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# Viromes in Xylariaceae fungi infecting avocado in Spain

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ABSTRACT

Four isolates of *Entoleuca* sp., family Xylariaceae, Ascomycota, recovered from avocado rhizosphere in Spain were analyzed for mycoviruses presence. For that, the dsRNAs from the mycelia were extracted and subjected to metagenomics analysis that revealed the presence of eleven viruses putatively belonging to families *Partitiviridae*, *Hypoviridae*, *Megabirnaviridae*, and orders *Tymovirales* and *Bunyavirales*, in addition to one ourmia-like virus plus other two unclassified virus species. Moreover, a sequence with 98% nucleotide identity to plant endornavirus Phaseolus vulgaris alphaendornavirus 1 has been identified in the *Entoleuca* sp. isolates. Concerning the virome composition, the four isolates only differed in the presence of the bunyavirus and the ourmia-like virus, while all other viruses showed common patterns. Specific primers allowed the detection by RT-PCR of these viruses in a collection of *Entoleuca* sp. and *Rosellinia necatrix* isolates obtained from roots of avocado trees. Results indicate that intra- and interspecies horizontal virus transmission occur frequently in this pathosystem.

## 1. Introduction

In the last years, widespread analysis of plant-pathogenic fungi metatranscriptomes has revealed the presence of complex infections by multiple viruses belonging to different taxonomic groups including families *Alphaflexiviridae*, *Chrysoviridae*, *Endornaviridae*, *Gammaflexiviridae*, *Hypoviridae*, *Megabirnaviridae*, *Narnaviridae*, *Deltaflexiviridae*, *Partitiviridae*, "Ourmiaviridae", *Totiviridae*, *Virgaviridae* and order *Bunyavirales*, whilst many others mycoviruses do not belong to known virus families (Marzano and Domier, 2016; Marzano et al., 2016; Osaki et al., 2016; Deakin et al., 2017; Xin et al., 2017; Rott et al., 2018; Arjona-Lopez et al., 2018; Mu et al., 2018). Among the viruses identified, most of them have double-stranded (ds) RNA or positive single-stranded (ss) RNA genomes, being scarce those with negative ssRNA genomes. Mycoviruses are often associated with families containing plant-infecting and/or invertebrate-infecting members as evidenced by recent metagenomics analysis (reviewed in: Dolja and Koonin, 2018), that is also an extremely helpful tool in clarifying taxonomic relationships (Shi et al., 2018).

Mycovirus research aims to control fungal pathogens by identifying mycoviruses capable of weakening or limiting fungal damages (Ghabrial and Suzuki, 2009; Xie and Jiang, 2014; Ghabrial et al., 2015). On the other hand, there is growing evidence for horizontal virus transmission (HVT) among species from different taxa as a major driver of virus evolution (Dolja and Koonin, 2018). Direct confirmation of plant-fungi HVT was provided recently when a plant virus, cucumber mosaic virus, was detected in natural infection in the plant-pathogenic fungus *Rhizoctonia solani* and HVT could be experimentally demonstrated (Andika et al., 2017).

Avocado cultivation was introduced in the southern coast of Spain in the '70s as an alternative to traditional Mediterranean crops such as olive, almond or grapevine (López-Herrera and Zea-Bonilla, 2007). Since then, avocado white root rot, caused by *R. necatrix*, has become one of the most serious threats to the cultivation of this crop in the area (López-Herrera, 1998). Chemical treatments have been ineffective in controlling the disease, especially when the harmful effects on the environment and human health of their application are considered. Thus, alternative methods are being considered, such as grafting on resistant rootstocks or the use of biological control agents. Among those, and in parallel with similar approaches used in other trees, the identification of mycoviruses capable of limiting the growth of fungal pathogens appeared to us as a potential control strategy. Mycoviruses infecting *Rosellinia necatrix*, a fungal species that severely infects many crops have been extensively studied in different plant-fungus pathosystems, including pears, apple (reviewed in: Kondo et al., 2013) and, more recently, avocado (*Persea americana* Mill.) (Arjona-Lopez et al., 2018; Velasco et al., 2018).

With this goal in mind, we investigated the presence of mycoviruses in avirulent fungal isolates supposed to be *R. necatrix* by high-

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throughput sequencing (HTS) on dsRNA extracts of four different isolates. Then, based on molecular tools, these avirulent isolates were shown to be another fungus species morphologically indistinguishable from *R. necatrix*, namely *Entoleuca* sp., a closely related member of family Xylariaceae (Arjona-Girona and López-Herrera, 2018). Xylariaceae comprise one of the largest and most diverse families of filamentous Ascomycota that includes saprotrophs occurring in wood, litter, soil, and dung, and a few plant pathogens that cause canker diseases, root rots, and needle blight in agricultural and natural systems (Whalley, 1996). Despite a member of the genus *Entoleuca* (*E. mammata*) is pathogenic for forest trees (Ostry and Anderson, 2009), the *Entoleuca* sp. isolated from avocado has not shown any pathogenicity on this crop yet but seems to compete with *R. necatrix* for root colonization (Arjona-Girona and López-Herrera, 2018). Therefore, and because of the multiple virus infection observed in the four isolates of *Entoleuca* sp. as revealed by HTS analysis, we extended the determination of the identified viruses to a collection composed of 19 isolates of *Entoleuca* sp. and 18 of *R. necatrix*.

## 2. Materials and methods

### 2.1. Virus sources and culture media

Four isolates of *Entoleuca* sp. (E97-14, E112-4, E115-15 and E117-4) were originally obtained from the rhizosphere of different avocado escape trees located in orchards and the mycelia grown on PDA medium (Difco) was used later for subsequent analyses. These isolates belong to a collection of 37 fungal isolates from avocado trees that were used for mycovirus detection afterward. The escape trees were normally vegetating in diseased patches located in orchards in the Málaga province (Spain) (Arjona-Girona and López-Herrera, 2018) and in the same orchard, other threes showed symptoms of white root rot due to *R. necatrix* infection.

### 2.2. dsRNA extraction for sequence analyses

For each *Entoleuca* sp. isolate, 1 g of fungal mycelium was grown for 10 days on cellophane membrane-covered PDA Petri plates. Then, dsRNAs were extracted using CF-11 cellulose as described by Morris and Dodds (1979). Quality and yield were determined using the NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies, USA). DsRNA bands sizes were analyzed in 1% agarose gels after RedSafe™ staining under UV light. After extraction, dsRNAs were eluted in 50 µl RNase-free water and stored at −70 °C until further use.

### 2.3. Sequence determination from high-throughput sequencing (HTS)

For HTS, 5-10 µg of dsRNA were used, denatured at 95 °C for 10 min and immediately cooled on ice. Next, RNA fragmentation and cDNA synthesis were carried out. Libraries were prepared following procedures developed at the CRG Genomic Unit (Barcelona, Spain). Illumina sequencing was performed using the Hiseq2000 for 50 nt reads. Reads were used to generate contigs by *de novo* assembly using the algorithm Velvet v.12.08 (k-mer = 31) at the SCBI Picasso supercomputing server (Malaga, Spain). Contig data sets were subjected to BLASTN and BLASTX in the NCBI GenBank through the software Geneious 7.1.9 (Biomatters).

### 2.4. 5′- and 3′-ends determination of the mycoviral genomes

For completion of several mycoviral genomic sequences we analyzed the 5′- and 3′- ends using a modification of the protocol developed by Potgieter et al. (2009) as described elsewhere (Velasco et al., 2018). Briefly, the T4 RNA ligase (Ambion, USA) ligates the PC3-T7 loop primer to the 3′ end of each dsRNA strand allowing the cDNA synthesis by reverse transcription. Amplification of cDNA ends is performed with the PC2 primer, that is internal to the PC3-T7 loop primer, and template specific primers.

### 2.5. Phylogenetic analyses

The genomic sequences obtained from scaffolds were translated accordingly to amino acids in the correct frame and aligned to sets of sequences retrieved from the GenBank employing the MAFFT algorithm (Katoh & Standley, 2013) available in Geneious R11.1 (Biomatters, New Zealand). Maximum likelihood (ML) phylogenetic trees were inferred for the different amino acid sequences using the IQ-TREE web server (http://iqtree.cibiv.univie.ac.at/) that also calculated the most appropriate substitution model for each case (Trifinopoulos et al., 2016). Estimation of branch support was done using the aBayes test as implemented in IQ-TREE.

### 2.6. RT-PCR detection of mycoviruses in Entoleuca sp. and Rosellinia necatrix isolates

The detection of the different hypothetical mycoviruses revealed by HTS was done using RT-PCR after the availability of specific primers based on the sequences for the different viral genomes. For that, dsRNA was extracted from *Entoleuca* sp. and *R. necatrix* collection (IAS, Córdoba, Spain) using the viral dsRNA extraction kit (Intron, South Korea). The synthesis of cDNA by reverse transcription was performed with 200 ng of dsRNA with the High-Capacity cDNA reverse transcription kit (Applied Biosystems, USA). PCR reactions were carried out with the AmpliTools master mix (Biotools, Spain) using 50 pmol each of forward and reverse primers (Table S1) in the following conditions: an initial denaturing cycle of 2 min at 94 °C, then 40 cycles of 30 s at 95 °C, 30 s at 55-60 °C and 40 s at 72 °C, and a final extension step of 5 min at 72 °C. The amplicons were cloned and subjected to Sanger sequencing and compared with the HTS derived sequences.

### 2.7. Phenotypic characterization of Entoleuca sp. isolates

Three parameters of mycelia were evaluated: colony aspect, radial growth, and fresh weight. Phenoloxidase activity was tested by using Bavendamm's medium. Each treatment consisted of four replicates per isolate, and each experiment was repeated once. To compare fungal radial growth, the values for the Standardized Area Under the Growth Curve (AUGCs) was calculated and statistical tests were performed.

## 3. Results

A collection of 37 isolates of Xylariaceae fungi isolated from avocado roots were used for dsRNA extraction. The dsRNA profiles showed a range of sizes, indicative of the probable occurrence of virus(es) in the fungal tissues (some representative dsRNA profiles are displayed in Fig. 1). Four fungal isolates were selected for HTS on the basis their dsRNA profiles, lack on virulence to avocado and differential characteristics regarding both hyphae phenotype, phenoloxidase activity, and colony morphology (see supplementary materials, Tables S7–S8 and Figs. S2–S5). Also, incompatibility barriers (IB) were observed for all isolate combinations (Fig. S6), indicating that the four isolates considered here belong to different vegetative compatibility groups (VCG).

### 3.1. Taxonomic adscription of the mycoviral sequences

Genomic sequences (complete or partial) were obtained by alignment of contigs from the different viruses, completion with Sanger sequencing of gaps or undetermined regions and, when necessary, determination of RNA ends. Phylogenetic analysis allowed their adscription to different virus families and genera. Eleven mycoviruses putatively belonging to different taxonomic groups resulted from the analysis (Table 1).

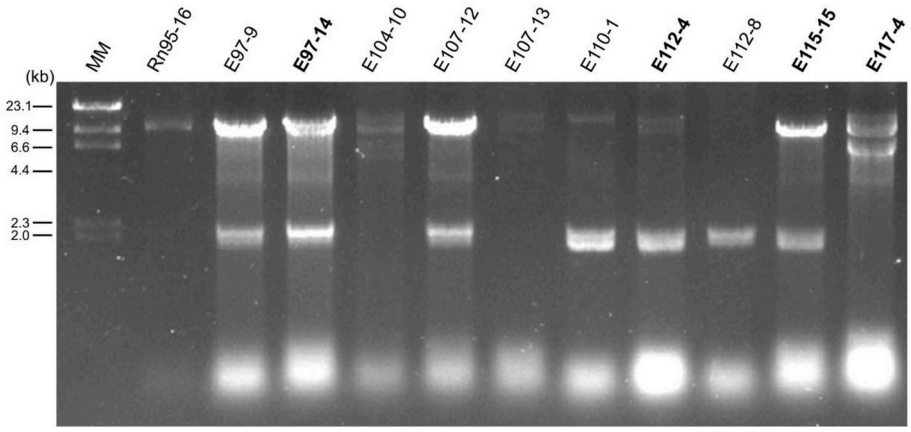


**Fig. 1.** Diversity in dsRNA profiles extracted from Xylariaceae fungi isolated from avocado roots. MM: λ-*Hin*dIII DNA marker, Rn95-16 corresponds to *R. necatrix,* while the rest of the dsRNAs belong to *Entoleuca* sp. isolates. Samples selected for HTS analysis are indicated in bold.

**Table 1**
Virome composition in the *Entoleuca* sp. isolates deduced from the number of reads obtained from dsRNA aligning with the genomic regions for the different virus species identified.

| | | | *Entoleuca* sp. isolate | | | |
|---|---|---|---|---|---|---|
| Virus taxonomy | Species acronym* | Genomic segment | 97-14 | 112-4 | 115-15 | 117-4 |
| *Gammaflexiviridae* | EnGFV1 | RdRp | 185,784 | 40ǂ | 3,915 | 16ǂ |
| | EnGFV2 | RdRp | 74,350 | 23ǂ | 1,751 | 16ǂ |
| *Endornaviridae* | PvEV1 | RdRp | 13,219 | 3,717 | 14,075 | 3,048 |
| *Partitiviridae* | EnPV1 | RdRp | 3,351 | 3,873 | 764 | 1,267 |
| | EnPV2 | RdRp | 215ǂ | 268ǂ | 52 ǂ | 71ǂ |
| *Hypoviridae* | EnHV1 | RdRp | 201,521 | 272,696 | 241,467 | 988,555 |
| *Megabirnaviridae* | EnMBV1 | RdRp | 145,724 | 216ǂ | 4,255 | 700,821 |
| "Fusagraviridae" | RnFSV1 | RdRp | 12,304,829 | 10,533 | 12,197,523 | 5,613 |
| *Bunyavirales* | EnPLV1 | RdRp | 0 | 0 | 5,589 | 0 |
| | EnPLV1 | CP/MP | 0 | 0 | 12,767 | 0 |
| "Ourmiaviridae" | EnOLV1 | RdRp | 0 | 2,224 | 0 | 1,441 |
| "Yadokariviridae" | YkV2 | RdRp | 136,635 | 156,788 | 151,046 | 3,024,844 |
| Mycoviral reads | | | 13,593,056 | 4,009,386 | 14,771,674 | 4,727,082 |
| Total reads | | | 25,405,202 | 28,056,398 | 26,562,997 | 28,337,624 |
| % Mycoviral dsRNA reads | | | 53.51% | 14.29% | 55.61% | 16.68% |

ǂ Confirmed by RT-PCR: most of the reads matched to core RdRp.
* Acronym definition in the text.

### *3.2. Two viral sequences related to positive single-strand RNA viruses, order Tymovirales*

An RNA virus resembling positive ssRNA viruses was first determined in *Entoleuca* isolate E97-14 and then detected in other fungal isolates (Tables 1 and 2). This new species was named Entoleuca gammaflexivirus 1 (EnGFV1). The complete genomic sequence was 9,221 nucleotides (nt) length, presenting three ORFs and no tRNA-like structure at both 5'and 3′ ends (Fig. 2). ORF1 consists of 5,790 nt starting at position 278 from the 5′ end. It encodes for a putative protein of 1,929 amino acids (aa) with a molecular mass of 213.38 kDa. NCBI's CDD search identified three putative domains: methyltransferase (pfam01660, E-value = 1.10e-37), helicase (pfam01443, E-value = 7.78e-14) and RNA dependent RNA polymerase (pfam00978, E-value = 2.37e-11). Compared to other members of order *Tymovirales*, the EnGFV1 replicase showed the highest similarity to Botrytis virus F (BVF), the only known member of the family *Gammaflexiviridae* in *Tymovirales* (Howitt et al., 2001), being the values of identity and similarity 18.8% and 33.6%, respectively. However, when the specific domains in the replicase (Met, Hel, RdRp) are considered, the homology scores varied greatly (Table S2). Phylogenetic analysis using the aa sequence of the core RdRp clustered this virus species in the family *Gammaflexiviridae* (Fig. 3A) Next, the ORF2 extends from positions 6,074 to 8,086. It encodes for a protein of 670 aa and a molecular mass of 17.41 kDa and presents a domain homologous to DEAD-like helicases (smart00487, E-value = 3.47e-11). This protein appears to be related to the putative movement proteins encoded by two recently described mycoviruses provisionally ascribed to family *Virgaviridae*, Macrophomina phaseolina tobamo-like virus 1 (MpTLV1; Marzano et al., 2016) and Podosphaera prunicola tobamo-like virus 1 (PPrTLV1; Pandey et al., 2018) (Fig. 3B). The ORF3 of EnGFV1 consists of 984 nts, and encodes for a putative coat protein of 327 aa and a molecular mass of 35.54 kDa. The aa sequence of the ORF3 showed the highest similarities with MpTLV1 and PPrTLV1 (41.0% and 40.5%, respectively) (Table S4) and in the phylogenetic tree resulted grouped as the ORF2 with these two same viruses (Fig. 3C).

Another mycovirus, originally identified in E97-14 and apparently belonging to a new species, was named Entoleuca gammaflexivirus 2 (EnGFV2). The complete genome consists of 7,321 nt and three possible ORFs were predicted (Fig. 2). The ORF1, extending from position 121 to 5,820, encodes for a putative replicase of 1,899 aa and molecular mass of 209.85 kDa. Three domains were identified in this replicase: methyltransferase (pfam01660, E-value = 1.12e-44), helicase (pfam01443, E-value = -6.02e-21) and RNA dependent RNA polymerase (pfam00978, E-value = 7.76e-15). Phylogenetic analysis of the aa sequence of the core

**Table 2**
Detection by RT-PCR of the different mycoviruses in isolates of *Entoleuca* sp. and *R. necatrix* recovered from avocado trees.

| Host | Isolate | EnPLV1 | EnGFV1 | EnGFV2 | EnHV1 | EnMBV1 | EnPLV1 | EnPV2 | RnFSV1 | YkV2 | EnOLV1 |
|---|---|---|---|---|---|---|---|---|---|---|---|
| *Entoleuca sp.* | 97–09 | | | | + | + | + | + | + | + | |
| *Entoleuca sp.* | 97–14 | | + | + | + | + | + | + | + | + | |
| *Entoleuca sp.* | 102–4 | | | | | + | + | + | + | + | + |
| *Entoleuca sp.* | 104–10 | | | | + | + | + | + | + | + | |
| *Entoleuca sp.* | 106–14 | | | | + | + | + | + | + | + | |
| *Entoleuca sp.* | 106–4 | | nt | nt | | | + | + | nt | + | |
| *Entoleuca sp.* | 107–12 | | + | + | + | + | + | + | + | + | |
| *Entoleuca sp.* | 107–13 | | | | + | + | + | + | + | + | |
| *Entoleuca sp.* | 108–8 | | | | + | + | + | + | + | + | |
| *Entoleuca sp.* | 110–1 | | | | + | + | + | + | + | + | |
| *Entoleuca sp.* | 110-15R | | | | | | + | + | + | + | |
| *Entoleuca sp.* | 112–4 | | + | + | + | + | + | + | + | + | + |
| *Entoleuca sp.* | 112–8 | | + | | + | + | + | + | + | + | |
| *Entoleuca sp.* | 114-15CH | | | | + | + | + | + | + | + | |
| *Entoleuca sp.* | 115–14 | | | | + | | | | + | + | |
| *Entoleuca sp.* | 115–15 | + | + | + | + | + | + | + | + | + | |
| *Entoleuca sp.* | 117–4 | | + | + | + | + | + | + | + | + | + |
| *Entoleuca sp.* | 117–7 | | | | | | + | + | + | + | |
| *Entoleuca sp.* | 118–7 | | | | | | + | + | | + | |
| *R. necatrix* | 95–12 | | | | | + | + | + | + | + | |
| *R. necatrix* | 95–16 | | | | | + | + | + | + | + | |
| *R. necatrix* | 97–11 | | nt | nt | | | + | + | nt | + | |
| *R. necatrix* | 103–7 | | | | + | + | + | + | + | + | |
| *R. necatrix* | 106–11 | | | | + | | + | + | | + | |
| *R. necatrix* | 108–10 | | | | | | + | + | + | | |
| *R. necatrix* | 108–15 | | | | + | + | ? | + | + | + | |
| *R. necatrix* | 110-15N | | | | + | | + | + | + | + | |
| *R. necatrix* | 114-15R | | | | | + | + | + | + | + | |
| *R. necatrix* | 114–16 | | | | + | | + | + | + | + | |
| *R. necatrix* | 114–4 | | | | | | + | + | + | + | |
| *R. necatrix* | 114–5 | | | | + | + | + | + | + | + | |
| *R. necatrix* | 118–8 | | | | + | | + | + | | + | |

nt: not tested.

RdRp allocated EnGFV2 in the *Gammaflexiviridae* (Fig. 3A; Table S3). On the contrary to BVF, the type species of family *Gammaflexiviridae*, the replicase-encoding ORFs of EnGFV1 and EnGFV2 do not present a leaky stop codon between the RdRp core and the helicase domains (Howitt et al., 2001). Next, the ORF2 extends from position 5,872 to 6,447. It encodes for a protein of 191 aa and a predicted molecular mass of 21.25 kDa. No

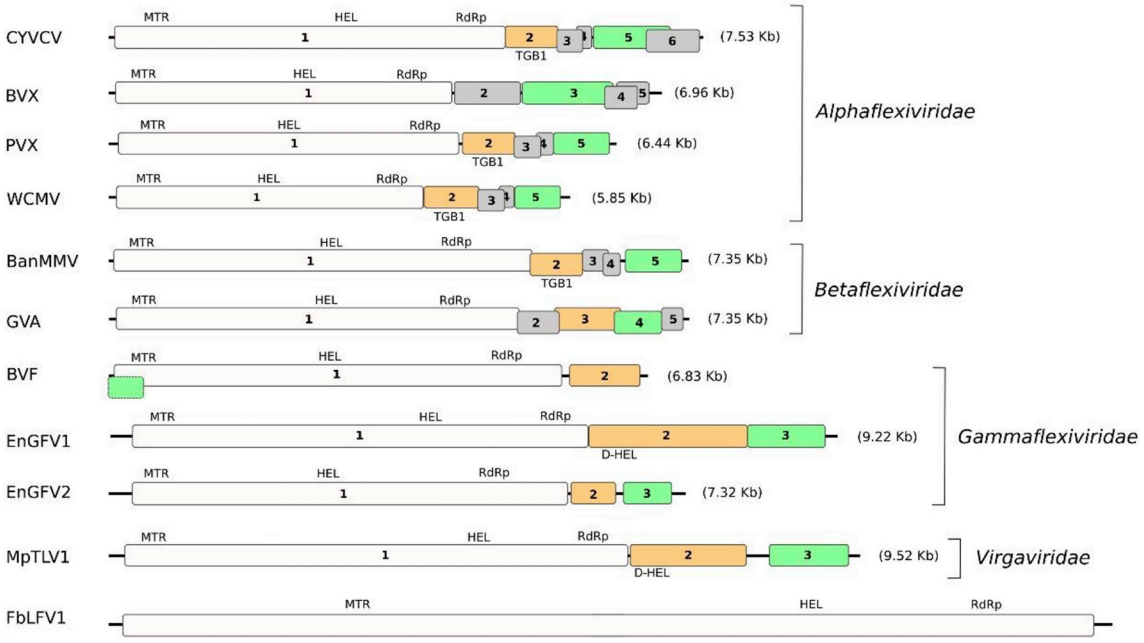


**Fig. 2.** Comparison of the genome organization and size in selected members of order *Tymovirales* with Entoleuca gammaflexivirus 1 (EnGFV1) and Entoleuca gammaflexivirus 2 (EnGFV2). CYVCV: citrus yellow vein clearing virus (KP313240), PVX: potato virus X (D00344), WCMV: white clover mosaic virus (X06728), BanMMV: banana mild mosaic virus (AF314662), GVA: grapevine virus A (X75433), BVF: Botrytis virus F (AF238884), FbLFV1: Fusarium boothii large flexivirus 1 (LC425115), MpTLV1: Macrophomina phaseolina tobamo-like virus 1 (KF537660). Color interpretation of protein homologies: blank: RdRp, orange: movement protein; green: coat protein, grey: unknown or other functions.

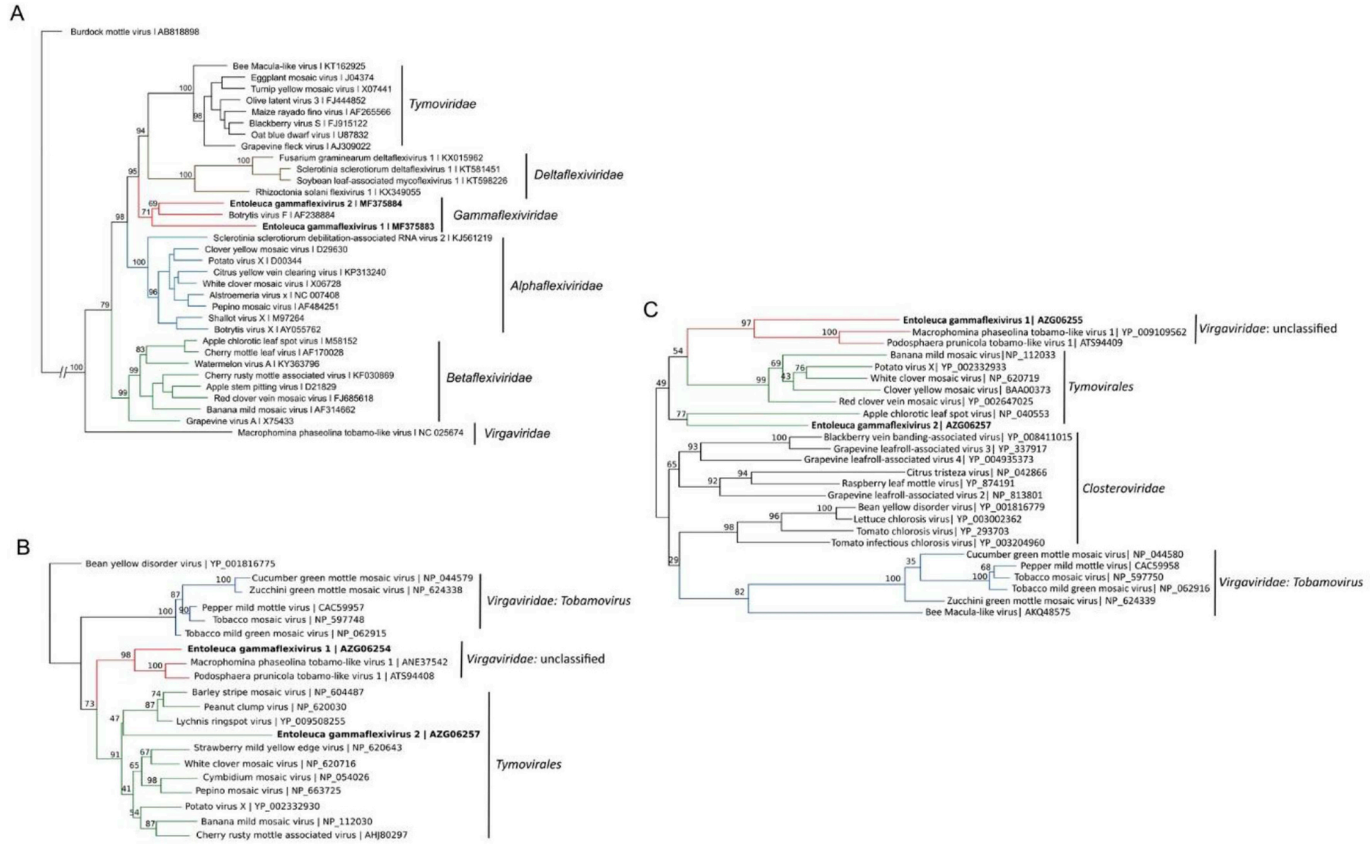


**Fig. 3.** ML phylogenetic tree of the aa sequences of the RdRp domain in the replicase (**A**), movement protein (**B**) and coat protein (**C**) of representative members of order *Tymovirales*, family *Virgaviridae* and other clades as generated with IQ-TREE. The best-fit model of substitution according to BIC for the replicases was the VT + F + R5. EnGFV1 and EnGFV2 resulted allocated in the same clade as BFV, the only known member of the family *Gammaflexiviridae* that consists of a single ORF. The best-fit model of substitution for MPs, according to BIC scores was the Blosum62 + F + I + G4. In this case, EnGFV1 MP protein resulted grouped with other tobamo-like mycoviruses ascribed to the *Virgaviridae* and close to MPs of tobamoviruses. In the case of EnGFV2, the CP clustered with members of order *Tymovirales*. For both the MP and the CPs, we introduced in the analysis members of family *Closteroviridae* as reference. In this last case, the best-fit model of substitution was the VT + G4. EnGFV1 CP grouped with mycoviruses ascribed to the *Virgaviridae*, while EnGFV2 CP resulted allocated with *Tymovirales*. Bootstrap support values (SH-aLRT) written on the branches.

domains or significant similarities with known proteins could be detected. Phylogenetic analysis clustered the putative protein encoded by ORF2 with *Tymovirales*, in the same branch of the betaflexivirus apple chlorotic leaf spot virus (ACLSV) (Fig. 3B) but showing the highest similarities with the alphaflexivirus white clover mosaic virus (WCMV) (15.6% and 37.2%, identity and similarity, respectively) (Table S4). Finally, ORF3 is comprised between positions 6,548 and 7,159 and encodes for a protein of 203 aa with a predicted molecular mass of 22.08 kDa. NCBI's CDD search allowed the detection of a domain with similarity to the superfamily of closterovirus' coat proteins (pfam01785, E-value = 4.09e-08). However, phylogenetic analysis grouped this protein with members of *Tymovirales*, in the same clade of the betaflexivirus apple chlorotic leaf spot virus (ACLSV) (Fig. 3C). The highest similarities were observed when compared with the betaflexivirus banana mild mosaic virus (BaMMV) and the alphaflexivirus potato virus X (PVX) (Table S4).

### 3.3. Two novel partitiviruses

The two partitiviruses, named EnPV1 and EnPV2, are shared by the four *Entoleuca* sp. isolates analyzed by HTS and resulted very common in the *Entoleuca* and *R. necatrix* isolate collection (Table 2). They belong to genus *Alpha-* and *Betapartitivirus*, respectively. Deduced aa sequences from the RNA1 segments of both viruses showed an RT-like superfamily domain (Acc. cl02808) (E-values = $2.62e^{-8}$ and $8.41e^{-6}$, for EnPV1 and EnPV2, respectively). These two partitiviruses are described in detail in a separate article and shall not be discussed here (Velasco et al., in preparation).

### 3.4. Entoleuca megabirnavirus 1

A sequence related to dsRNA1 segment of megabirnaviruses was identified by HTS in all four *Entoleuca* sp. isolates and named Entoleuca megabirnavirus 1 (EnMBV1). RT-PCR using consensus primers (Table S1) showed that this virus was common in the Xylariaceae fungi in our work collection (Table 2). The complete genome sequence of EnMBV1 consists of 8,927 nt and present two ORFs, putatively encoding the CP and the RdRp proteins. The sequenced strain from isolate E97-14 showed 87.1% of nucleotide identity compared with *Rosellinia necatrix* megabirnavirus 3 (RnMBV3), previously described in *R. necatrix* isolate Rn454 of the same fungal collection (Arjona-Lopez et al., 2018). The coat protein sequence is 78.8% identical among these two viruses while for the RdRp the nucleotide identity is 81.2%. Coat protein derived amplicons of 529 bp were sequenced for ten EnMBV1 strains isolated from *Entoleuca* sp. and *R. necatrix* and compared with the corresponding sequence of RnMBV3. The nucleotide distances ranged between 77.2% and 99.8% (Table S5). Interestingly, the partial sequence of the CP region of EnMBV1 Rn114-15R strain from *R. necatrix* and EnMBV1 E94-14 from *Entoleuca* sp. are 100% identical (Table S5). Hence, RnMBV3 and EnMBV1 are strains of the same virus species. No contigs with homology to the dsRNA2 segment of megabirnaviruses could be identified after BLASTX and BLASTN searches.

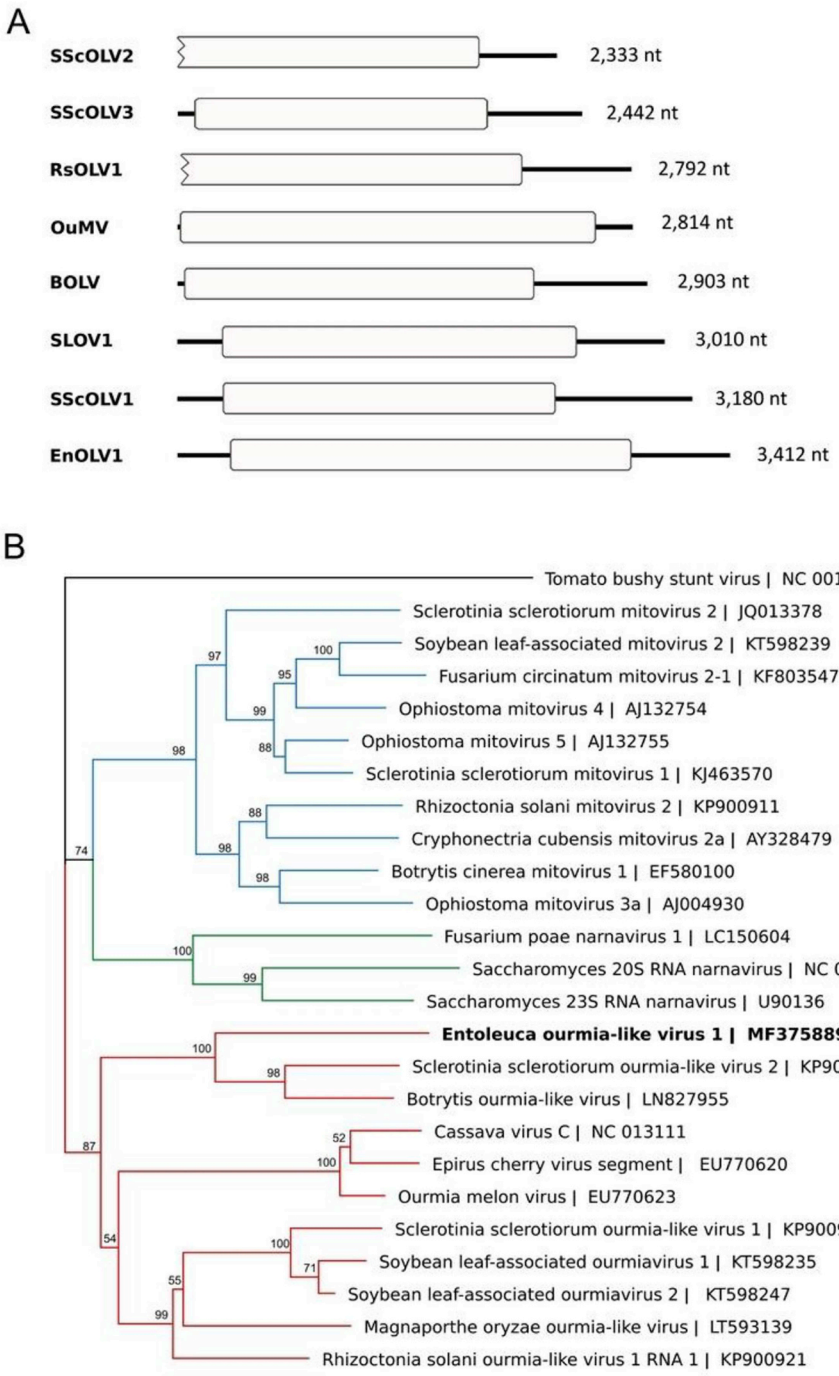


**Fig. 4. A**. Comparisons of the genome sizes and organization for Botrytis ourmia-like virus (BOLV), Entoleuca ourmia-like virus 1 (EnOLV1), Ourmia melon virus (OuMV), rhizoctonia solani ourmia-like virus 1 RNA 1 (RsOLV1), Sclerotinia sclerotiorum ourmia-like virus 1 (SScOLV1), Sclerotinia sclerotiorum ourmia-like virus 2 (SScOLV2), Sclerotinia sclerotiorum ourmia-like virus 3 (SScOLV3), and soybean leaf-associated ourmiavirus 1 (SLOV1). **B**. ML phylogenetic tree of the amino acid sequences of RNA-dependent RNA polymerase (RdRp) for selected members of family *Narnaviridae* (genera *Narnavirus* and *Mitovirus*), and genus *Ourmiavirus* as generated with IQ-TREE. The best-fit model of substitution according to BIC was the Blosum62 + F + R4. EnOLV1 was grouped with other ourmiaviruses. Tomato bushy stunt virus, a *Tombusvirus* was used as outgroup. Bootstrap support values written on the branches.

### 3.5. *Entoleuca hypovirus 1*

A sequence resembling members of family *Hypoviridae* was identified as shared by the four *Entoleuca* sp. isolates and named Entoleuca hypovirus 1 (EnHV1). It has been described previously (Velasco et al., 2018) and is no longer considered here.

### 3.6. *A sequence related to ourmia-like viruses*

A viral sequence with similarity to ourmia-like viruses was identified in *Entoleuca* sp. isolates E112-4 and E117-4. This virus was named Entoleuca ourmia-like virus 1 (EnOLV1) and its genome has 3,412 nt, longer than any other fungal ourmia-like virus described so far (Fig. 4A). In this work, we characterized the EnOLV1 strain from E112-4. A single ORF of 2,556 nts was identified starting at position 323 from the 5′ end. It encodes for a putative protein of 851 aa and a predicted molecular mass of 97.5 kDa that showed no significant domains in NCBI's CDD. It probably encodes for the replicase. After the ORF a long 3′UTR of 534 nt was present. The highest similarity of the putative protein of EnOLV1 ORF was observed with the aa sequence of Botrytis ourmia-like virus (BOLV; Donaire et al., 2016) (23.5% of identity, 40.2% of similarity) followed by Sclerotinia sclerotiorum ourmia-like virus 2 (SScOLV2; Marzano et al., 2016) (21.4%, 34.5%) and Sclerotinia sclerotiorum ourmia-like virus 3 (SScOLV3; Mu et al., 2018) (20.4%; 33.8%). It is remarkable that the protein encoded by the ORF in EnOLV1 is 12% larger to that from BOLV (722 aa) but comparable to the one of OuMV (860 aa) a plant virus more distant phylogenetically. Given that in the sequence available for SScOLV2 the 5′ end is lacking

we cannot compare with EnOLV1. On the other hand, the ORF of the SScOLV3 replicase encodes for a protein of only 645 aa. A highly conserved region among ourmiaviruses was present in the middle part of the protein (Fig. S1). EnOLV1 aa sequence features conserved residues in the RdRp shared by positive ssRNA viruses (Fig. S1). A phylogenetic tree grouped EnOLV1 in the same clade (87% bootstrap support) of BOLV and SScOLV2 and separated from other ourmiaviruses (Fig. 4B). The 5′ end of EnOLV1 RNA presents neither a secondary structure nor the 5′—CCC terminal sequence, both typical in other ourmiaviruses, so the genomic sequence obtained for EnOLV1 may be incomplete.

### 3.7. A virus related to negative single-stranded RNA viruses, order Bunyavirales

In *Entoleuca* sp. isolate E115-5 two viral sequences were identified and attributed to a novel mycovirus species, namely Entoleuca phenuilike virus 1 (EnPLV1). Its genome consists of two RNA sequences. RNA1 has a size of 7,256 nt and presents one single ORF of 7,062 nt that starts at position 96 from the 5'end. NCBI's CDD search identified a domain from nt position 2,066 (aa position 652) to position 4,106 (aa 1,333) with homology to Bunya-RNA dependent RNA polymerases superfamily (pfam04196, E-value = 2.6e-32). The alignment with other viral sequences showed the six conserved motifs (premotif A and motifs A–E) typical of bunyaviruses (Fig. 5A): motif A (DATKWC), motif B (QGILHDASC), motif C (VQGSDDSA; including the SDD amino acids), motif D (GIWCSEAKSS), and finally motif E (EYNSEWYMNG) that includes the tetrapeptide E(F/Y)xS, which is specific in polymerases of segmented negative-sense RNA viruses (Kormelink et al., 2011). Besides, we identified three basic residues (K, R, and R/K) in premotif A and a glutamic acid (E) residue downstream of premotif A (Fig. 5A), which are commonly conserved in RdRps of bunyaviruses (Elbeaino et al., 2009). A phylogenetic analysis of the aa sequences of RdRp allocated EnPLV1 to the group IV of negative ssRNA viruses, order *Bunyavirales* within the family *Phenuiviridae* (Fig. 5B), that includes members consisting of either two or three genomic segments (L, M, S) (Fig. 6A).

EnPLV1 RNA2 is related to the M and S genomic segments of bunyaviruses and consists of 2,816 nt, presenting two ORFs in opposite directions (Fig. 6A). ORF1, from nt 120-1,178 encodes a putative protein of 352 aa and a predicted molecular mass of 38.6 kDa, with similarity to nucleocapsid proteins of bunyaviruses. A domain typical of tenuivirus-phlebovirus nucleocapsid proteins (pfam05733, E-value: 1.13e-07) was predicted between the aa positions 92-240. ORF2 is homologous to MPs. It ends 285 nt downstream ORF1 and consists of 1,221 nt, encoding for a putative protein of 406 aa and a molecular mass of 45.27 kDa. The MP protein of EnPLV1 (403 aa) is comparable in size to the corresponding of watermelon crinkle leaf-associated virus 2 (WCLaV2). The highest and lowest similarities were observed in comparison to WCLaV2 and Rift Valley fever virus (RVFV), respectively (Table S6). The intergenic region (IR) in ambisense RNA segments are highly rich in A- and U- stretches and predicted to fold into a stable hairpin structure (Kiening et al., 2017). In the case of the RNA2 of EnPLV1, the IR is rich in A-U, and shows strong secondary hairpin structure (Fig. 6B). Interestingly, the IR of RVFV, which is proximal phylogenetically to EnPLV1, has no A-U predominance; on the contrary, it has a high C-content (56%) and is considerably shorter (82 nt) than that of EnPLV1. The terminal sequences of the RNA1 and RNA2 of EnPLV1 at the 5′ and 3’ ends resemble those of phleboviruses and tenuiviruses (Fig. 6C). Like other segmented negative-sense RNA viruses (Pettersson and Bonsdorff, 1975), the inverted repeats of their terminal sequences conform a panhandle structure (Fig. 6C).

### 3.8. Other positive ssRNA and dsRNA viruses

A mycoviral sequence was identified in the *Entoleuca* isolates resembling the recently described Rosellinia necatrix fusagravirus 1 (RnFSV1; LC333734). RnFSV1 was identified in *R. necatrix* Rn459 isolated in the same avocado orchards of this study (Arjona-Lopez et al., 2018). The mycoviral sequence identified in *Entoleuca* sp. presented a genome of 9,327 nt in length and includes two putative ORFs. ORF1 encodes for a protein of 1,422 aa and does not present similarity with known sequences or predicted domains. ORF2 encodes for a 1,311 aa protein and includes a RT-like domain (cl02808; E-value = $4.04e^{-11}$). The genomic sequence of this mycovirus showed high nucleotide identity (89.0%) with RnFSV1. Considering that this identity is 92% along the coding regions (not shown), this mycovirus can be considered as a strain of RnFSV1, ascribed to the newly proposed family “Fusagraviridae” (Wang et al., 2016).

Another mycovirus sequence could be identified in *Entoleuca* consisting of 5,860 nt in length. The only predicted ORF presented a RT-like domain (cl02808; E-value = 1.00e-19). This ORF showed similarities with the so-called Yado-kari viruses (YkV1, -2, -3 and -4), recently identified in *R. necatrix* isolates from the same pathosystem (Arjona-Lopez et al., 2018). The predicted aa sequence of this ORF showed 84.8% and 39.8% of identity compared to YkV2 and YkV4, respectively. In the case of the 5′UTR the nucleotide identity between this mycovirus and YkV2 was 85.6% and in the case of the 3′UTR it was 91.3%. Thus, the new mycovirus detected originally in *Entoleuca* probably represents a different strain of YkV2. The published sequences for the three Yado-kari viruses showed a C/U indetermination at the 5′ terminal nucleotide of the RNA sequences that could not be defined either in the genomic sequence of this mycovirus after specific sequencing.

### 3.9. Phaseolus vulgaris alphaendornavirus 1, strain E97-14

A sequence showing 98.1% of nucleotide identity (98.6% identity for the deduced aa sequence of the polyprotein) to *Phaseolus vulgaris alphaendornavirus 1* (PvEV1; AB719397; Okada et al., 2013) was identified in the four *Entoleuca* sp. isolates (Table 1). Therefore, this endornavirus represents a new strain of PvEV1. The nucleotide sequence of PvEV1 E97-14 was identical in the four *Entoleuca* sp. isolates. RT-PCR with specific primers (Table S1) allowed the detection of this virus from dsRNA extracts of *Entoleuca* sp. isolates but not in *R. necatrix*. Moreover, PCR with the same primer set using extracts of *Entoleuca* sp. E97-14 total DNA showed that the virus was not integrated into the fungal genome (not shown). Up to our knowledge, this endornavirus species has not been detected previously in any other fungal host.

### 3.10. Multiple virus infections in Entoleuca sp. isolates

An in-depth analysis of the contigs and scaffolds obtained from HTS revealed that only single virus strains of the families *Hypoviridae*, *Megabirna-*, *Partiti-*, “Ourmia-” and order *Bunyavirales* were present in the *Entoleuca* sp. isolates (not shown). For each sample, the total number of Illumina reads matching to the mycoviral genomes detected in this work is shown in Table 1. Differential virome composition among the four isolates arises from these results (Table 1). There are nine virus species shared by the four *Entoleuca* sp. isolates and two other viruses that appear differentially. Specifically, the ourmia-like virus is present in isolates E112-4 and E117-4. In contrast, the bunya-virus-like virus is present only in *Entoleuc*a E115-15, whiles both viruses are absent in E97-14. Also, there are huge differences in the number of reads, ranging from 83.1% ($5.95 \times 10^6$ reads) in the case of the RnFSV1 from isolate 97-14 to below 0.01% (16 reads) for RnFSV1 in isolate E117-4, regarding the total of mycovirus-related reads. RT-PCR confirmed the presence of viruses or viral genomic segments displaying a low number of reads in some fungal isolates (e.g. EnGFV1, EnGFV2, EnMBV1, EnPV1 or EnPV2) from dsRNA and total RNA extracts (not shown). The presence of circular DNA viruses was discarded after the RCA analysis of DNA extractions from the fungal isolates tested negative (not shown).

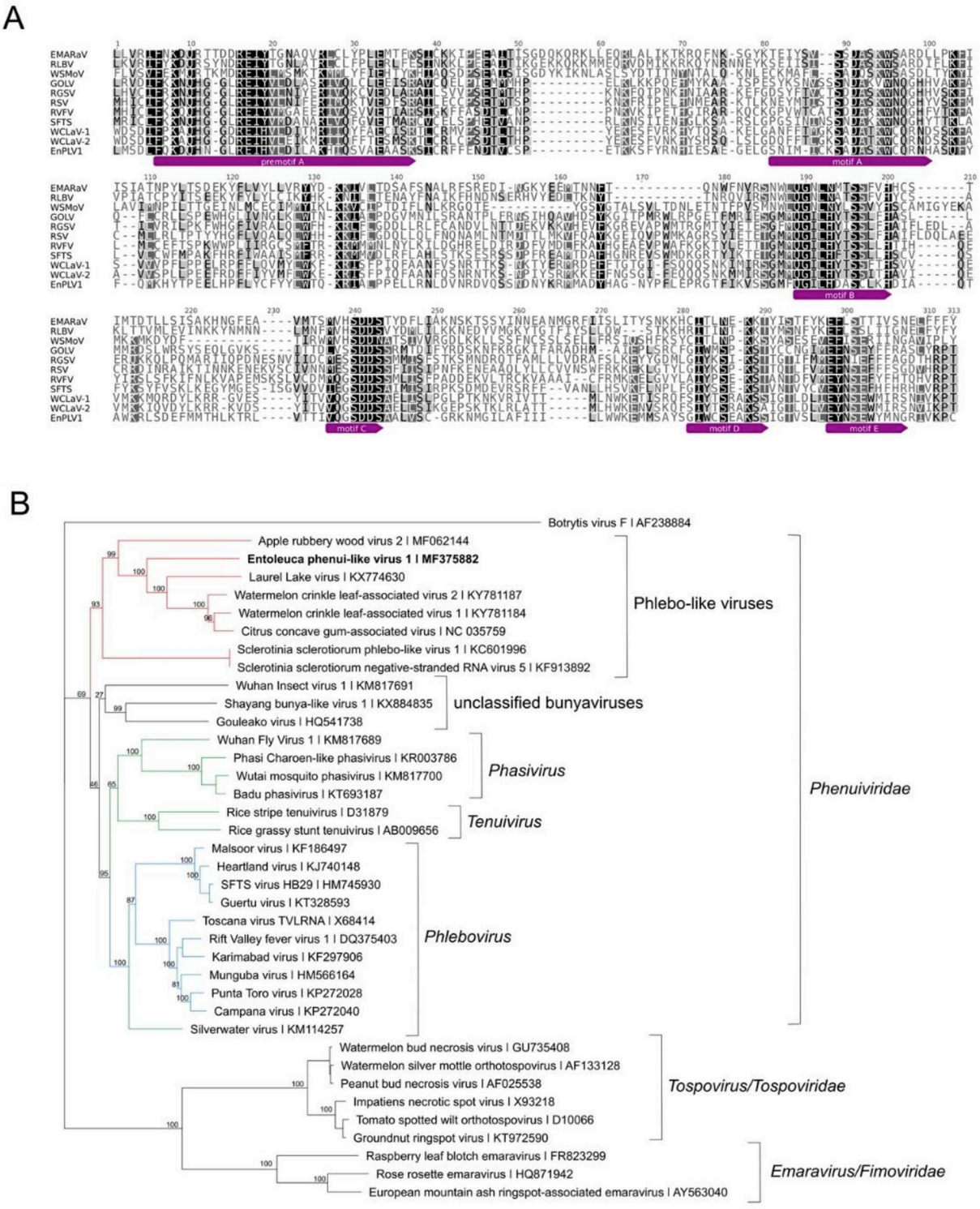


**Fig. 5. A**. Amino acid alignment between RdRp domains showing the premotif A and motifs A–E of EnPLV1 and selected negative single stranded RNA viruses. EMARaV, European mountain ash ringspot-associated virus (AY563040); RRV, rose rosette virus (HQ871942); RLBV, raspberry leaf blotch virus (FR823299); WSMoV, watermelon silver mottle virus (AF133128); RSV, rice stripe virus (D31879); RVFV, Rift Valley fever virus (DQ375403); SFTSV, severe fever with thrombocytopenia syndrome (SFTS) virus (HM745930); RGSV, rice grassy stunt virus (AB009656), GOLV, Gouleako virus (HQ541738), SFTSV, severe fever with thrombocytopenia syndrome virus (HM745930), WCLaV-1 and -2, watermelon crinkle leaf-associated viruses 1 and 2 (KY781184, KY781187). **B.** ML phylogenetic tree of the amino acid sequences of RNA-dependent RNA polymerase (RdRp) for a selection of bunyaviruses. The best-fit model of substitution according to BIC was the rtREV + F + R6. Botrytis virus F (BVF) was predicted as out-group by IQ-TREE. Bootstrap support values written on the branches.

### *3.11. Screening mycoviruses in the Entoleuca sp. and R. necatrix collection*

The availability of partial sequences for the viruses described in this work allowed the design of specific primers for RT-PCR detection. For that, consensus primers were obtained based on the genomic sequences obtained for each virus in the four *Entoleuca* sp. isolates (Table S1). A collection of *Entoleuca* sp. and *R. necatrix* isolates recovered from avocado trees in orchards in Spain was investigated for the presence of the

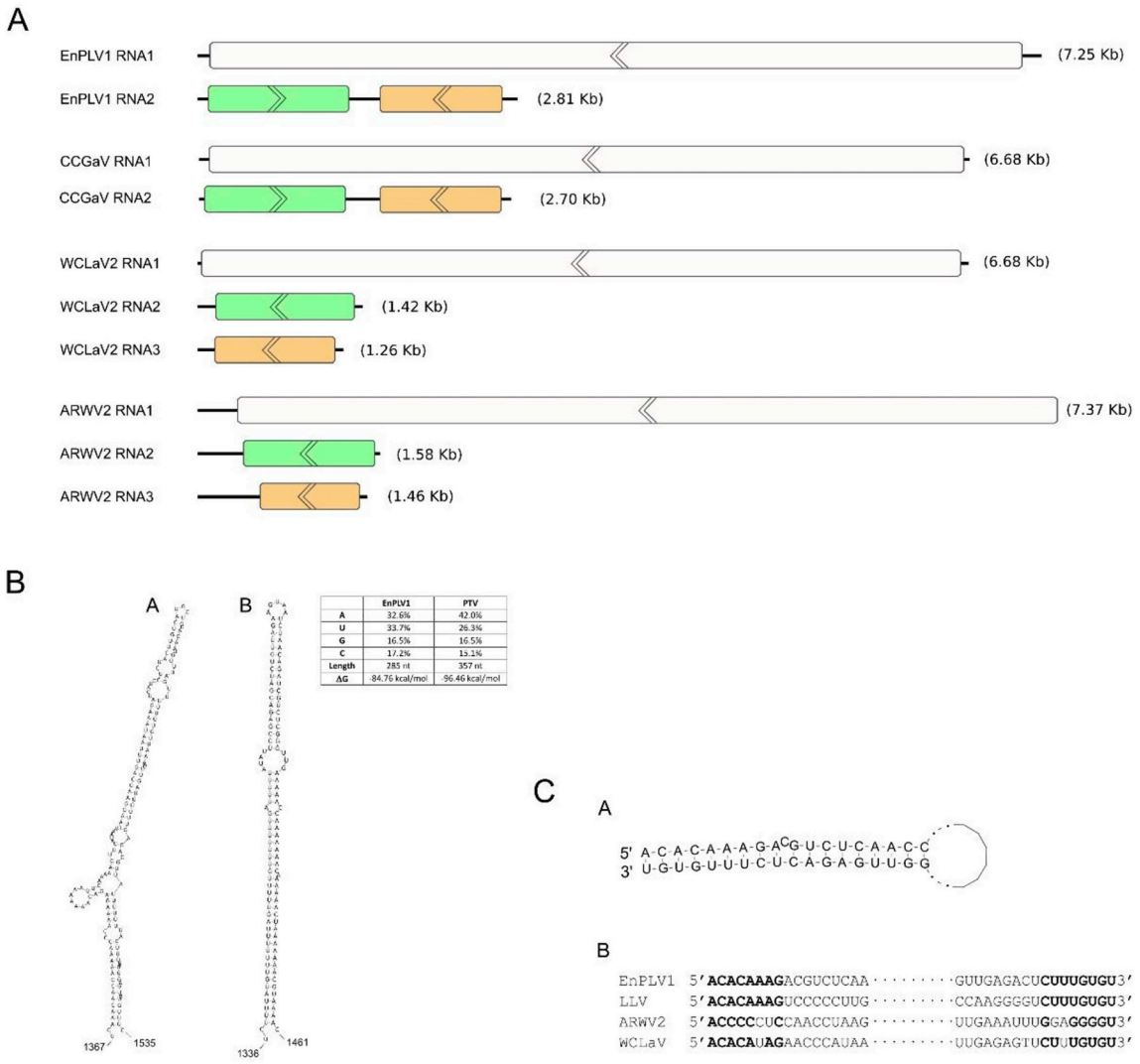


**Fig. 6. A**. Comparisons of the genome organization and sizes of Entoleuca bunyavirus 1 (EnPLV1), citrus concave gum-associated virus (CCGaV), apple rubbery wood virus 2 (ARVV2) and watermelon crinkle leaf-associated virus 2 (WCLaV1). Blank boxes represent the RdRp ORF, green boxes refer to the MP ORF and orange boxes represent the CP ORF. The double arrows in the ORF boxes represent the sense of the RNA transcription. **B**. Secondary structure and analysis of the intergenic regions of the RNA2 of (a) EnPLV1 and (b) Citrus concave gum-associated virus (CCGaV) (Navarro et al., 2018), a phlebo-like virus isolated from orange trees. **C**. Structural characteristics of EnPLV1 RNA1 terminal sequences. (a) Panhandle structure formed by the 3′ and 5′ termini. (b) Comparison of the terminal sequences of the L segments for representative bunyaviruses (see text for virus acronyms). Matching base pairing is indicated in bold.

mycoviruses described in this work (Table 2). The result of this survey showed that the mycoviruses are well spread in these fungi, showing recurrent multiple virus infections. Moreover, several viruses are present simultaneously in *Entoleuca* sp. and *R. necatrix*, being the most frequent EnHV1, and EnMBV1, RnFSV1, YkV2 and the two partitiviruses.

## 4. Discussion

### *4.1. Novel mycoviruses detected in Xylariaceae species isolated from avocado*

In the four *Entoleuca* sp. isolates that are the main object of this work we could identify 14 sequence segments apparently belonging to eleven different mycovirus species. The number of reads obtained after HT sequencing matching to the reconstructed genomes varied greatly depending not only on the specific virus species but also on the fungal isolate from which they were isolated. These differences must be related to virome composition, the replication strategy for each virus species as well as the characteristics of the fungal isolate. Given the variability observed in the virus genomic sequences, the RT-PCR primers designed in this work could be not suitable to detect all the variants, as some unspecific amplification bands have been observed in some cases (results not shown). Strains of four of the pool of virus species have been already identified in *R. necatrix* (Arjona-Lopez et al., 2018), including EnHV1, RnFSV1 and YkV2. Another megabirnavirus identified, EnMBV1, appears to be the same species as RnMBV3 according to genome structure comparison and genetic analysis. The two flexiviruses reported here showed striking genome structure and features. EnGFV1 replicase corresponds to gammaflexiviruses but the MP and the CP shares homologies with virgaviruses. The polyphyletic origin of the CPs and MPs of alpha-like viruses, such as the two novel gammaflexiviruses described here, complicates the elaboration of a coherent evolutionary history for this group of viruses (Dolja and Koonin, 2018). Recently, a novel virus with similarity to gammaflexiviruses (FbLFV1) has been reported presenting a single ORF encoding for the replicase (Mizutani et al., 2018). Clearly, more genomic sequences from different phyla are necessary to elucidate the evolution of this important group of viruses. The ourmia-like virus identified in this study (EnOLV1) was phylogenetically grouped in a clade with other two ourmia-like viruses whose

hosts are fungi, separated from mitoviruses and fungal narnaviruses and allocated proximal to other plant and fungal ourmia-like viruses that have a genome structure consisting of a single ORF. Another viral species, EnPLV1, has been ascribed to negative ssRNA viruses order *Bunyavirales*, according to genetic and phylogenetic analysis. The structure of its genome is bipartite, like that of CCGaV, recently described in orange trees (Navarro et al., 2018), although the genetic sequence is more similar to phlebo-like viruses with tripartite genomes, reflecting in-parallel genome arrangements in plant and fungal phlebo-like viruses. Ambisense RNA segments are relatively scarce in viruses and up to now found only in members of the family *Arenaviridae*, in order *Bunyavirales* (genus *Tenuivirus* and *Phlebovirus*) and tospoviruses (Nguyen and Haenni, 2003). The detection of an endornavirus species in the fungal isolates that it is 98% homologous to the endornavirus PvEV1 originally identified in common bean (Okada et al., 2013), suggests that in some cases the plant host may act as a bridge between incompatible fungal species or isolates for inter-specific virus transmission. In their interaction, plant cells and colonizing fungi exchange macromolecules that may include virion particles or large dsRNA molecules (Roossinck, 2019). Whereas endornaviruses are found in plants, fungi or oomycetes (Fukuhara et al., 2006), up to our knowledge, there are no reports of a same endornavirus species present across kingdoms, even though it has been suggested that endornaviruses have originated in fungi (Fermin et al., 2018). Phylogenetic analyses of endornaviruses point out to extensive horizontal transfer between plants and fungi (Roossinck, 2019). An endornavirus-like dsRNA (Persea americana endornavirus 1, PaEV1) different from the one described here, has been isolated from leaves of an avocado tree belonging to a germplasm collection in Spain (Estación Experimental “La Mayora”, Algarrobo-Costa, Málaga, Spain) (Villanueva et al., 2012). However, preliminary RT-PCR of Spanish avocado trees using PvEV1-derived primers tested negative (results not shown).

### 4.2. Virome composition and host phenotype

Mycoviruses infecting *R. necatrix* have been extensively investigated (Kondo et al., 2013; Zhang et al., 2014; Yaegashi and Kanematsu, 2015; Chiba et al., 2016) especially in Japanese fruit tree pathosystems. The presence of simple and multiple mycovirus infections has been related with hypovirulence phenomena in *R. necatrix* (Chiba et al., 2009; Sasaki et al., 2015). Virus effects on their hosts can include limitation of hyphae development and/or a decrease of the fungus virulence, but in most cases, there is a negligible impact in the fungus-plant host interaction (Kondo et al., 2013). The biological characterization of all the viruses identified in this work is far for being completed. Curing these viruses in the fungal isolates has been unsuccessful after hyphal tip cultivation combined with antiviral compounds (Velasco et al., 2018). From the complete virome analysis of the four *Entoleuca* isolates studied in the present work, it became evident that three different sets of viruses (viromes) appeared. On the other hand, variation in the biological characteristics of the four *Entoleuca* sp. isolates resulted high, however, we cannot definitively attribute those differences to virome composition or intrinsic biological characteristics of each fungal isolate (or a combination of both).

### 4.3. Horizontal virus transmission in Xylariaceae fungi from avocado

We found vegetative incompatibility among the four *Entoleuca* sp. isolates object of this work. In *R. necatrix*, the isolates collected from avocado roots in Spain present different VCGs (Pérez-Jiménez et al., 2002). Despite that, a same set of mycoviruses is shared by isolates of *Entoleuca* sp. and *R. necatrix* that are colonizing the same avocado trees, evidencing the probable horizontal virus transmission intra- and interspecies. Moreover, a population analysis in one of the mycovirus species (EnHV1) revealed polymorphism among strains not linked to host origin (Velasco et al., 2018), that has also been observed in the other virus species investigated in this work (EnMBV1). Transmission of mycoviruses intra- and interspecies of the same genera of fungi in natural environments has been reported previously (Liu et al., 2003; Ikeda et al., 2004; Vainio et al., 2011; Liu et al., 2012). Artificial transmission of the mycoviruses between different fungal species has been achieved (Kanematsu et al., 2010). Moreover, it has been experimentally demonstrated the transmission of a virus between vegetatively incompatible *R. necatrix* isolates using zinc ions in the growth medium (Ikeda et al., 2013). Interestingly, Yaegashi et al., (2013) showed a virus infection of a traceable *R. necatrix* isolate from an unknown vector source after the inoculation during a three-year period in the soil of an apple orchard. Thus, it is not surprising that we could detect the partitiviruses EnPV1 and EnPV2 in a *Fusarium* sp. isolate collected from the same avocado orchards (results not shown). Avocado, the perennial plant that harbors the two species of fungi studied here, conforms a particular ecological niche in the rhizosphere that might include mycophagous species (insects, *Trichoderma*, etc.) as possible vectors for mycoviruses. We foresee that these same viruses or some others will be detected in future surveys in other fungal species or oomycetes collected in this pathosystem. Finally, this work provides supporting evidence for HVT among two sympatric fungal species in natural conditions involving several mycovirus species belonging to different taxa.

Although the *R. necatrix* isolates identified so far in Spain are virulent to avocado, the eventual finding of a virus species capable of conferring hypovirulence and the probable horizontal transmission of the mycoviruses observed in this work suggests that virocontrol of *R. necatrix* in avocado is plausible. Finally, high-throughput sequencing technologies that enable the determination of complete viral infection status in fungal hosts, help to clarify the role of viruses in the mycovirus-fungal-plant interaction, to explore virocontrol strategies for fungal diseases, and to better understand virus evolution.

## Accessions numbers

The sequences described in this work were deposited in GenBank under the following accession numbers: EnPLV1 (MF375882; MK140653), EnGFV1 (MF375883), EnGFV2 (MF375884), EnHV1 (MF375885), EnMBV1 (MF375886), RnFSV1 (MF375887), YkV2 (MF375888), EnOLV1 (MF375889), EnPV1 (MF375890), EnPV2 (MF375891) and PvEV1 (MF375892).

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## Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.virol.2019.03.021.

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