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Brief Communication

Molecular characterization of Botrytis ourmia-like virus, a mycovirus close to the plant pathogenic genus *Ourmiavirus*

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ABSTRACT

The molecular characterization of a novel single-stranded RNA virus, obtained by next generation sequencing using Illumina platform, in a field grapevine isolate of the plant pathogenic fungus *Botrytis*, is reported in this work. The sequence comparison of this virus against the NCBI database showed a strong identity with RNA dependent RNA polymerases (RdRps) of plant pathogenic viruses belonging to the genus *Ourmiavirus*, therefore, this novel virus was named Botrytis ourmia-like virus (BOLV). BOLV has one open reading frame of 2169 nucleotides, which encodes a protein of 722 amino acids showing conserved domains of plant RNA viruses RdRps such as the most conserved GDD active domain. Our analyses showed that BOLV is phylogenetically closer to the fungal *Narnavirus* and the plant *Ourmiavirus* than to *Mitovirus* of the family *Narnaviridae*. Hence, we proposed that BOLV might represent the link between fungal viruses of the family *Narnaviridae* and the plant ourmiaviruses.

Introduction

Mycoviruses or fungal viruses are widespread in all taxonomic groups of fungi and to date have been isolated from mushrooms, plant-pathogenic and medical fungi. However, the first report of a virus infecting a fungus dates back about to only fifty years, relatively recent comparing the discovery of animal or plant viruses; the fact that the infection of most mycoviruses is latent, with no clear appreciable symptoms in the fungal host (Ghabrial and Suzuki, 2009), precluded an early discovery. Now, it is known that symptoms caused by mycoviruses infecting plant pathogenic fungi are diverse. They can induce severe symptoms causing economical loses in cultivated mushrooms or plant hosts (Ghabrial and Suzuki, 2009); be beneficial for both, the fungi and the infected plant, increasing their surveillance under stress conditions (Márquez et al., 2007); or may cause hypovirulence or attenuation of fungal virulence in the plant (Nuss, 2005; Xie and Jiang, 2014). Mycoviruses have been broadly studied both for their potential use as biocontrol agents of phytopathogenic fungi of different species, as well as to understand the ecology and evolution of viruses; indeed, currently more than 250 mycoviral sequences have been registered in public databases (Xie and Jiang, 2014).

Most mycoviruses have positive single-stranded RNA ((+) ssRNA) and double-stranded RNA (dsRNA) genomes (Ghabrial and Suzuki, 2009); nevertheless it has been also reported mycoviruses with single-stranded DNA (ssDNA) genomes, such as the *Sclerotinia sclerotiorum* hypovirulence associated DNA virus 1 (SsHADV-1) (Yu et al., 2010) and *Hypericum japonicum*-associated circular DNA virus (HJasCV) (Du et al., 2014) and more recently, a mycovirus with negative ssRNA genome isolated from a hypovirulent isolate of the plant pathogenic fungus *S. sclerotiorum* (Liu et al., 2014). Mycoviruses belonging to Narnaviridae family, with (+) ssRNA genome of approximately 2–3 kb, have the simplest genome organization among the 13 described families of mycoviruses. They only encode for the RNA dependent RNA polymerase (RdRp), enzyme involved in their own replication, and have no capside structure (Hillman and Cai, 2013). Remarkably, the most phylogenetically-close viruses to the Narnaviridae family (composed by the genera *Narnavirus* and *Mitovirus*), are members of the plant genus Ourmiavirus (Hillman and Cai, 2013; Rastgou et al., 2009). As other plant viruses, *ourmiaviruses* have tripartite, (+) ssRNA genomes with the characteristic that each one of the three genes (encoding the RdRp, the coat protein (CP) or the movement protein (MP)) is located in a different genome segment (Rastgou et al., 2009). Based on phylogenetic analyses, it was proposed that ourmiaviruses represent a link between mycoviruses and viruses of higher organisms (Hillman and Cai, 2013). Since most of the mycoviruses identified are closely related to plant viruses the origin and evolution of mycoviruses have been explained by two main hypotheses (Ghabrial, 1998): the ancient coevolution and the plant virus hypothesis. The ancient coevolution hypothesis is based on the suggestion that the life cycle of mycoviruses is limited to an

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intracellular phase; as a result, the association between the virus and the fungus must be ancient. The plant virus hypothesis proposes that plant-pathogenic fungi have acquired recently the viruses from their infected plant hosts. This hypothesis is based on the similarity between fungal and plant viruses with the important difference that no mycovirus encodes a movement protein and some of them not even a coat protein. According to this hypothesis, mycoviruses would have evolved from plant viruses and would have lost those genes encoding dispensable proteins for their survival inside the fungal host, so most of them only encode the RdRp.

Several groups, including ours, have reported the presence of mycoviruses in several species of the phytopathogenic fungus *Botrytis*, but only a few of them have been sequenced and assigned as new virus species (Howitt et al., 2001, 2006; Pearson and Bailey, 2013; Rodríguez-García et al., 2014; Wu et al., 2010, 2012). In this work, we have used high-throughput sequencing, and we have identified a new mycovirus from a field isolate of *Botrytis* infecting grapevine. We found that this new mycovirus, Botrytis ourmia-like virus (BOLV), is phylogenetically close to plant ourmiaviruses, results that provides a new insight into the relationship between fungal and plant viruses.

Results

Discovering of a novel mycovirus of Botrytis by deep-sequencing

In a previous study, the presence of dsRNA elements and virus like particles (VLPs) from several isolates of the plant pathogenic fungus *Botrytis* was reported (Rodríguez-García et al., 2014). From this work, it was selected a field isolate of the fungus obtained from grapevine (isolate V446) to further analyze the identity of infecting mycoviruses. Total RNA isolated from the fungus was used for cDNA library preparation and subsequent next generation sequencing (NGS) using Illumina platform. To identify viral sequences, a total of 19,732 contigs (with an average length of 600 nucleotides (nts)), which do not match with *Botrytis* databases, were subjected to BLAST search against NCBI non-redundant (nr) DNA and protein databases. Among other mycoviral sequences (the blast search identified a variant of the *S. sclerotiorum* dsRNA mycovirus-L and a possible endornavirus, unpublished results), a sequence of 968 amino acids (aa) including stop codons, translated from a single contig of 2903 nts, had 22% and 21% of identity with two plant viruses belonging to genus *Ourmiavirus*, Ourmia melon virus (OuMV) and Epirus cherry virus (EpCV), respectively (*E*-value $< 3 \times 10^{-4}$). However, the corresponding nucleotide sequence (2903 nts long) did not show similarity with viral sequences deposited in the NCBI database. Since, BLAST analyses did not reveal high identity with known mycoviruses, and the most closely related sequence was the RdRp of OuMV, this viral sequence was considered as new specie and it was tentatively named Botrytis ourmia-like virus (BOLV) for its similarity with members of this genus of plant viruses. The presence of BOLV in the fungal isolate was corroborated by Northern blot analysis (Fig. 1A). A BOLV 3′-terminal negative-stranded specific riboprobe hybridized with total RNA and total nucleic acids extracted from our *Botrytis* grapevine isolate (V446), but not with other *Botrytis* isolates (Pi258.8 and V448) (Fig. 1A). A band corresponding to a size of approximately 3 kb can be observed in the Northern blot analysis (Fig. 1A). However, several bands of smaller sizes were also detected in the hybridization that likely represent degradation products of the BOLV genome. BOLV was also detected with specific primers by RT-PCR in extracts of total nucleic acids, total RNA and dsRNA obtained from the grapevine fungal isolate, indicating that indeed the virus is infecting the fungus (data not shown).

BOLV sequence analysis

BOLV genome is a (+) ssRNA molecule of 2903 nts with a 45.9% GC content. The viral genome had a unique open reading frame (ORF) from position 42 to 2210 (Fig. 1A). The translation of this ORF of 2169 nts gave a polypeptide of 722 aa with a theoretical molecular weight of 81.88 kDa, and with the high identity to the RdRp of OuMV (Identity=27%; *E*-value=5×10^{-9}) and EpCV (Identity=25%; *E*-value=1×10^{-4}). The BLASTP search also showed a certain degree of identity, but with lower *E*-value, with RdRp of another ourmiavirus, Cassava virus C (CsVC) (Identity=25%; *E*-value=1) and with RdRps of two mycoviruses of the *Narnaviridae* family, Ophiostoma mitovirus 7 (Identity=23%; *E*-value=1.1) and Saccharomyces 23S RNA narnavirus (Identity=25%; *E*-value=2.8). In addition, the conceptual protein encoded by BOLV contained the conserved domains of the viral RdRps of (+) ssRNA viruses (Koonin, 1991), including the highly conserved core domain GDD (motif VI) (Fig. 1C). These results indicate that the ORF of BOLV encode a putative RdRp. The determined sequence had a 5′ non coding region (NCR) of 41 nts and a 3′ NCR of 693 nts. Its alignment with the 5′ NCR of the three ourmiavirus sequences showed that all viruses start with three C residues and have conserved nucleotides in this region, being closer to the 5′ NCR of EpCV than to the other two ourmiavirus sequences (Fig. 2A). On the other hand, the 3′ end of BOLV had three G residues exactly as the sequence of the three ourmiavirus (Fig. 2A). There were also some conserved nucleotides in a stretch of 30 nts of the 3′ NCR, but only the three G residues were conserved among all viruses (Fig. 2A). These conserved nucleotides at both ends could be implicated in initiation of the replication step. The predicted secondary structure of the 5′ NCR of BOLV and the three ourmiaviruses indicated the presence of a stable terminal stem-loop structure only for the first 29 nts of EpCV (ΔG value of -8.9 kcal/mol) and the first 28 nts of BOLV (ΔG value of -11 kcal/mol) (Fig. 2B). The last 30 nts of the 3′ NCR of the three ourmiavirus OuMV, EpCV and CsVC could be folded into stable stem-loop structures with ΔG values of -15.80, -16.80 and -13.20 kcal/mol, respectively (Fig. 2C). However, the last 50 nts of the 3′ end of BOLV folded into a less stable but more complex structure (ΔG value of -9.10 kcal/mol) (Fig. 2C). These structures could also play a role in viral replication.

Phylogenetic analysis of mitovirus, narnavirus and ourmiavirus RdRps

Although the RdRp protein sequence of BOLV only showed significant identity with the RdRps of OuMV and EpCV, a more detailed computational analysis using PSI-BLAST program showed its possible relationship with RdRps of mycoviruses belonging to *Narnaviridae* family. Full-length amino acid sequences of 48 RdRps were selected in the fourth iteration of the PSI-BLAST run (*E*-value $< 10^{-4}$) and multiple aligned (using MUSCLE; Fig. S1) to construct a phylogenetic tree to infer the evolutionary history of BOLV (Fig. 3). The RdRp of the *Tobamovirus* Tobacco mosaic virus (TMV) was used as an outgroup in the tree. The maximum likelihood (ML) phylogenetic tree was inferred from the amino acid sequences of viral RdRp after removing positions with less than 50% site coverage. The phylogenetic analyses clearly showed a clade (93% bootstrap support) which includes two groups, one of them grouping the two *Saccharomyces narnavirus* and *Phytophthora infestans* RNA virus (43% bootstrap support), and the second one grouping BOLV and the three members of the plant ourmiavirus group, OuMV, EpCV and CsVC (73% bootstrap support) (Fig. 3). Similar results were obtained in the ML phylogenetic tree inferred in basis of the MAFFT and COBALT multiple sequence alignments (yielding a 61% and 97% bootstrap support, respectively, in the clade formed by BOLV and the three plant ourmiavirus, trees not shown). Bayesian-based phylogenetic trees inferred with the three multiple sequence alignment methods, supported the ML phylogenetic trees results (posterior

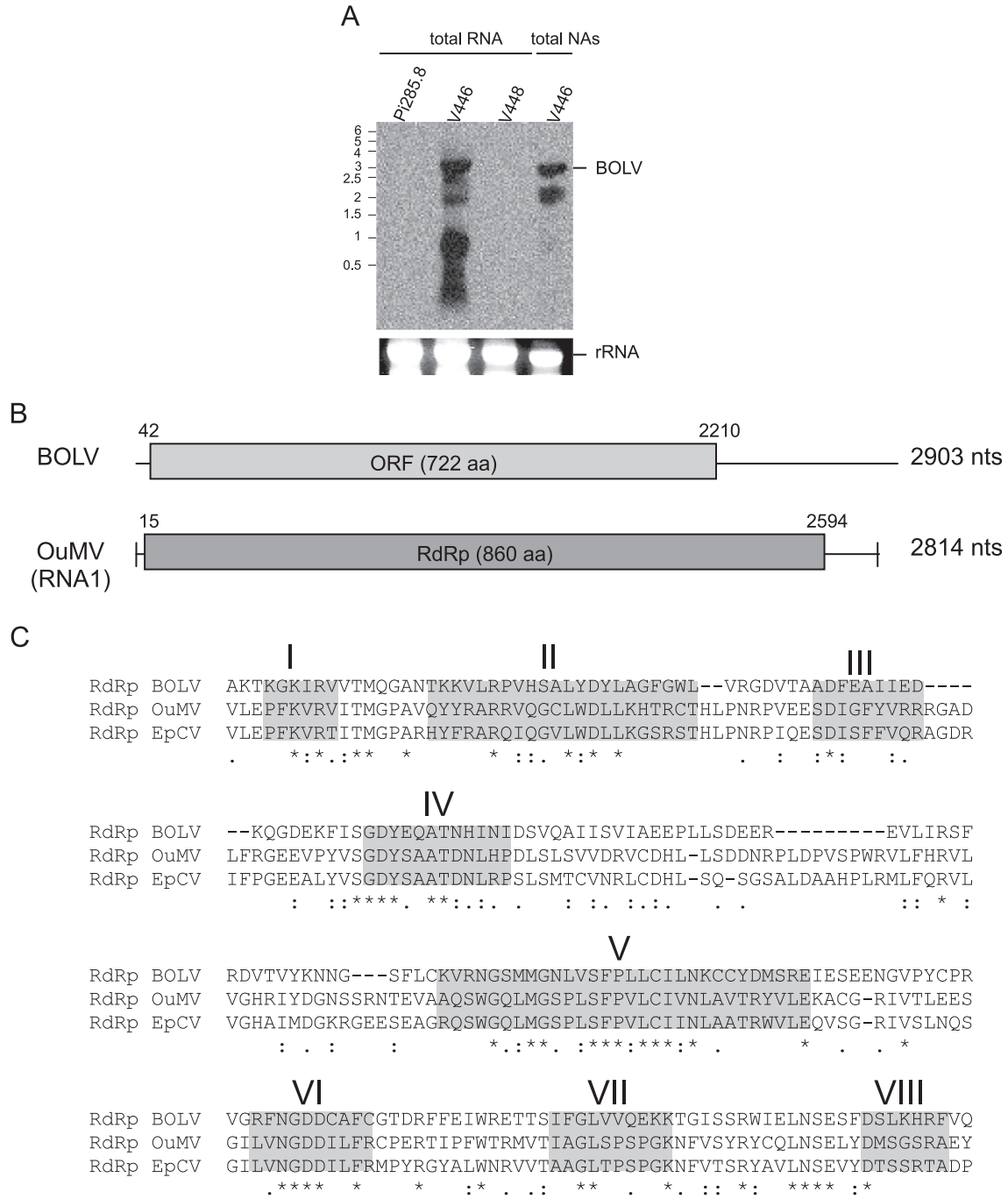


Fig. 1. Sequence properties of BOLV. (A) Northern blot hybridization analysis of BOLV-specific RNA using a negative-stranded riboprobe for detecting the 3′-terminal region of the genome. Position of each band of the Millennium RNA marker (Ambion) is shown on the left. Ethidium bromide staining of the gel prior to transfer is shown as loading control. (B) Schematic representation of BOLV RNA genome and OuMV RNA1 (Rastgou et al., 2009) showing location of ORFs. (C) Alignment showing conserved motifs I to VIII with RdRps of BOLV (LN827955), OuMV (YP_002019757.1) and EpCV (YP_002019754.1).

probabilities for all nodes higher than 0.6389), with the exception of the MAFFT-based tree that generated two clades, one including all three plant ourmiavirus, and the other grouping BOLV, *P. infestans* RNA virus and the two *S. narnavirus* (trees not shown). Then, the ML analyses indicate that BOLV is more closely related to plant virus species in the genus *Ourmiavirus*; and among mycoviruses of the family *Narnaviridae*, BOLV and plant ourmiaviruses are closer related to members of genus *Narnavirus* than to members of genus *Mitovirus* (Fig. 3). These results suggest that BOLV could be the closer link between plant ourmiaviruses and fungal viruses.

Discussion

NGS experiments have been previously applied to search for mycoviruses using purified dsRNA as template for cDNA library construction and high-throughput sequencing (Al Rwahnih et al., 2011; Coetzee et al., 2010). This methodology allows the detection of mycoviruses with dsRNA genome as well as dsRNA intermediates originated during replication of ssRNA mycoviruses. Since the level of dsRNA intermediates could be in some cases undetectable, we have used direct sequencing of total RNA, an

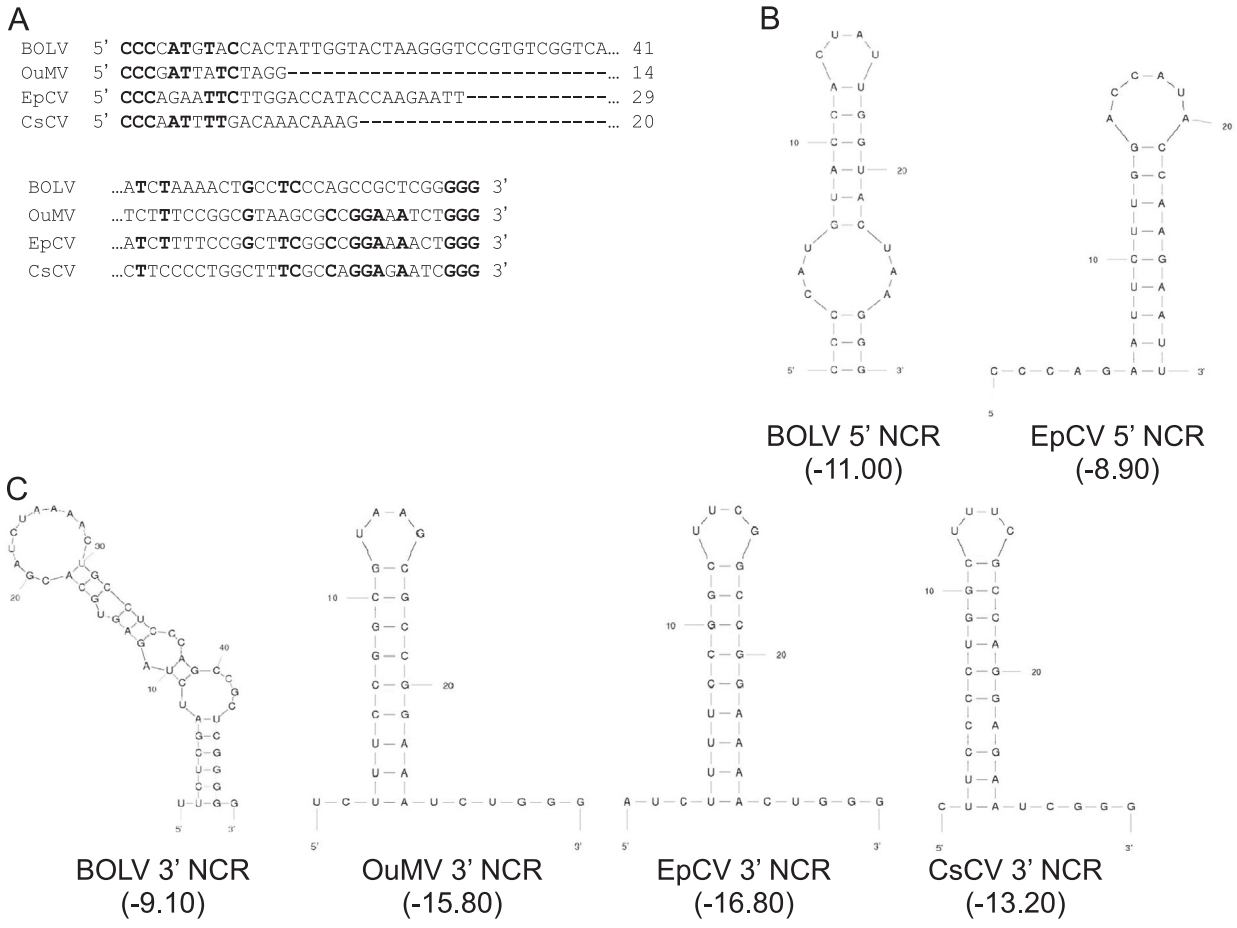


Fig. 2. Comparison of 5′ and 3′ RNA ends between BOLV and ourmiaviruses. (A) Alignment of 5′ and 3′ end regions of BOLV and the three plant ourmiaviruses, OuMV, EpCV and CsCV, with conserved nucleotides marked in bold. (B) Predicted secondary structures of the 5′ terminal region of EpCV and BOLV with their corresponding initial ΔG in kcal/mol. (C) Predicted secondary structures of the 3′ terminal regions of BOLV and plant ourmiaviruses with their corresponding initial ΔG in kcal/mol.

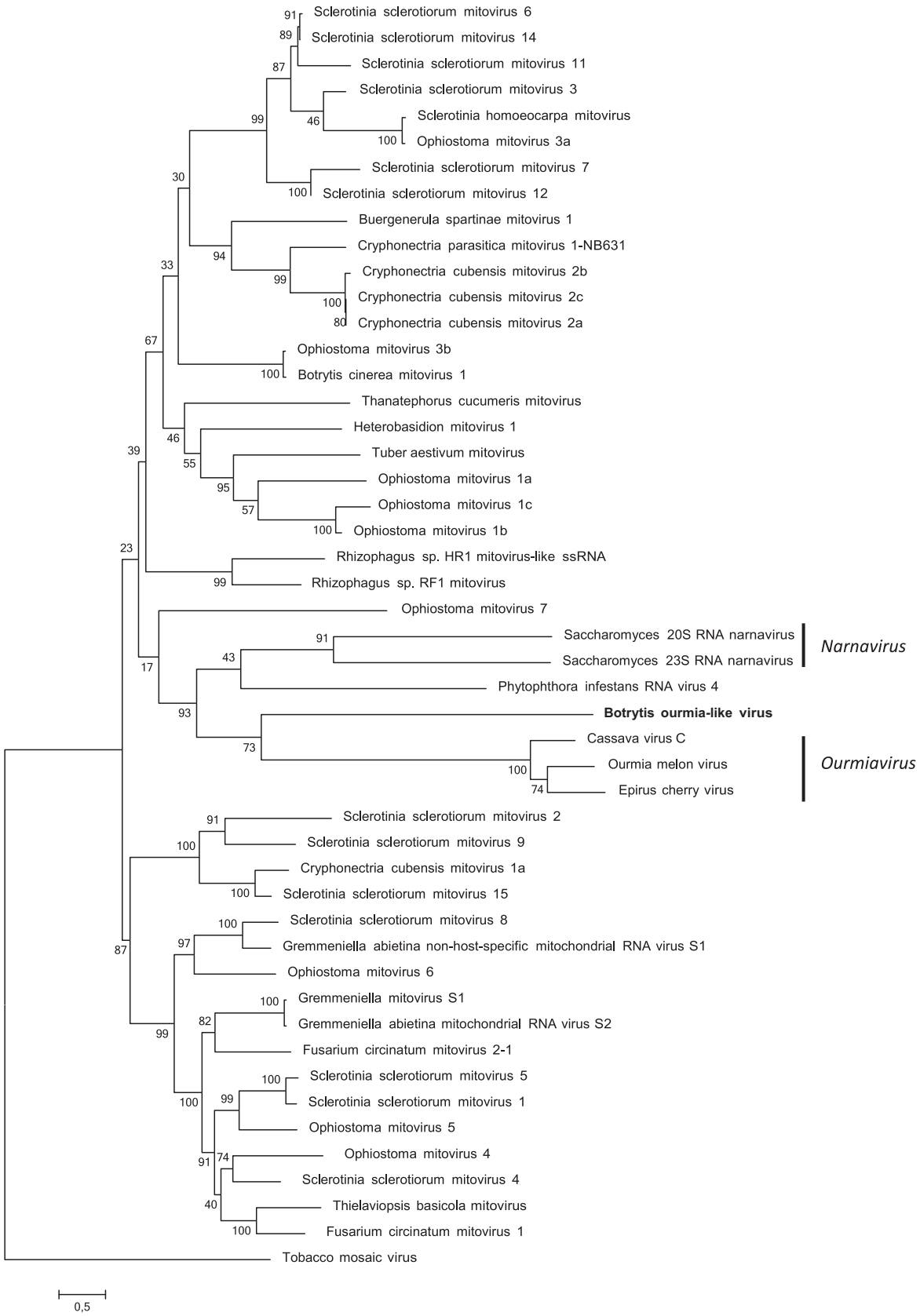
approach used to identify ssRNA mitovirus (Schoebel et al., 2014) that also permits the detection of mycoviruses with dsRNA and DNA genomes. A great advantage of this strategy is that it also allows the in-parallel sequencing of host transcripts and hence, a transcriptomic analysis of fungal mRNAs during mycoviral infections. In this work, we have followed an in silico approach using NGS assembled contigs to identify (via BLAST) a new viral sequence in a field isolate of *Botrytis*. Moreover, the presence of the virus identified in this study was confirmed experimentally by Northern blot analysis using a specific 3′ negative-sense riboprobe and by RT-PCR using specific primers.

Although at the nucleotide level there was no significant similarity between BOLV and any viral sequence deposited in public databases, at the amino acid level we found significant results with ourmiaviruses, indicating a certain degree of protein conservation among plant and fungal viruses. The unique ORF in BOLV likely encodes an RdRp with common characteristics with RdRps of (+) ssRNA viruses, and specifically it shares high identity with RdRps of Ourmiavirus. Regardless the slightly smaller size of the RdRp of BOLV (closer to the mitovirus RdRps size in comparison with the RdRp of *ourmiaviruses*; 722 aa of BOLV versus 860 aa of OuMV), we identified RdRps conserved motifs between BOLV and *Ourmiavirus* (Fig. 1C). Remarkably, we also identified some of the invariable residues found in RdRps of (+) ssRNA viruses: a lysine in Motif I, two aspartic acids in Motif IV (although in BOLV only one is conserved), and other two aspartic acids in Motif VI that are part of the known active GDD domain (Koonin, 1991).

Rastgou et al. (2009) showed that the genome sequence of the RNA1 of the three ourmiaviruses, OuMV, EpCV and CsVC, start with three consecutive cytosines (CCC), and end with three guanines (GGG), and they suggested that the polymerase might use the same CCC sequences as initiation site for positive- and negative-strand synthesis. In this case, the BOLV polymerase might also use the same sequence to initiate the synthesis of both strands, indicating that the 3′ end of BOLV sequence (GGG) and the 5′ end (CCC), determined by 5′ and 3′ RACE analyses, are correct. The stem-loop structures at the 5′, but mainly, at the 3′ terminal regions may play a role in the replication of BOLV and ourmiaviruses acting as recognition sites for the RdRp; but for BOLV may have also a protector function of the naked RNA genome for the degradation as has been proposed for mitoviruses (Xie and Ghabrial, 2012).

Our phylogenetic analysis shows that BOLV and plant ourmiaviruses have a common ancestor more recent than narnaviruses. Indeed, we found that BOLV and ourmiaviruses form a clade (73% bootstrap support), whereas narnaviruses and *P. infestans* RNA virus constitute other separate clade (43% bootstrap support), forming altogether a monophyletic clade (93% bootstrap support). Our results are in agreement with previous reports indicating that members of the *Narnavirus* genus (*Narnaviridae* family) are more closely related to the *Ourmiavirus* than to members of the *Mitovirus* genus (Cai et al., 2012; Rastgou et al., 2009). Although, there are also examples of virus genera composed of viruses infecting different host taxons, for instance, inside the genus *Narnavirus* it have been characterized two members infecting the yeast *Saccharomyces cerevisiae* (Hillman and Cai, 2013) and a member infecting the oomycete *P. infestans* (proposed narnavirus, Cai et al., 2012); we propose that BOLV should be classified in the same viral

family as ourmiaviruses but in a different genus. *Ourmiavirus* is a genus of plant viruses (unassigned family) where the three viral proteins are codified in different genome segments (Rastgou et al., 2009). A hypothesis of their origin suggests that ourmiaviruses might have evolved by the reassortment of at least two or three viruses belonging to different RNA virus groups (Cai et al., 2012; Rastgou et al., 2009). According to these authors, the origin of ourmiaviruses could be from a fungal virus, most probably a



Narnavirus, with a ssRNA genome encoding only the RdRp protein, which acquired the MP gene from a member of *Tombusviridae* family, whereas the CP gene was likely acquired from a different plant virus, to codify the proteins for virion formation and systemic spread of the virus in the plant host (Rastgou et al., 2009).

According with our results BOLV might represent the closest link between fungal narnaviruses and plant ourmiaviruses. Our results might be explained assuming that the common ancestor of the ourmiaviruses was a fungal virus (with certain BOLV characteristics), that infected a plant host, to further evolve acquiring the other two RNA genomic segments from different plant viruses. However, combining all pieces of evidences (phylogenetic and sequence analyses) of our data, the origin of BOLV can be more parsimoniously explained by the so-called plant virus hypothesis (Ghabrial, 1998). Under this scenario an ancestor of the current *Botrytis* fungus might have acquired an ancient ourmiavirus from its infected plant host; later the virus might have lost dispensable genes (those encoding proteins dispensable for their survival inside the fungal host), while maintaining just the RdRp encoding gene. Indeed, the Bayesian-based phylogenetic tree based on the MAFFT alignment might reinforce that hypothesis (although this phylogenetic analysis is not well supported). This analysis showed two clades, one including the plant ourmiavirus, and the other including BOLV, *P. infestans* RNA virus and the two *S. narnavirus* , suggesting that the plant ourmiavirus might in fact be the common ancestor of BOLV and the narnavirus. However, we cannot completely exclude that a fungal precursor was really the common ancestor of ourmiaviruses and BOLV. Nonetheless, our results points to BOLV could be a closer link between plant ourmiaviruses and fungal viruses. In our opinion, the discovery of BOLV has significantly contributed to elucidated the complex relationship between fungal and plant ourmiaviruses.

During the course of the final revision of this work, Marzano and Domier (2015) found two soybean leaf-associated ourmiavirus in the analysis of the metatranscriptomes of soybean leaf samples. The finding of new ourmia-like sequences associated with fungus and plants will help us in the future to understand more precisely the evolution of the genus *Ourmiavirus*.

Materials and methods

Fungal isolate and culture conditions

The Botritys isolate V446 was collected in a field vineyard in Roa de Duero (Burgos, Castilla y León) in central Spain by Dr. Ernesto P. Benito (CIALE, Spain) and stored in 20% glycerol at −80 °C as described (Rodríguez-García et al., 2014). Stock culture was maintained in potato dextrose agar (PDA) plates at 4 °C. Fresh mycelia of the fungus was obtained by placing mycelial agar plugs of the stock culture in 100 ml of potato dextrose broth (PDB) and incubated in the darkness at 23 °C during 10 days.

Total RNA extraction and nucleotide sequencing

Fresh mycelia was dried by pressing with sterile filter paper and total RNA was purified from 1 g of dry mycelia using TRIZOL reagent (Invitrogen) following the protocol provided by the manufacturer but repeating the chloroform extraction and the subsequent centrifugation step until no protein interface was observed. For virus detection, 5–10 μg of total RNA was used for library preparation as described by Al Rwahnih et al. (2011) and subjected to high-throughput deep-sequencing using the Illumina platform (HiSeq2000, 2 × 100 bp length, IGA Technology Services, Udine, Italy, http://www.igatechnology.com).

Northern blot analysis and RNA Ligase Mediated Rapid Amplification of cDNA Ends (RLM-RACE)

Extracts of total nucleic acids (5 μg) (Rodríguez-García et al., 2014) and total RNA (10 μg) from our *Botrytis* isolate and other *Botrytis* isolate used as negative control were analyzed by Northern blot hybridization using 3′-terminal negative-stranded RNA specific digoxigenin-labeled riboprobe. A riboprobe of 345 nt (from nt 2547 to nt 2891) was generated by RT-PCR using specific primers BC44 (positive sense) (5′ GTGTTACTGAAACCGTCCTG 3′) and BC43 (negative sense) (5′ ATTTAATACGACTCACTATAGGTGGGAGGCAGTTTTAGATCGTGC 3′) including the T7 polymerase promoter sequence (underlined). The 3′ ribroprobe was transcribed and marked with digoxigenine directly from the RT-PCR product using T7 RNA polymerase and DIG RNA labeling mix (Roche) following the manufacturer's recommendations. Extracts of dsRNA (Rodríguez-García et al., 2014), total nucleic acids and total RNA were used as templates to detect the viral sequence by RT-PCR using specific primers BC43 and BC44.

5′ and 3′ RACE were performed with FirstChoice RLM-RACE kit according to the manufacturer's instructions (Life Technologies). Total RNA was poly-adenylated using Yeast Poly-A Polymerase (Affymetrix) before conducting 3′ RLM-RACE. PCRs were performed with specific primers and Taq DNA polymerase (Roche). For 5′ RACE BC39 outer primer (negative sense) (5′GGATACAAACTGCAAGCAACTC 3′) and BC40 inner primer (negative sense) (5′TCCCACTGACTGCGTAATTC 3′) were used; and for 3′ RACE BC41 outer primer (positive sense) (5′CTCCTGTCGAAACCCATATCC 3′) and BC42 inner primer (positive sense) (5´ ATGCCTCGCGGTGATTATCG 3′) were used. The PCR products were cloned using the vector TA dual promoter cloning kit pCRII (Invitrogen) and, after transformation in *Escherichia coli* INVαF′ competent cells, plasmid were amplified with M13 universal primers from individual colonies and sequenced (htpp://www.macrogen.com).

Bioinformatic and phylogenetic analysis

Sequenced reads were *de novo* assembled using CLC Genomics Workbench software with a minimum contig length of 61 nts and other parameters as default. To search for viral sequences, large contigs with no match with B. cinerea databases (isolates B05.10

Fig. 3. Phylogenetic tree obtained by a Maximum Likelihood method with the RdRp sequences of 45 mycoviruses, including BOLV, and 3 plant ourmiaviruses. The RdRp sequence of TMV (ABN79257.1) was included as an outgroup. All bootstrap values (%) are represented at each node of the tree (test with 1000 replicates). Branch lengths are proportional to the number of amino acid substitutions and are measured by the scale bar. Sequence accession numbers of viruses used are: *Sclerotinia sclerotiorum* mitovirus 6 (AHX84133.1), *Sclerotinia sclerotiorum* mitovirus 14 (AHF48630.1), *Sclerotinia sclerotiorum* mitovirus 11 (AHF48627.1), *Sclerotinia sclerotiorum* mitovirus 3 (AGC24232.1), *Sclerotinia hooeocarpa* mitovirus (AAO21337.1), Ophiostoma mitovirus 3a (NP_660176.1), *Sclerotinia sclerotiorum* mitovirus 7 (AHE13866.1), *Sclerotinia sclerotiorum* mitovirus 12 (AHF48628.1), Ophiostoma mitovirus 3b (CAJ32468.1), Botrytis cinerea mitovirus 1 (YP_002284334.1), *Buergenerula spartinae* mitovirus 1 (AHY03257.1), *Cryphonectria parasitica* mitovirus 1-NB631 (NP_660174.1), *Cryphonectria cubensis* mitovirus 2b (AAR01974.1), *Cryphonectria cubensis* mitovirus 2c (AAR01975.1), *Cryphonectria cubensis* mitovirus 2a (AAR01973.1), *Thanatephorus cucumeris* mitovirus (AAD17381.1), Heterobasidion mitovirus 1 (AIF33766.1), Ophiostoma mitovirus 1a (CAJ32466.1), Tuber aestivum mitovirus (YP_004564622.1), Ophiostoma mitovirus 1c (AGT55876.1), Ophiostoma mitovirus 1b (CAJ32467.1), Rhizophagus sp. HR1 mitovirus-like ssRNA (BAN85985.1), Rhizophagus sp. RF1 mitovirus (BAJ23143.2), *Sclerotinia sclerotiorum* mitovirus 2 (AEX91879.1), *Sclerotinia sclerotiorum* mitovirus 9 (AHE13865.1), *Cryphonectria cubensis* mitovirus 1a (AAR01970.1), *Sclerotinia sclerotiorum* mitovirus 15 (AHF48631.1), *Sclerotinia sclerotiorum* mitovirus 8 (AHE13867.1), *Gremmeniella abietina* mitochondrial RNA virus S2 (AEY76153.1), Ophiostoma mitovirus 6 (NP_660181.1), Gremmeniella mitovirus S1 (AAN05635.1), *Gremmeniella abietina* mitochondrial RNA virus S2 (YP_077184.1), *Fusarium circinatum* mitovirus 2-1 (AHI43534.1), *Sclerotinia sclerotiorum* mitovirus 5 (AHX84130.1), *Sclerotinia sclerotiorum* mitovirus 1 (AEX91878.1), Ophiostoma mitovirus 5 (NP_660180.1), Ophiostoma mitovirus 4 (NP_660179.1), *Sclerotinia sclerotiorum* mitovirus 4 (AGC24233.1), Thielaviopsis basicola mitovirus (AAZ95419.1), *Fusarium circinatum* mitovirus 1 (AHI43533.1), Ophiostoma mitovirus 7 (AGT55877.1), *Phytophthora infestans* RNA virus 4 (AEM89293.1), Saccharomyces 20S RNA narnavirus (NP_660178.1), Saccharomyces 23S RNA narnavirus (NP_660177.1), Cassava virus C (CsVC, YP_003104770.1), Ourmia melon virus (OuMV, YP_002019757.1) and Epirus cherry virus (EpCV, YP_002019754.1).

and T4) were subjected to BLASTN and BLASTX alignments against the NCBI database (Altschul et al., 1997) at the nucleotide and the protein level, respectively. Conserved motifs in BOLV protein sequence were searched by alignments with related RdRps proteins using Clustal Omega (McWilliam et al., 2013). Potential secondary structures of the 5′ and 3′ terminal regions were predicted and the free energy (ΔG) was estimated using MFOLD software (http://mfold.rna.albany.edu/; Zuker, 2003). Related sequences for phylogenetic analysis were obtained by PSI-BLAST (Altschul et al., 1997). Evolutionary analysis was conducted using the software MEGA6 (Tamura et al., 2013). Multiple sequence alignments of amino acid sequences of viral RdRps were obtained using MUSCLE (Multiple sequence comparison by log-expectation, (Edgar, 2004)), MAFFT (Multiple Alignment using Fast Fourier Transform, (Katoh et al., 2002)) and COBALT (Constraint-based multiple protein alignment tool, (Papadopoulos and Agarwala, 2007)) programs, under the default parameters. The phylogenetic relationships were inferred by using the ML method based on the WAG+G+F protein evolution model (Whelan and Goldman, 2001), chosen by ProtTest 3.4 (Abascal et al., 2005) as the best-fit amino acid substitution model that was implemented in MEGA6, and using the bootstrap phylogeny test with 1000 replicates. All positions with less than 50% site coverage were eliminated. Bayesian-based trees were inferred using the resulting alignments from MUSCLE, MAFFT or COBALT programs and utilizing the Bayesian Markov Chain Monte Carlo (MCMC) method, available in MrBayes v3.2.2 (Ronquist and Huelsenbeck, 2003), based in WAG+G+F protein evolution model. MrBayes analyses were run until two runs converged onto the stationary distribution. Tree topology was drawn from the consensus tree generated in the MrBayes analysis using Figtree (http://tree.bio.ed.ac.uk/software/figtree/). Bayesian posterior probability values provided a measure of statistical support at each node. BOLV sequence was deposited in the EMBL-EBI database under the accession number LN827955.

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Appendix A. Supplementary material

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.virol.2015.11.027.

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