolish the genus *Mandarivirus* and create the subgenus *Mandarivirus* within the genus *Potexvirus* [17].

Alphaflexiviruses have a polyadenylated genome of 5.9-9.0 kb in size with 1 or 5 open reading frames (ORFs) in those fungi-associated viruses and 5 to 7 ORFs in those plant-infecting viruses. The gene encoding the replicase (REP) is the only conserved among all alphaflexiviruses. In plant-infecting alphaflexiviruses, the gene encoding the capsid protein (CP) and, with the exception of platypuviruses, the module of the three overlapping ORFs 2 to 4, which encode the triple gene block (TGB) that is involved in the cell-to-cell movement, are conserved [16].

The family *Betaflexiviridae* is composed by plant-infecting viruses, classified into two subfamilies, *Quinvirinae* and *Trivirinae*, distinguished by the cell-to-cell type movement protein (MP), 30K-like or TGB, that are classified in thirteen genera. *Carlavirus*, *Foveavirus*, and *Robigovirus* genera belong to the *Quinvirinae* subfamily, while *Capillovirus*, *Chordovirus*, *Citrivirus*, *Divavirus*, *Prunevirus*, *Ravavirus*, *Tepovirus*, *Trichivirus*, *Tepovirus*, and *Wamavirus* genera belong to the *Trinvirinae* subfamily [15].

Betaflexiviruses have a polyadenylated genome of 6.5-9.5 kb in size with 2 to 6 ORFs. Each betaflexivirus typically codes for a REP, a MP, and a CP; while some viruses also code for a nucleic acid binding protein (NABP) [15]. The viruses belonging to the subfamily *Quinvirinae* have 5 to 6 ORFs, while those viruses in the subfamily *Trivirinae* have 2 to 5 ORFs [15]. Among viruses belonging to the later subfamily there is variation in their genome organization. For instance, the MP encoded by capilloviruses and divaviruses is nested within the ORF1, while that one of citriviruses and trichoviruses overlap with the REP [15].

92 The family *Deltaflexiviridae* contains one genera, named as *Deltaflexivirus*, which is comprised by fungi-
 93 associated viruses [18-20]. Deltaflexiviruses have a polyadenylated genome of 8.1-8.3 kb in size with 4 to
 94 5 ORFs. The gene encoding the replicase is the only one conserved among all deltaflexiviruses [18-20].
 95 The family *Tymoviridae* contains three genera the *Marafivirus* and the *Maculavirus*, which have a 3'
 96 terminal poly (A) tail, and *Tymovirus*, whose genomes are not polyadenylated [21]. The genus
 97 *Marafivirus* is exclusively composed of plant-infecting viruses, but several tymoviruses have been
 98 associated to fungi or insects [22-24]. Tymoviruses have a genome of 6.0-7.5 kb in size with 1 to 4 ORFs,
 99 and the gene encoding the replicase is the only conserved among all tymoviruses [21].
 100 In this study we queried the publically available plant transcriptome datasets in the transcriptome shotgun
 101 assembly (TSA) database and their corresponding SRA datasets hosted at NCBI and identified 31 novel
 102 tymovirids from 27 plant species, showing structural, functional and evolutionary cues to be classified in
 103 new and existing genera within the families *Alphaflexiviridae*, *Betaflexiviridae*, *Deltaflexiviridae* and
 104 *Tymoviridae* in the order *Tymovirales*. The discovery of these new viruses increase the knowledge of
 105 tymovirids and greatly expands the number of viruses associated to the order *Tymovirales*.
 106

107 **Materials and methods**

108 **Identification of tymovirid sequences from public plant transcriptome datasets**

109 The amino acid sequences corresponding to the replicase proteins of several known viruses belonging to
 110 the order *Tymovirales*, such as Cnidium virus X (QVW10166), Fusarium graminearum deltaflexivirus 1
 111 (ANS13830), alfalfa virus F (AWB13379), eggplant mosaic virus (P20126), apple stem grooving virus
 112 (QVX32671), Diuris virus A (AFV57238), fig fleck-associated virus 2 (AOF41056), Helenium virus S
 113 (QQX32728), and garlic yellow mosaic-associated virus (AZM69107), were used as query in tBlastn
 114 searches with parameters word size = 6, expected threshold = 10, and scoring matrix = BLOSUM62,
 115 against the viridiplantae (taxid:33090) TSA database. The obtained hits were explored by hand and based
 116 on percentage identity (<90%), query coverage (>50%) and E-value (>1e-5), shortlisted as likely
 117 corresponding to novel virus transcripts, which were further analyzed. Given the redundant nature of
 118 many retrieved hits, a step of contig clustering was implemented using the CD-hit suite with standard
 119 parameters available at http://weizhongli-lab.org/cdhit_suite/cgi-bin/index.cgi?cmd=cd-hit. In addition,
 120 the raw data corresponding to the SRA experiments associated with the different target NCBI Bioprojects
 121 (Table 1) was retrieved for further analyses.

122 **Sequence assembly and identification**

123 The nucleotide (nt) raw sequence reads from each analyzed SRA experiment linked to the TSA projects
 124 returning tymovirids-like hits were downloaded and pre-processed by trimming and filtering with the
 125 Trimmomatic tool as implemented in <http://www.usadellab.org/cms/?page=trimmomatic>, and the
 126 resulting reads were assembled *de novo* with Trinity release v2.11.00 using standard parameters. The
 127 transcripts obtained from *de novo* transcriptome assembly were subjected to bulk local BLASTX searches
 128 (E-value < 1e⁻⁵) against the TSA database obtained hits and a Refseq virus protein database available at
 129 <ftp://ftp.ncbi.nlm.nih.gov/refseq/release/viral/viral.1.protein.faa.gz>. The resulting viral sequence hits of
 130 each bioproject were visually explored. TSA and *de novo* assembled overlapping transcripts
 131 corresponding to tymovirids were re assembled and curated by mapping of SRA reads to the obtained

132 sequences using Bowtie2 available at <http://bowtie-bio.sourceforge.net/bowtie2/index.shtml>, which was
133 used to calculate total and mean coverage and reads per million (RPM) of each assembled virus sequence.

134 **Bioinformatics tools and analyses**

135 *Sequence analyses*

136 ORFs were predicted with ORFfinder (<https://www.ncbi.nlm.nih.gov/orffinder/>), functional domains and
137 architecture of translated gene products were determined using InterPro
138 (<https://www.ebi.ac.uk/interpro/search/sequence-search>) and MOTIF search
139 (<https://www.genome.jp/tools/motif/>). Importin- α dependent nuclear localization signals were predicted
140 using cNLS Mapper available at <http://nls-mapper.iab.keio.ac.jp/>, and transmembrane domains were
141 predicted using the TMHMM version 2.0 tool (<http://www.cbs.dtu.dk/services/TMHMM/>).

142 *Percent identity matrices*

143 Percentage amino acid (aa) sequence identity of the predicted ORFs of each virus identified in this study
144 based on available tymovirids genome sequences were calculated using
145 https://www.ebi.ac.uk/Tools/psa/emboss_needle/.

146 *Phylogenetic analysis*

147 Phylogenetic analysis based on the predicted replicase and capsid protein of the tymovirids detailed in
148 Table S1, was done using MAFFT 7 <https://mafft.cbrc.jp/alignment/software> with multiple aa sequence
149 alignments using FFT-NS-i as the best-fit model. The aligned aa sequences were used as input in MegaX
150 software [25] to generate phylogenetic trees by the maximum-likelihood method using the best fit method
151 which is detailed in the legend describing each phylogenetic tree. Local support values were computed
152 using bootstrap with 1,000 replicates.

153 *Recombination analysis*

154 The sequences assembled in this study were examined for potential recombination events using the
155 recombination detection program (RDP) version 5.05 with default settings [26]. Seven algorithms,
156 BootScan, RDP, SiScan, GENECONV, MaxChi, Chimaera and Philpro were employed with a bonferroni
157 corrected *P* value cut-off of <0.05. An event was considered as positive if was identified by at least three
158 or more algorithms.

159

160 **Results**

161 **Summary of discovered tymovirid sequences**

162 The complete coding regions of 29 novel tymovirids were identified, in addition, partial genomic
163 sequences for two novel viruses were assembled. These viruses were associated with 27 plant host species
164 (Table 1). Bioinformatic and source data of each of the 29 viral sequences, as well as the GenBank
165 accession numbers and proposed classification are listed in Table 1; the summary of the assembly
166 statistics of each virus of the tymovirid sequences identified from the transcriptome data available in the
167 NCBI database are presented in Table S2. Based on phylogenetic relatedness, genome organization and
168 sequence identity, the novel viruses were tentatively assigned to the established tymovirales families:
169 *Alphaflexiviridae*, *Betaflexiviridae*, *Deltaflexiviridae*, and *Tymoviridae*. 10 herbaceous dicots, 8
170 herbaceous monocots, and 9 woody dicots plant were identified as putative hosts of the viruses described
171 in this study. Most of the woody dicot plants (6/9) appeared to be infected by betaflexiviruses belonging

172 to the *Trivirinae* subfamily (Table 1). The conserved domains of deduced proteins encoded by each
173 tymovirid sequence were determined by predictive algorithms, and are shown in Table S3. Genomic
174 architecture and evolutionary placement of the 29 discovered viruses are described below, grouped by
175 affinity to members of the diverse families within the order *Tymovirales*.

176

177 *Alphaflexiviridae*

178 The complete coding region of six putative alphaflexiviruses, tentatively named *Agave tequilana* virus 2
179 (ATV2), *Cymodocea nodosa* virus 1 (CyNoV1), indian gooseberry virus 1 (InGoV1), ferula virus 1
180 (FerV1), peltate yam virus 1 (PeLYV1) and *Yucca gloriosa* virus 1 (YuGIV1), were assembled in this
181 study (Table 1). ATV2, InGoV1, FerV1, PeLYV1 and YuGIV1 genomes have five ORFs in the order
182 5'Rep-TGB1-TGB2-TGB3-CP-3' (Fig.1A); while CyNoV1 genome has two ORFs in the order 5'-Rep-
183 MP-3' (Fig. 1A), which is a distinctive genomic organization among plant alphaflexiviruses.
184 Blast P searches showed that ATV2, InGoV1, FerV1, PeLYV1 and YuGIV1 ORFs 1, 2, 3, 4 and 5
185 encoded proteins are related with replicases, TGB1, TGB2, TGB3 and capsid proteins encoded by other
186 potexviruses (Table 1); while the BlastP search of CyNoV1 ORF1 and ORF2 encoded proteins showed
187 that are related with the replicase and the MP encoded by the alphaflexivirus donkey orchid symptomless
188 virus (DOSV) (Table 1).

189 The viral methyltransferase, viral helicase and the RdRP motifs were identified in the replicases encoded
190 by ATV2, CyNoV1, InGoV1, FerV1, PeLYV1 and YuGIV1. The 3A/RNA2 MP family conserved domain
191 was identified in the MP encoded by the CyNoV1. Whereas the conserved domains viral helicase, plant
192 viral MP, triple gene block 3 and flexi_CP conserved domains were identified in the TGB1, TGB2, TGB3
193 and CP, respectively, encoded by ATV2, InGoV1, FerV1, PeLYV1 and YuGIV1 (Table S3).

194 Based on the ATV2, InGoV1, FerV1, PeLYV1 and YuGIV1 aa sequences of the replicase and CP
195 proteins, these viruses shared the highest sequence similarity with Vanilla virus X (VVX) (54.3%) and
196 Cassava virus X (CsVX) (46.6%), Euonymus yellow mottle associated virus (EuYMaV) (58.6%) and
197 EuYMaV (52.5%), Cnidium virus X (CnVX) (66.9%) and VVX (57.7%), Yam virus X (YVX) (57.8%)
198 and YVX (50.1%), Potato virus X (PVX) (53.8%) and EuYMaV (47.2%), respectively. While, the aa
199 sequence of the CyNoV1 replicase shared the highest sequence similarity with DOSV (50.1%).

200 No recombination event was detected in the ATV2, CyNoV1, InGoV1, FerV1, PeLYV1 and YuGIV1
201 genomic sequences.

202 In a phylogenetic tree based on the replicase aa sequence, PeLYAV1 clustered together with the
203 potexvirus ambrosia asymptomatic virus 1, InGoV1 clustered together with the potexvirus EuYMaV and
204 FerV1 clustered together with the potexvirus CnVX, while ATV2 and YuGIV1 clustered together in a
205 sister group to the potexviruses (Fig.2). Whereas, CyNoV1 clustered together with the platypuvirus
206 DOSV (Fig.2). In the phylogenetic tree based on the CP aa sequences the same clustering were observed
207 for ATV2, InGoV1, FerV1, PeLYV1 and YuGIV1 (Figure S1).

208 *Betaflexiviridae*

209 The complete coding region of 15 putative betaflexiviruses, tentatively named as avocado virus 1
210 (AvoV1), avocado virus 2 (AvoV2), breadfruit virus 1 (BreFV1), *Corylus avellana* virus 1 (CorAvV1),
211 *Daiswa yunannensis* virus 1 (DaYuV1), *Gymnadenia rhellicani* (GymRhV1), korean chestnut virus 1

(KoChV1), *Melampyrum roseum* virus 2 (MelRoV2), *Quercus castanea* virus 1 (QueCaV1), *Rhodiola rosea* virus 1 (RhRoV1), *Rhododendron delavayi* virus 2 (RhoDeV2), *Silene dioica* virus 1 (SiDiV1), *Suaeda fruticosa* virus 1 (SuFruV1), sweetleaf virus 1 (SLeV1) and *Tagetes erecta* virus 2 (TaEV2), well as the partial genome of two other betaflexiviruses, named *Cymodocea nodosa* virus (CyNoV2) and German iris virus 2 (GerIV1), were assembled in this study (Table 1). BreFV1, CorAvV1, GerIV1, KoChV1, QueCaV1, RhRoV1, SiDiV1 and SuFruV1 genomes have two ORFs, named as Polyprotein-MP, where the ORF encoding the MP is overlapped (Fig.1B). GymRhV1 and MelRoV2 genomes have three ORFs in the order 5'-Polyprotein-MP-NABP-3' where the ORF encoding the MP is overlapped (Fig.1B). AvoV2 and DaYuV1 genomes have three ORFs in the order 5'-Rep-MP-CP-3' (Fig.1B), while RhoDeV2 has three ORFs in the order 5'-Rep-P2-P3-3' (Fig.1B). AvoV1 genome has five ORFs in the order 5'-Rep-MP-CP-P4-NABP-3' (Fig.1B). CyNoV2 genome has five ORFs in the order 5'-Rep-TGB1-TGB2-TGB3-CP-3' (Fig.1B); while SLeV1 and TaEV2 genome have six ORFs in the order 5'-Rep-TGB1-TGB2-TGB3-CP-NABP-3' (Fig. 1B).

Blast P searches showed that BreFV1, CorAvV1, GerIV1, GymRhV1, KoChV1, MelRoV2, QueCaV1, RhRoV1, SiDiV1 and SuFruV1 ORFs 1 and 2 encoded proteins are similar to the polyprotein and movement proteins of other divaviruses, capilloviruses and unclassified betaflexiviruses (Table 1), while the GymRhV1 and MelRoV2 ORF3 showed identity with the NABPs of trichoviruses (Table 1). Blast P searches showed that AvoV2 and DaYuV1 ORFs 1, 2 and 3 encoded proteins are similar to the replicases, movement proteins and capsid proteins of other betaflexiviruses (Table 1); while Blast P searches showed that RhoDeV2 ORF1 encoded protein is more similar to the replicase of other trichoviruses, but no hits were found when ORFs 2 and 3 encoded proteins were searched (Table 1). Blast P searches showed that AvoV1 ORFs 1, 2, 3 and 5 encoded proteins are similar to the replicases, movement proteins, capsid proteins and nucleic acid binding proteins of other betaflexiviruses, whereas no hits were found when AvoV1 ORF4 encoded protein was subjected to a BlastP search (Table 1). Blast P searches showed that CyNoV2 ORFs 1, 2, 3, 4 and 5 encoded proteins are similar to the replicases, TGB1, TGB2, TGB3 and capsid proteins of other foveaviruses (for CyNoV2) (Table 1). BlastP searches showed that SLeV1 and TaEV2 ORFs 1, 2, 3, 4, 5 and 6 encoded proteins are similar to the replicases, TGB1, TGB2, TGB3, capsid proteins and nucleic acid binding proteins of other carlaviruses (Table 1).

The viral methyltransferase, viral helicase and the RdRP motifs were identified in the replicases encoded by Avo1, Avo2, RhoDeV2, SLeV1 and TaEV2, and in the polyproteins encoded by BreFV1, CorAvV1, DaYuV1, GymRhV1, KoChV1, MelRoV2, QueCaV1, RhRoV1, SiDiV1 and SuFruV1. The viral methyltransferase motif was not found in the CyNoV2 encoded replicase and GerIV1 encoded polyprotein, probably because they were only partially assembled and its N-terminal region is missing, but the viral helicase and RdRP motifs were identified. Furthermore, the carlavirus endopeptidase conserved domain was found in the replicases encoded by SLeV1 and TaEV2. Whereas the trichovirus coat protein motif was found in the polyprotein encoded by BreFV1, CorAvV1, GerIV1, GymRhV1, KoChV1, MelRoV2, QueCaV1, RhRoV1, SiDiV1 and SuFruV1 and in the CP encoded by AvoV1, AvoV2 and DaYuV1; while the DUF1717 conserved domain was identified in the polyprotein encoded by BreFV1, CorAvV1, GerIV1, RhRoV1 and SiDiV1 and the 2OG-FeII_Oxy_2 motif was identified in the replicase encoded by AvoV2 and RhoDeV2. Moreover, the conserved domain viral MP was identified

in the MP encoded by AvoV1, AvoV2, BreFV1, CorAvV1, DaYuV1 GerIV1, GymRhV1, KoChV1, MelRoV2, QueCaV1, RhRoV1, SiDiV1 and SuFruV1. Whereas the conserved domains viral helicase, plant viral MP, triple gene block 3 and flexi_CP conserved domains were identified in the TGB1, TGB2, TGB3 and CP, respectively, encoded by CyNoV2, SleV1 and TaEV2. Furthermore, the Carlavirus coat motif was identified in the CP encoded by SLeV1 and TaEV2, and the carlavirus putative NABP conserved domain was identified in the NABP encoded by AvoV1, SLeV1 and TaEV2 (Table S3), while a viral_NABP conserved domain was identified in the NABP encoded by GymRhV1 and MelRoV2 (Table S3). No conserved domains were identified in the proteins 2 and 3 encoded by RhoDeV2, and in the protein 4 encoded by AvoV1 (Table S3), but a bipartite NLS was detected in the P3 encoded by RhoDeV2 and in the P4 encoded by AvoV1.

Based on the AvoV1, AvoV2, CyNoV2, DaYuV1, SleV1 and TaEV2 aa sequences of the replicase and CP proteins, these viruses shared the highest sequence similarity with grapevine Kizil Sapak virus (GKSV) (50.1%) and fig latent virus 1 (FLV1) (48.6%), citrus leaf blotch virus (CLBV) (55.6%), and Camellia ringspot-associated virus 2 (CRSaV2) (52.8%), Asian prunus virus 3 (APV3) (43.0%), and Grapevine rupestris stem pitting-associated virus (GRSPaV) (44.7%), Agapanthus virus A (AgVA) (54.4%) and AgVA (51.7%), Helenium virus S (HelVS) (60.6%) and HelVS (63.9%), HelVS (60.9%) and (HelVs) (65.7%), respectively. Whereas the aa sequence of the BreFV1, CorAvV1, GerIV1, GymRhV1, KoChV1, MelRoV2, QueCaV1, RhRoV1, RhoDeV2, SiDiV1 and SuFruV1 replicase protein, shared the highest sequence similarity with Apple stem grooving virus (ASGV) (69.4%), ASGV (58.8%), ASGV (53.6%), diuris virus B (DiVB) (52.2%), diuris virus A (DiVA) (53.5%), DiVB (47.7%), DiVB (52.7%), Hobart betaflexivirus 1 (HoBFV1) (47.1%), cherry mottle leaf virus (CMLV) (44.1%), ASGV (49.7%) and Camellia ringspot-associated virus 3 (CRSaV3) (52.8%), respectively.

No recombination event was detected in the betaflexiviruses assembled in this study.

In a phylogenetic tree based on the replicase aa sequence of viruses belonging to the subfamily *Quinvirinae*, SleV1 and TaEV2 clustered together and this group was related with the carlavirus HelVS. On the other hand, CyNoV2 formed a monophyletic group that was grouped with the unclassified betaflexiviruses yam virus Y and sugarcane striate mosaic-associated virus (Fig. 3). Whereas, in the phylogenetic tree based on the CP aa sequence, SleV1 and TaEV2 clustered together and this group was related with the carlavirus HelVS; while CyNoV2 clustered together with the foveavirus rubus virus 1 (Figure S2).

In a phylogenetic tree based on the replicase aa sequence of viruses belonging to the subfamily *Trinvirinae*, AvoV1 was placed in the same clade as GKSV and FLV1, that is separated from the clades formed by other genera belonging to the *Trinvirinae* subfamily (Fig.4). RhoDeV2 formed a monophyletic cluster that is related with the *Chordovirus* clade and the ravavirus Ribes americanum virus A (Fig. 4). GymRhV1 clustered together with MelRoV2, and KoChV1 with QueCaV1 and this two clades were grouped with the *Divavirus* clade (Fig. 4). AvoV2 formed a monophyletic cluster that is related with the *Citrivirus* clade and the unclassified betaflexivirus citrus yellow spot virus (Fig. 4). DaYuV1 clustered together with AgVA and this cluster is separated from the clades formed by members of other genera belonging to the *Trinvirinae* subfamily (Fig. 4). SuFruV1 clustered together with CRSaV3 and this clade grouped with the capilloviruses (Fig. 4). RhRoV1 clustered together with HoBFV1 and SiDiV1 and this

cluster grouped with capilloviruses (Fig. 4). Whereas, BreFV1, CorAvV1 and GerIV1 clustered with the capilloviruses ASGV and yacon virus A (Fig. 4). In the phylogenetic tree based on the CP aa sequence, AvoV1 was placed in the same clade as GKSv and FLV1 (Figure S3). GymRhV1 clustered together with MelRoV2, and KoChV1 with QueCaV1 and this two clades were grouped with the *Divavirus* clade (Figure S3). AvoV2 formed a monophyletic cluster that is related with the *Prunevirus* clade (Supp. Figure 3). DaYuV1 clustered together with AgVA (Figure S3). SuFruV1 is related with CRSaV3 and capilloviruses (Figure S3). RhRoV1 clustered together with HoBFV1 and SiDiV1 and this cluster grouped with known capilloviruses, while, CorAvV1 and GerIV1 clustered with the capilloviruses yacon virus A and BreFV1 with the capillovirus ASGV (Figure S3).

301 ***Deltaflexiviridae***

302 The complete coding region of two putative deltaflexiviruses, tentatively named Agave tequilana virus 3
303 (ATV3) and sesame virus 1 (SesV1), were assembled in this study (Table 1). ATV3 and SesV1 genomes
304 consist of five ORFs in the order 5'Rep-P2-P3-P4-P5-3' (Fig.1C).

305 Blast P searches showed that ATV3 and SesV1 ORF1 encoded proteins are similar to the replicases of
306 other deltaflexiviruses (Table 1); while BlastP searches showed SesV1 ORFs 4 and 5 are similar to the P4
307 and P5 proteins of other deltaflexiviruses (Table 1). No hits were found when ATV3 ORFs 2, 3, 4 and 5
308 encoded proteins, as well as SesV1 ORFs 2, 3 encoded proteins, were subjected to a BlastP search (Table
309 1).

310 The typical viral methyltransferase and the RdRP motifs were identified in the replicase encoded by
311 ATV3, and these two conserved domains as well a viral helicase conserved domain was identified in the
312 SesV1 encoded replicase; while no conserved domains were identified in the other proteins encoded by
313 ATV3 and SesV1 (Table S3).

314 Based on the ATV3 and SesV1 aa sequences of the replicase, these viruses shared the highest sequence
315 similarity with *Fusarium graminearum* deltaflexivirus 1 (FgDFV1) (55.7%) and FgDFV1 (58.2%),
316 respectively.

317 No recombination event was detected in the deltaflexiviruses assembled in this study.

318 In a phylogenetic tree based on the replicase aa sequence, ATV3 and SesV1 were grouped with other
319 known deltaflexiviruses (Fig.5).

320 ***Tymoviridae and tymo-like virus***

321 The complete coding region of six putative tymoviruses, tentatively named *Davidia involucrata* virus 1
322 (DaInvV1), *Davidia involucrata* virus 2 (DaInvV2), kava virus 1 (KaV1), polish wheat virus 1 (PolWhV1),
323 watercress associated virus 1 (WaCraV1) and yellow poplar virus 1 (YePoV1) were assembled in this
324 study (Table 1). DaInvV1 and DaInvV2 genomes have one ORF (Fig.1D), YePoV1 has two ORFs in the
325 order 5'Rep-CP-3' (Fig.1D); while KaV1 and PolWhV1 genomes have three ORFs in the order 5'-Rep-
326 P2-P3-3', while WaCraV1 genome has three ORFs in the order 5'-Rep-CP-P3-3' (Fig. 1D).

327 Blast P searches showed that DaInvV1 and DaInvV2 ORF encoded protein are similar to the polyprotein of
328 other marafiviruses (Table 1). Blast P searches showed that KaV1, PolWhV1, WaCraV1 and
329 YePoV1ORF1 encoded proteins are similar to the replicases of other tymo-like viruses, while WaCraV1
330 and YePoV1 ORF 2 encoded proteins are similar to the CP of other tymo-like viruses, and WaCraV1
331 ORF3 encoded protein is similar to the P3 encoded by other tymo-like viruses (Table 1). No hits were

found when KaV1, PolWhV1 and WaCraV1 ORFs 2 and 3 encoded proteins were subjected to a BlastP search (Table 1).

The viral methyltransferase, viral helicase and the RdRP motifs were identified in the DaInV1 and DaInv2 polyproteins and in the replicase encoded by KaV1, PolWhV1, WaCraV1 and YePoV1, while the tymovirus endopeptidase conserved domain was identified in the DaInV1 and DaInv2 polyproteins and in the replicase encoded by PolWhV1 and WaCraV1. Whereas the tymovirus coat protein conserved domain was identified in the DaInV1 and DaInv2 polyproteins and in the capsid protein encoded by WaCraV1 (Table S3). Moreover, a transmembrane domain was identified in the protein 2 and protein 3 encoded by KaV1 and WaCraV1, respectively.

Based on the nearly complete assembled genomes of DaInv1, DaInv2, KaV1, PolWhV1, WaCraV1 and YePoV1, these viruses shared the highest sequence similarity with *Medicago sativa marafivirus 1* (MsMV1) (57.2%), *citrus sudden death-associated virus* (CSDaV) (59.1%), *Ullucus tymovirus 1* (UTyV1) (42.3%), *Fusarium graminearum mycotymovirus 1* (FgMTV1) (51.6%), *bee macula-like virus 2* (BeeMLV2) (55.2%) and *prunus yellow spot-associated virus* (PYSaV) (59.7%), respectively. While the DaInv1, DaInv2, WaCraV1 and YePoV1 CP shared the highest amino acid sequence identity with MsMV1 (57.36%), CSDaV (59.09%), BeeMLV2 (48.70%) and PYSaV (44.94%).

No recombination events were detected in the DaInV1, DaInV2, KaV1, PolWhV1, WaCraV1 and YePoV1 genomic sequences.

In a phylogenetic tree based on the replicase aa sequence, DaINV1 and DaINV2 clustered with marafiviruses. DaINV1 was related to two clades, one formed by *Medicago sativa marafivirus 1* and alfalfa virus F, and the other by citrus virus C and grapevine red globe virus; while DaInv2 was closely related with blackberry virus S (Fig.6). WaCraV1 clustered together with the maculaviruses BeeMLV2 and *Bombyx mori macula-like virus*. KaV1 formed a monophyletic cluster that was distantly related with known tymoviruses, while PolWhV1 clustered together with the mycotymoviruses FgMTV1 and this viruses grouped with the cluster formed by the mycotymoviruses *Sclerotinia sclerotiorum mycotymovirus 1*, *Sclerotinia sclerotiorum mycotymovirus 2* and *Botrytis cinerea mycotymovirus 1* (Fig.6). Whereas, YePoV1 clustered together with the tymo-like viruses PYSaV and grapevine associated tymo-like virus (GaTLV) (Fig.6).

Discussion

In the last few years, the employment of HTS, allowed the identification and characterization of several novel viruses associated to the order *Tymovirales*, which may be asymptomatic [27-33]. Moreover, some tymovirids were also discovered mining publically available transcriptome datasets at the NCBI [34-40]. Thus, many tymovirales-like sequences could be hidden in publicly available plant transcriptome datasets. Therefore, we conducted an extensive search of tymovirales-like sequences querying the transcriptome shotgun assembly (TSA) which resulted in the identification of 31 novel tymovirids. After extensive structural and functional annotation and evolutionary analyses we suggest these viruses correspond to potential new members of new virus species, which expand the diversity of the complete *Tymovirales* order by ca. 30%.

Conserved domains

All the replicase proteins assembled in this study contained the methyltransferase (MTR), RNA helicase (Hel), and RNA-dependent RNA polymerase (RdRp) domains. These domains have been identified in a wide range of ssRNA viruses, where MTR is involved in capping, which enhances mRNA stability [41], the RdRp corresponds to the main viral replicase, an essential protein encoded in all RNA viruses, while the HEL domain plays multiple roles at different stages of viral RNA replication [42]. Transmembrane domains were identified in the TGB2 and TGB3 proteins encoded by ATV2, CyNoV2, FerV1, InGoV1, PelYV1, SLeV1, TaEV2 and YuGIV1, which also were reported for the TGB2 and TGB3 proteins encoded by other foveaviruses, carlaviruses, potexviruses, robigoviruses and allexiviruses [43], thus supporting the membrane-association and involvement in viral movement of these two proteins [44-46].

Genome organization and phylogenetic relationships of the novel tymovirids

Alphaflexiviridae

ATV2, InGoV1, FerV1, PelYV1 and YuGIV1 showed a genomic organization 5'-Rep-TGB1-TGB2-TGB3-CP-3', that is typical of potexviruses [16] and these five viruses are phylogenetically related with other potexviruses, but the highest nt identities when the Rep and CP genes were compared with other potexviruses was below 72%, which is the criteria to demarcate species in the *Alphaflexiviridae* family [16]. Thus, based on sequence similarities, genomic organization and phylogenetic relationships, ATV2, InGoV1, FerV1, PelYV1 and YuGIV1 should be classified as new members of the genus *Potexvirus* in the family *Alphaflexiviridae*.

On the other hand, CyNoV1 has a distinctive genomic organization among alphaflexiviruses because only two ORFs, encoding a replicase and MP, were identified in its genome, likely being the first alphaflexivirus reported so far that has only two ORFs. Like the sclerodarnaviruses, CyNoV1 does not encode a putative CP. Moreover, its replicase and MP are distantly related with that one encoded by the platypovirus DOSV. Similarly to the DOSV MP, the CyNoV1 encoded MP is related with the 3A-like MPs of viruses belonging to the families *Tombusviridae* and *Virgaviridae* [47], which is distinctive among alphaflexiviruses, where the TGB proteins are the MPs [16]. Despite that the replicase nt sequence similarity between CyNoV1 and DOSV is above the 45% threshold value to demarcate genus in the family *Alphaflexiviridae* [16], and that the Rep of these two viruses grouped together in the phylogenetic tree, CyNoV1 has a genomic organization different than that one reported for DOSV [47]; thus CyNoV1 could be classified as a member of a novel genus belonging to the family *Alphaflexiviridae*.

Betaflexiviridae

SLeV1 and TaEV2 showed a genomic organization 5'-Rep-TGB1-TGB2-TGB3-CP-NABP-3', that is typical of carlaviruses [15]. Moreover, these two viruses are phylogenetically related with other carlaviruses, but the highest nt identities when the Rep and CP genes were compared with other carlaviruses was well below 72%, which is the criteria to demarcate species in the *Betaflexivirus* family [15]. Thus, based on sequence similarities, genomic organization and phylogenetic relationships, SLeV1 and TaEV2 should be classified as novel members of the genus *Carlavirus* in the family *Betaflexiviridae*. BreFV1, CorAvV1, GerIV1, RhRoV1, SiDiV1 and SuFruV1 showed a genomic organization with two overlapping ORFs; where one encodes a Rep protein fused to the CP, while the other encodes a MP, which is typical of capilloviruses [30]. The highest nt identity when the Rep and CP proteins were

411 compared with other betaflexiviruses was below 72%, which is the criteria to demarcate species in the
 412 *Betaflexiviridae* family [15]. The BreFV1, CorAvV1, GerIV1, RhRoV1, SiDiV1 Rep and CP clustered
 413 together with known capilloviruses, while SuFruV1 Rep and CP was similar to the one of CRSaV3 and
 414 these two viruses are related with known capilloviruses. Interestingly, the CRSaV3 genomic organization
 415 is different than the one of SuFruV1 and known capilloviruses [48]. Moreover, the conserved domain
 416 DUF1717 was identified in the polyprotein of BreFV1, CorAvV1, GerIV1, RhRoV1 and SiDiV1, but not
 417 in the SuFruV1 polyprotein. The DUF1717 domain, which function is unknown, was found in the
 418 polyprotein of the capilloviruses ASGV and cherry virus A [49, 50] as well as in the polyprotein of the
 419 unclassified betaflexivirus HoBFV1; however this domain was not found in the polyprotein encoded by
 420 CRSaV3. Sequence similarities, genomic organization and phylogenetic relationships supports that
 421 BreFV1, CorAvV1, GerIV1 RhRoV1, SiDiV1, and SuFruV1 as well as the previously reported HoBFV1
 422 (Roberts et al., 2018) and CRSaV3 [48] should be classified as members of the genus *Capillovirus* in the
 423 family *Betaflexiviridae*, where CRSaV3 would be the first capillovirus with three ORFs instead of two,
 424 while HoBFV1 is likely the first insect-associated capillovirus described so far.

425 KoChV1 and QueCaV1 genomes have two overlapping ORFs, one is the fused Rep-CP ORF and the
 426 other one encodes the MP, which is typical of divaviruses [34]; while GymRhV1 and MelRoV2 also have
 427 a third ORF which encodes a putative NABP. These four viruses were distantly related with other
 428 divaviruses, and the highest nt identity when the Rep and CP genes were compared with other
 429 betaflexiviruses was below 72%, which is the criteria to demarcate species in the *Betaflexiviridae* family
 430 [15]. Moreover, these viruses clustered together with divaviruses both in the Rep and CP phylogenetic
 431 trees, thus they likely share a similar evolutionary history. Thus, based on sequence similarities, genomic
 432 organization and phylogenetic relationships, KoChV1 and QueCaV1 should be classified as novel
 433 members of the genus *Divavirus* in the family *Betaflexiviridae*. Despite that GymRhV1 and MelRoV2
 434 have a different genomic organization than known divaviruses, based on sequence similarities and
 435 phylogenetic relationships these two viruses should also be classified as novel members of the genus
 436 *Divavirus*. The genus *Trichovirus* also contains viruses that display different genomic organizations [15],
 437 which support the potential classification of GymRhV1 and MelRoV2 as divaviruses.

438 The genomic organization of AvoV1 5'-Rep-MP-CP-P4-NABP-3', is similar to that reported for the
 439 betaflexivirus GKSV [52]. The highest nt identity when the Rep and CP proteins were compared with
 440 other betaflexiviruses was below 72%, which is the criteria to demarcate species in the *Betaflexiviridae*
 441 family [15]. Neither conserved domains were identified, nor any BlastP hits were obtained when the
 442 AvoV1 ORF4 encoded protein was analyzed, which is similar to that reported for the encoded protein by
 443 the GKSV ORF4 [52]. Interestingly, in this protein of unknown function, a NLS was found in the one
 444 encoded by AvoV1, but no NLS was detected in the protein encoded by GKSV ORF4. Further studies
 445 should be conducted to characterize the putative function of this protein. AvoV1 and GKSV, along with
 446 FLV1, which full sequence has not been obtained yet [53], were phylogenetically related in a distinct
 447 clade from other genera belonging to the subfamily *Trivirinae*. GKSV and FLV1 were proposed to belong
 448 to a new genus named as *Fivivirus* [52]. Based on sequence similarities, genomic organization and
 449 phylogenetic relationships, AvoV1 should be classified as novel member of this genus, which name could
 450 be changed to Avofivirus (derived from **A**vo**c**ado-**F**icus-**V**itis-virus).

451 CyNoV2 genome organization 5'-Rep-TGB1-TGB2-TGB3-CP-3', resembles the one described both for
 452 foveaviruses [54] and for the unclassified betaflexiviruses banana mild mosaic virus [55], sugarcane
 453 striate mosaic-associated virus [56] and yam virus Y [57]. The identities between the nt sequence of the
 454 replicase and CP of CyNoV2 with other betaflexiviruses is below 45%, that is the genus demarcation
 455 criteria for betaflexiviruses [15]. CyNoV2 replicase clustered together with the unassigned
 456 betaflexiviruses sugarcane striate mosaic-associated virus and yam virus X, while its CP was
 457 phylogenetically related with known foveaviruses. Based on sequence similarities, and the incongruences
 458 in the phylogenetic relationships observed between Rep and CP proteins, which likely indicates a distinct
 459 evolutionary history of CyNoV2 Rep and CP, this virus may represents a novel member of a new genus
 460 within the *Quinvirinae* subfamily in the *Betaflexiviridae* family, which could be tentatively named as
 461 Cynovirus.

462 The AvoV2 genome has three ORFs with a genomic organization 5'-Rep-MP-CP-3', that is similar to that
 463 reported for the unclassified betaflexivirus citrus yellow spot virus [58], almost identical to that of
 464 citriviruses, where the ORF that encodes the MP is located adjacent to the ORF that encodes the Rep
 465 protein [15]. However, in the AvoV2 genomes, the ORF encoding the CP is overlapped with that one
 466 encoding the MP [58]; while in the citriviruses these two ORFs are separated by an intergenic region [15,
 467 32, 59]. Similarly to the AvoV2 genome, in the pruneviruses the ORFs encoding the MP and CP are
 468 overlapped [15]; however most of the pruneviruses have four ORFs instead of three [48]. AvoV2 Rep and
 469 CP were distantly related with other citrivirus and pruneviruses, respectively, and the replicase and CP nt
 470 sequence similarity is above the 45% threshold value to demarcate genus in the family *Betaflexiviridae*
 471 [15]. However, this virus formed a monophyletic cluster that was related with citrus yellow spot and the
 472 *Citrivirus* clade, and the *Prunevirus* clade when the Rep and CP proteins were analyzed, respectively.

473 Therefore, there is incongruences in the phylogenetic relationships observed between Rep and CP
 474 proteins, which likely indicates a distinct evolutionary history of AvoV2 Rep and CP. Thus, based on its
 475 genomic organization and phylogenetic relationships, AvoV2 should be classified as a novel member of a
 476 new genus within the subfamily *Trivirinae* in the family *Betaflexiviridae*, which could be tentatively
 477 named as Avovirus.

478 It is tempting to speculate that the discrepancy regarding phylogenetic tree topology between the one
 479 inferred from the Rep sequences and the one inferred from the CP sequences found for CyNoV2 and
 480 Avo2 could be linked to ancestral recombination events, or that these genes have different mutation rates.
 481 Similar hypotheses were speculated for other betaflexiviruses where discrepancies on the REP and CP
 482 evolutionary relationships have also been observed [58, 60].

483 The DaYuV1 genome has three ORFs, with a genomic organization 5'-Rep-MP-CP-3', that is similar to
 484 the unclassified betaflexivirus AgVA, and reminiscent to the one reported for those trichoviruses with
 485 three ORFs, where the ORF that encodes the MP is slightly overlapped with the ORF that encodes the
 486 Rep [15]. However, in the DaYuV1 and AgVA genomes the ORF encoding the CP is separated from the
 487 ORF encoding the MP by a short intergenic region, while in the trichoviruses, these two ORFs are
 488 slightly overlapped [15, 61, 62]. DaYuV1 is distantly related with AgVA, and their replicase and CP nt
 489 sequence similarity is below the 72% threshold value to demarcate species in the family *Betaflexiviridae*
 490 [15]. DaYuV1 and AgVA Rep and CP proteins were phylogenetically related in a distinct clade from

other viruses belonging to genera within the subfamily *Trivirinae*. Thus, based on sequence similarities, genomic organization and phylogenetic relationships, DaYuV1 and AgVA should be classified as novel members of a new genus within the subfamily *Trivirinae* in the family *Betaflexiviridae*, which could be tentatively named as Daisagavirus.

The RhoDeV2 genome has three ORFs, with a genomic organization 5'-Rep-P2-P3-3', that is similar to the one reported for the carrot chordoviruses [63]. Its replicase is distantly related with the one encoded by other betaflexiviruses, and the nt sequence similarity is below the 45% threshold value to demarcate genus in the family *Betaflexiviridae* [15]. Neither hits were obtained nor conserved domains were identified when the proteins encoded by ORFs 2 and 3 were analyzed, but a bipartite NLS was detected in the sequence of the ORF 3 encoded protein. Thus, RhoDeV2 ORF2 and ORF3 encoded proteins have an unknown function and are not likely a MP and CP, which are the putative proteins encoded by carrot chordoviruses ORFs2 and 3 [63], which likely indicate that RhoDeV2 is unique among betaflexiviruses. The RhoDeV2 replicase formed a monophyletic cluster that was related with the *Chordovirus* and *Ravavirus* clades. Thus, sequence similarities, the evolutionary relationships with other betaflexiviruses and the unknown function of its putative P2 and P3 proteins suggest that RhoDeV2 is a unique novel betaflexivirus that should belong to a novel genus within the subfamily *Trivirinae* in the family *Betaflexiviridae*, which could be tentatively named as Rhodovirus.

Deltaflexiviridae

The ATV3 and SesV1 genomic organization 5'-Rep-P2-P3-P4-P5-3', resembles the one described for the deltaflexiviruses FgDFV1 [18]. Their replicase is distantly related with the one encoded by other deltaflexiviruses, and phylogenetic analysis showed that ATV3 and SesV1 grouped together with the deltaflexiviruses. Thus, based on sequence similarities, genomic organization and phylogenetic relationships, ATV3 and SesV1 should be classified as novel members of the genus *Deltaflexivirus* in the family *Deltaxiviridae*.

Recombination

Interestingly, despite that the recombination is a key process that strongly impact the evolution of many plant virus species [26] and has been mentioned as a driving force in the evolution of flexiviruses [64], no recombination event, using as threshold a p-value < 0.05 in at least three algorithms, was detected in the alphaflexiviruses, betaflexiviruses and deltaflexiviruses identified in this study.

Tymoviridae and tymo-like virus

DaInvV1 and DaInv2 genome organization resemble the one described for marafiviruses [65], and these viruses are phylogenetically related with other marafiviruses. The highest aa identity when their CP was compared with other marafiviruses was below 80%, which is the criteria to demarcate species in the *Tymoviridae* family [21]. Thus, based on sequence similarities, genomic organization and phylogenetic relationships, DaInvV1 and DaInv2 should be classified as novel members of the genus *Marafivirus* in the family *Tymoviridae*.

YePoV1 genomic organization 5'-Rep-CP-3', resembles the one reported for the tymovirids PYSaV [66] and GaTLV [67]. YePoV1 grouped together with these two viruses in the phylogenetic tree, and their CP shared an aa identity below 80%, which is the threshold value to demarcate species in the *Tymoviridae* family [21]. Thus, based on sequence similarities, genomic organization and phylogenetic relationships,

531 YePoV1 would be the third member of the recently proposed genus *Gratylovirus*, that belongs to an
 532 unassigned family within the order *Tymovirales* [66, 67].
 533 WaCraV1 has three ORFs and its genomic organization 5'-Rep-CP-P3-3', is similar to the one reported
 534 for the invertebrate-associated macula-like viruses BeeMLV2 [68] and Bombyx mori Macula like-virus
 535 (BmMLV) [69], which differs from the one reported for the plant-associated maculaviruses [70, 71].
 536 Interestingly, the P3 encoded by the WaCraV1 genome has a transmembrane domain, while no
 537 transmembrane domains were identified in the P3 encoded by BeeMLV2 and BmMLV genomes; thus the
 538 function of WaCraV1 P3 protein could be different than the BeeMLV2 and BmMLV P3s. WaCraV1
 539 grouped together with BeeMLV2 and BmMLV in the phylogenetic tree, but their CP shared an aa identity
 540 lower than 80%, which is the criteria to demarcate species in the *Tymoviridae* family [21]. Phylogenetic
 541 relationships suggest that WaCraV1 is likely an invertebrate-infecting virus derived from insect RNA that
 542 was eventually co-purified with the targeted plant RNA. The identification of invertebrate-infecting
 543 viruses from plant transcriptome datasets was already reported elsewhere suggesting that some undetected
 544 invertebrate was contaminating the plant samples [72]. Based on sequence similarities, the distinct
 545 genomic organization and phylogenetic relationships WaCraV1, BeeMLV2 and BmMLV should be
 546 classified as members of a novel genus within the *Tymoviridae* family, which could be tentatively named
 547 as *Inmaculavirus*.

548 The KaV1 and PolWhV1 genome has three ORFs, 5'-Rep-P2-P3-3,' where ORFs 1 and 2 are separated
 549 by a short intergenic region, while ORF3 is slightly overlapped with ORF2, which is likely a unique
 550 genomic organization among tymoviruses. KaV1 and PolWhV1 replicases are distantly related with other
 551 unclassified tymoviruses and mycotymoviruses, respectively, and the nt sequence similarity of its nearly
 552 complete genome is below the 80% threshold value to demarcate species in the family *Tymoviridae* [21].
 553 No hits were obtained when the proteins encoded by ORFs 2 and 3 of KaV1 and PolWhV1 were
 554 analyzed, but a transmembrane domain was identified in the KaV1 encoded P2. Thus, KaV1 and
 555 PolWhV1 P2 and P3 have an unknown function and is tempting to speculate that KaV1 P2 could have a
 556 membrane associated function. Despite that Kav1 and PolWhV1 share a distinctive genomic organization
 557 among tymoviruses, their evolutionary history is different because the KaV1 replicase virus formed a
 558 monophyletic cluster that was distantly related with other tymoviruses, while PolWhV1 replicase
 559 clustered together with mycotymoviruses. Thus, the evolutionary relationship of KaV1 with other known
 560 members of the family *Tymoviridae*, and the unknown function of its putative P2 and P3 proteins suggest
 561 that KaV1 likely represents a novel genus in the *Tymoviridae* family which could be tentatively as
 562 Kavavirus. On the other hand, the evolutionary history of PolWhV1 suggest that this virus should be
 563 classified as a novel member of the proposed genus *Mycotymovirus* [23], despite its genomic organization
 564 is different than the reported mycotymoviruses [23, 73].

565 **Fungal RNA present in plant transcriptome datasets**

566 It is tempting to speculate that fungal RNA was also present in the *Agave tequilana*, *Sesamum indicum*
 567 and *Triticum polonicum* transcriptomes given that the evolutionary analyses of ATV3, SesV1 and
 568 PolWhV1 suggest they could be derived from a fungus associated to these plant samples. Furthermore, an
 569 isolate of the fungi-associated deltaflexiviruses *Erysiphe necator* associated deltaflexivirus 2 and
 570 *Alternaria alternate* deltaflexivirus 1 were identified in the transcriptome of *Agrostis stolonifera*

(MW328744). Recently the putative gammaflexivirus pistacia-associated flexivirus 1 was identified analysing a publically available transcriptome dataset of pistacia [39]. Thus, the identification of mycoviruses from plant transcriptome dataset its plausible and thus tentative host assignment should consider the potential impact of eventual mixed sampling of transcriptome datasets..

Conclusion remarks

In summary, this study shows the complexity and diversity of the genome organization of viruses belonging to the order *Tymovirales* and reveal that analyzing SRA public data is a valuable tool not only to speed up the identification of novel viruses but also to increase our understanding of their evolution and taxonomical classification. Nevertheless, our inability to go back to the biological material which was employed to generate the transcriptome datasets analyzed, in order to check the viral genome sequences assembled and link the virus to a specific host, is the main limitation of using the data mining as a tool for virus discovery. This constraint may lead to a potential misidentification of the host species linked to the virus. Therefore a cautious attitude must be carried out before making strong statements related to viruses discovered from the analysis of transcriptome datasets publically available. This study includes the identification and characterization of a diverse set of tymovirids, presenting striking genome organization flexibility and interesting phylogenetic cues which expands the evolutionary history of the order incorporating 31 new tentative members of species and genera within the order, highlighting the importance of HTS in virus discovery and its potential in virus taxonomy.

589

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853 **Ethics declaration**

854 **Conflict of interest**

855 The authors declare that they have no conflict of interest.

856 **Human and animal rights statement**

857 This manuscript presents original work that does not involve studies with humans or animals.

Table 1. Summary of the tymovirids contigs identified from plant transcriptome data available in the NCBI database

Virus family/genus	Plant host; monocot/dicot	Virus name	Abbreviation	Bioproject ID	Data Citation	Lenght (nt)	Accession number	ORF (nt position)	Protein ID	Lenght (aa)	Highest scoring virus-protein/E-value/query coverage% (Blast P)
<i>Alphaflexiviridae</i> /unclassified	Neptune grass (<i>Cymodocea nodosa</i>); monocot	<i>Cymodocea nodosa</i> virus 1	CyNoV1	PRJNA275569	[74]	5508	MW128740	1 (107-4438) 2 (4480-5325)	Replicase MP	1443 281	DOSV-Rep/0.0/76 DOSV-MP/2e-22/73
<i>Alphaflexiviridae</i> / <i>Potexvirus</i>	Spanish dagger (<i>Yucca gloriosa</i>); monocot	<i>Yucca gloriosa</i> virus 1	YuGIV1	PRJNA374584	[75]	6341	MW328742	1 (109-4278) 2 (4317-4988) 3 (4975-5364) 4 (5198-5437) 5 (5440-6279)	Replicase TGB1 TGB2 TGB3 CP	1389 223 129 79 279	EuYMaV-Rep/0.0/98 LVX-TGB1/2e-9/99 YVX-TGB2/3e-8/73 MaMV-TGB3/7e-09/70 EuYMaV-CP/1e-50/90
<i>Alphaflexiviridae</i> / <i>Potexvirus</i>	Peltate yam (<i>Dioscorea zingiberensis</i>); monocot	Peltate yam virus 1	PeLYV1	PRJNA243000	[76]	6253	MW328724	1 (82-4232) 2 (4232-4948) 3 (4926-5255) 4 (5125-5367) 5 (5418-6119)	Replicase TGB1 TGB2 TGB3 CP	1382 238 109 80 233	YVX-Rep/0.0/95 YVX-TGB1/1e-8/94 VVX-TGB2/4e-6/94 YVX-TGB3/3e-09/4 YVX-CP/8e-48/90
<i>Alphaflexiviridae</i> / <i>Potexvirus</i>	Indian gooseberry (<i>Phyllanthus emblica</i>); dicot	Indian gooseberry virus 1	InGoV1	PRJNA431341	[77]	6269	MW328725	1 (66-4055) 2 (4165-4890) 3 (4865-5218) 4 (5151-5351) 5 (5357-6157)	Replicase TGB1 TGB2 TGB3 CP	1329 241 117 66 266	YVX-Rep/0.0/99% EuYMaV-TGB1/5e-41/99 EuYMaV-TGB2/7e-24/99 YVX-TGB3/1e-07/8 EuYMaV-CP/2e-60/80
<i>Alphaflexiviridae</i> / <i>Potexvirus</i>	Devil's dung (<i>Ferula assafoetida</i>); dicot	Ferula virus 1	FerV1	PRJNA476150	[78]	5891	MW328726	1 (129-4034) 2(4051-4749) 3(4727-5071) 4(4977-5198) 5(5146-5769)	Replicase TGB1 TGB2 TGB3 CP	1301 232 114 73 207	CnVX-Rep/0.0/99 SMYEV-TGB1/3e-32/100 YVX-TGB2/6e-21/100 ZVX-TGB3/1e-01/70 VVX-CP/2e-65/97
<i>Alphaflexiviridae</i> / <i>Potexvirus</i>	Blue agave (<i>Agave tequilana</i>); monocot	<i>Agave tequilana</i> virus 2	ATV2	PRJNA193469	[79]	6555	MW328740	1 (97-4440) 2(4474-5151) 3(5132-5521) 4(5328-5636) 5(5603-6433)	Replicase TGB1 TGB2 TGB3 CP	1447 225 129 102 276	VVX-Rep/0.0/98 LVX-TGB1/5e-35/96 YVX-TGB2/1e-18/72 NMV-TGB3/1e-06/63 EuYMaV-CP/5e-48/95
<i>Betaflexiviridae</i> /unclassified	Avocado (<i>Persea americana</i>); dicot	Avocado virus 2	AvoV2	PRJNA391003	[80]	8242	MW328736	1 (82-6183) 2 (6183-7490) 3 (7126-7794)	Replicase MP CP	2033 435 222	CLBV-Rep/99/0.0 CLBV-MP/4e-123/71 CRSaV2-CP/2e-47/99
<i>Betaflexiviridae</i> /	Neptune grass	<i>Cymodocea</i>	CyNoV2	PRJNA275569	[74]	6292*	MW328749	1 (7-4248)	Replicase	1415*	GarYSaV-Rep/0.0/79

unclassified	(<i>Cymodocea nodosa</i>); monocot	<i>nodosa</i> virus 2						2 (4273-4965) 3 (4940-5269) 4 (5205-5420) 5 (5455-6120)	TGB1 TGB2 TGB3 CP	230 109 71 221	APV2-TGB1/3e-43/97 PCMV-TGB2/2e-23/97 ASPV-TGB3/3e-09/54 GRSPaV/6e-52/83
<i>Betaflexiviridae</i> / unclassified	Dianchonglou (<i>Daiswa yunannensis</i>); monocot	<i>Daiswa yunannensis</i> virus 1	DaYuV1	PRJNA352766	[81]	6592	MW328734	1 (44-5038) 2 (4947-5909) 3 (5947-6558)	Replicase MP CP	1663 320 203	MaBV-Rep/0.0/95 MaTV1-MP/3e-94/83 GINV-CP/7e-24/74
<i>Betaflexiviridae</i> / unclassified	Red rhododendron (<i>Rhododendron delavayi</i>); dicot	<i>Rhododendron delavayi</i> virus 2	RhoDeV2	PRJNA358123	[82]	7763	MW328735	1 (129-5747) 2 (6115-6903) 3 (6623-7201)	Replicase P2 P3	1872 262 192	CMLV-Rep/0.0/81 no hits no hits
<i>Betaflexiviridae</i> / <i>Capillovirus</i>	Breadfruit (<i>Artocarpus artilis</i>); dicot	Breadfruit virus 1	BreFV1	PRJNA311339	[83]	6501	MW328738	1 (42-6359) 2 (4829-5785)	Replicase CP MP	1890 215 318	ASGV-polyprot/0.0/100 ASGV-MP/0.0/100
<i>Betaflexiviridae</i> / <i>Capillovirus</i>	Common hazel (<i>Corylus avellana</i>); dicot	<i>Corylus avellana</i> virus 1	CorAvV1	PRJNA316492	[84]	6430	MW328729	1 (21-6305) 2 (4712-5707)	Replicase CP MP	1926 168 331	ASGV-polyprot/0.0/100 ASGV-MP/6e-105/83
<i>Betaflexiviridae</i> / <i>Capillovirus</i>	German iris (<i>Iris germanica</i>); monocot	German iris virus 1	GerIV1	PRJNA638517	[85]	5286*	MW328757	1 (1-5178) 2 (3402-4523)	Replicase CP MP	1556* 169 373	ASGV-polyprot/0.0/94 YVA-MP/2e-112/87
<i>Betaflexiviridae</i> / <i>Capillovirus</i>	Roseroot (<i>Rhodiola rosea</i>); dicot	<i>Rhodiola rosea</i> virus 1	RhRoV1	PRJNA398393	[86]	6260	MW328730	1 (45-6194) 2 (4676-5920)	Replicase CP MP	1887 162 414	HoBFV1-polyprot/0.0/77 HoBFV1-MP/2e-77/94
<i>Betaflexiviridae</i> / <i>Capillovirus</i>	Red campion (<i>Silene dioica</i>); dicot	<i>Silene dioica</i> virus 1	SiDiV1	PRJNA356362	[87]	7035	MW328728	1 (62-6841) 2 (5242-6336)	Replicase CP MP	2068 191 364	ASGV-polyprot/0.0/77 ASGV-MP/4e-53/61
<i>Betaflexiviridae</i> / <i>Capillovirus</i>	Shrubby seablite (<i>Suaeda fruticosa</i>); dicot	<i>Suaeda fruticosa</i> virus 1	SuFruV1	PRJNA279892	[88]	6639	MW328748	1 (1-6639) 2 (5043-6299)	Replicase CP MP	2050 162 418	CRSaV3-polyprot/0.0/92 CRSaV3-MP/5e-123/88
<i>Betaflexiviridae</i> / <i>Carlavirus</i>	Sweetleaf (<i>Stevia rebaudiana</i>); dicot	Sweetleaf virus 1	SLeV1	PRJNA591974	[89]	8477	MW328722	1 (46-5919) 2 (5950-6645) 3 (6623-6949) 4 (6942-7127) 5 (7176-8066) 6 (8063-8401)	Replicase TGB1 TGB2 TGB3 CP NABP	1957 231 108 61 296 112	HeIVS-Rep/0.0/100 HeIVS-TGB1/4e-99/100 GLV-TGB2/1e-42/100 GLV-TGB3/4e-09/78 HeIVS/CP/3e-135/100 GLV-NABP/2e-42/94

<i>Betaflexiviridae/</i> <i>Carlavirus</i>	African marigold (<i>Tagetes erecta</i>); dicot	<i>Tagetes</i> <i>erecta</i> virus 2	TaEV2	PRJNA431782	[90]	8499	MW328723	1 (70-5952) 2 (5984-6679) 3 (6657-6986) 4 (6976-7161) 5 (7209-8096) 6 (8093-8498)	Replicase TGB1 TGB2 TGB3 CP NABP	1960 231 109 61 295 111	HelVS-Rep/0.0/99 HelVS-TGB1/6e-101/99 PhlVS-TGB2/3e-41/99 SLV-TGB3/7e-06/90 HelVS-CP/1e-136/100 GLV-NABP/1e-34/100
<i>Betaflexiviridae/</i> <i>Divavirus</i>	Dark vanilla orchid (<i>Gymnadenia</i> <i>rhellicani</i>); monocot	<i>Gymnadenia</i> <i>rhellicani</i> virus 1	GymRhV1	PRJNA504609	[91]	7490	MW328732	1 (104-6814) 2 (5170-6291) 3 (6834-7268)	Replicase CP MP NABP	2084 167 373 144	DiVB-polyprot/0.0/93 ObRV1-MP/9e-66/80 CMLV-P4/3e-04/46
<i>Betaflexiviridae/</i> <i>Divavirus</i>	Korean chestnut (<i>Castanea</i> <i>crenata</i>); dicot	Korean chestnut virus 1	KoChV1	PRJNA534190	Park, E.J., South Korea; unpublished	7348	MW328731	1 (45-7040) 2 (5285-6484)	Replicase CP MP	2160 171 399	DiVA-polyprot/0.0/99 ObRV1-MP/3e-47/77
<i>Betaflexiviridae/</i> <i>Divavirus</i>	Asian cow-wheat (<i>Melampyrum</i> <i>roseum</i>); dicot	<i>Melampyru</i> <i>m roseum</i> virus 2	MelRo V2	PRJDB5395	[92]	7094	MW328733	1 (50-6403) 2 (4723-5826) 3 (6426-6911)	Replicase CP MP NABP	1954 163 367 161	DiVB-polyprot/0.0/99 DiVA-MP/3e-63/84 CMLV-P4/6e-04/41
<i>Betaflexiviridae/</i> <i>Divavirus</i>	Capulincillo oak (<i>Quercus</i> <i>castanea</i>); dicot	<i>Quercus</i> <i>castanea</i> virus 1	QueCaV1	PRJNA532454	[93]	7761	MW328737	1 (30-7025) 2 (5282-6469)	Replicase CP MP	2150 181 395	DiVA-polyprot/0.0/100 DiVA-MP/1e-42/67
<i>Betaflexiviridae/</i> <i>Fivirus</i>	Avocado (<i>Persea</i> <i>americana</i>); dicot	Avocado virus 1	AvoV1	PRJNA391003	[80]	8002	MW328739	1 (115-5499) 2 (5560-6498) 3 (6281-6922) 4 (6997-7815) 5 (7640-7978)	Replicase MP CP P4 NABP	1794 312 213 272 112	GKSV-Rep/0.0/90 FLV1/2e-32/88 FLV1/4e-4/88 no hits GKSV-NABP/0.009/88
<i>Deltaflexiviridae/</i> <i>Deltaflexivirus</i>	Blue agave (<i>Agave</i> <i>tequilana</i>); monocot	<i>Agave</i> <i>tequilana</i> virus 3	ATV3	PRJNA193469	[79]	8565	MW328745	1 (13-6405) 2 (6732-7043) 3 (7126-7425) 4 (7427-7951) 5 (7945-8487)	Replicase P2 P3 P4 P5	2130 103 99 174 180	FgDFV1-Rep/0.0/97 no hits no hits no hits no hits
<i>Deltaflexiviridae/</i> <i>Deltaflexivirus</i>	Sesame (<i>Sesamum</i> <i>indicum</i>); dicot	Sesame virus 1	SesV1	PRNJA74261	[94]	8362	MW328747	1 (54-6350) 2 (6535-6732) 3 (6803-7099) 4 (7276-7788)	Replicase P2 P3 P4	2098 65 98 170	FgDFV1-Rep/0.0/99 no hits no hits FgDFV1-P4/2e-28/100

								5 (7772-8248)	P5	158	FgDFV1-P5/1e-07/97
<i>Tymoviridae/</i> <i>Marafivirus</i>	Dove-tree (<i>Davidia</i> <i>involucrata</i>); dicot	<i>Davidia</i> <i>involucrata</i> virus 1	DaInvV1	PRJNA513477	[95]	7109	MW328750	1 (164-6955)	Replicase CP	2073 190	MsMV1-polyprot/0.0/93
<i>Tymoviridae/</i> <i>Marafivirus</i>	Dove-tree (<i>Davidia</i> <i>involucrata</i>); dicot	<i>Davidia</i> <i>involucrata</i> virus 2	DaInvV2	PRJNA513477	[95]	6775	MW328752	1 (126-6632)	Replicase CP	1971 197	CSDaV-polyprot/0.0/97
<i>Tymoviridae/</i> <i>Maculavirus</i>	Watercress (<i>Nasturtium</i> <i>officinale</i>); dicot	Watercress associated virus 1	WaCraV1	PRJNA284126	[96]	6744	MW328754	1 (253-5637) 2 (5505-6317) 3 (6229-6648)	Replicase CP P3	1794 270 139	BmMLV-Rep/0.0/99 BmMLV-CP/5e-49/8 BmMLV-P3 /6e-13/6
<i>Tymoviridae/</i> unclassified	Kava (<i>Piper</i> <i>methysticum</i>), dicot	Kava virus 1	KaV1	PRJNA494686	[97]	9491	MW328755	1 (218-7048) 2 (7121-8215) 3 (8016-9395)	Replicase P2 P3	2276 364 459	UTyV1-Rep/3e-62/4 no hits no hits
<i>Tymoviridae/</i> unclassified	Polish wheat (<i>Triticum</i> <i>polonicum</i>); monocot	Polish wheat virus 1	PolWhV1	PRJNA304266	[98]	7702	MW328751	1 (99-6752) 2 (6756-7148) 3 (7093-7644)	Replicase P2 P3	2217 130 183	FgMTV1-Rep/0.0/75 no hits no hits
Unassigned <i>/Gratylivirus</i>	Yellow poplar (<i>Liriodendron</i> <i>tulipifera</i>); dicot	yellow poplar virus 1	YePoV1	PRJNA556244	[99]	6069	MW328760	1 (35-5443) 2 (5515-6057)	Replicase CP	1802 180	PYSaV-Rep/0.0/94 PRSaV-CP/2e-50/98

*partial sequence

Figure 1. Diagrams depicting the genomic organization of each (A) alphaflexivirus (B) betaflexivirus, (C) deltaflexivirus and (D) tymovirus sequence identified in this study. Abbreviations: MP, movement protein CDS; TGB1, triple gene block protein 1 CDS; TGB2, triple gene block protein 2 CDS; TGB3, triple gene block protein 3 CDS; P capsid protein CDS; NABP, nucleic acid binding protein CDS.

Figure 2. A Maximum Likelihood phylogenetic based on a multiple amino acid alignment of the replicase sequences of alphaflexiviruses, was constructed with the WAG+G+F model. Bootstrap values following 1000 replicates are given at the nodes, but only values above 40% are shown. Apple stem pitting virus, apricot latent virus, Helenium virus S and Chrysanthemum virus B replicase aa sequences were used as outgroup. The scale bar indicates the number of substitutions per site. Viruses identified in this study are noted with a green square. Accession numbers of every virus used to construct the ML tree are listed in Table S1.

Figure 3. A Maximum Likelihood phylogenetic based on a multiple amino acid alignment of the replicases sequences of viruses of the subfamily *Quinvirinae*, was constructed with the LG+G+F model. Bootstrap values following 1000 replicates are given at the nodes, but only values above 40% are shown. Apricot pseudo-chlorotic leaf spot virus, cherry mottle leaf virus and Grapevine Berry Inner necrosis virus replicases aa sequences were used as outgroup. The scale bar indicates the number of substitutions per site. Viruses identified in this study are noted with a green square. Viruses identified with an * correspond to unpublished sequences derived from the analysis of RNA-Seq data publically available at the NCBI. Accession numbers of every virus used to construct the ML tree are listed in Table S1.

Figure 4. A Maximum Likelihood phylogenetic based on a multiple amino acid alignment of the replicase sequences of viruses of the subfamily *Trinivirinae*, was constructed with the LG+G+F model. Bootstrap values following 1000 replicates are given at the nodes, but only values above 40% are shown. Cnidium virus X, lily virus X and vanilla virus X replicase aa sequences were used as outgroup. The scale bar indicates the number of substitutions per site. Viruses identified in this study are noted with a green square. Viruses identified with an * correspond to unpublished sequences derived from the analysis of RNA-Seq data publically available at the NCBI. Accession numbers of every virus used to construct the ML tree are listed in Table S1.

Figure 5. A Maximum Likelihood phylogenetic based on a multiple amino acid alignment of the replicase sequences of deltaflexiviruses, was constructed with the JTT+G model. Bootstrap values following 1000 replicates are given at the nodes, but only values above 40% are shown. The gammaflexiviruses Botrytis virus F, Entoleuca gammaflexivirus 1, Entoleuca gammaflexivirus 2 and pistacia-associated flexivirus 1 replicases aa sequences were used as outgroup. The scale bar indicates the number of substitutions per site. Viruses identified in this study are noted with a green square. Accession numbers of every virus used to construct the ML tree are listed in Table S1.

Figure 6. A Maximum Likelihood phylogenetic based on a multiple amino acid alignment of the replicase sequences of tymovirids, was constructed with the WAG+G+F model. Bootstrap values following 1000 replicates are given at the nodes, but only values above 40% are shown. . Apricot pseudo-chlorotic leaf spot virus, cherry mottle leaf virus and Grapevine Berry Inner necrosis virus replicases aa sequences were used as outgroup. The scale bar indicates the number of substitutions per site. Viruses identified in this study are noted with a green squares. Accession numbers of every virus used to construct the ML tree are listed in Table S1.