Characterization of the first Rubus yellow net virus genome from blackberry

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Abstract
Rubus yellow net virus (RYNV) is a badnavirus that infects Rubus spp. Mixed infections with black raspberry necrosis virus and raspberry leaf mottle virus cause raspberry mosaic, a disease that leads to significant losses and even plant death. RYNV has been reported in several European countries and the Americas yet there is substantial lack of knowledge, especially when it comes to virus diversity and the evolutionary forces that affect virus fitness outside its primary host, raspberry. Herein, we report the first RYNV episomal genome isolated from blackberry and this is the first report of the virus in Bosnia and Herzegovina. The isolate has five open reading frames (ORFs) and, when compared with other fully sequenced counterparts, showed 82–97% nucleotide pairwise identity. This communication adds to our limited knowledge on RYNV and addresses some of the gaps in RYNV genetics when it comes to the coding capacity of episomal isolates and the probability of the first fully sequenced isolate of the virus being integrated in the raspberry genome.

Keywords Badnavirus · Episomal · Rubus fruticosus L · Evolutionary analysis

Rubus yellow net virus (RYNV) is a member of the genus Badnavirus (family Caulimoviridae) [1, 2] and has been detected in several European countries and the Americas (USA, Canada and Chile) [3, 4]. RYNV host range is, to date, restricted to Rubus spp [5]. The most common symptoms elicited by RYNV on susceptible genotypes are net-like chlorosis along the veins, giving the plant a pale green appearance with some leaves being slightly cupped downwards. While there may be distortion or stunting in some, most genotypes remain symptomless [6]. Synergistic interactions with black raspberry necrosis virus and raspberry leaf mottle virus cause raspberry mosaic disease (RMD) [7], one of the most devastating virus diseases of raspberry [4].

In the field, RYNV spreads by the European large raspberry aphid, Amphorophora idaei Börner in Europe and the large raspberry aphid, A. agathonica Hottes, in North America, most likely in a semi-persistent manner [6]. Moreover, these vectors are also able to transmit the other two viruses in the RMD complex [8].

As other members of the genus Badnavirus, RYNV may be found in two forms: endogenous—integrated in the host genome, or episomal—forming virions [9]. The endogenous pararetroviruses (EPRVs) are inactive and do not cause symptoms, yet they cause major issues in international plant movement and trade given that plants test positive in PCR assays [9–11]. However, there is evidence that, at least in the case of banana streak virus, integrated forms are able to reactivate and form virions, initiating replication and cause disease [12].

There are only two full-length RYNV sequences available in GenBank (as of May 2022), both from raspberry. RYNV-Ca from Canada [2] and RYNV-BS, an isolate that likely originated in Europe but was characterized in the United States [13]. Here, we describe the first RYNV episomal genome from blackberry (Rubus fruticosus L.; cultivar Jumbo) collected in Bosnia and Herzegovina (BiH).

During the 2018 growing season, asymptomatic blackberry samples were collected from orchards in Banja Luka,
Bugojno, and Teslic. Total nucleic acids (TNA) from 28 samples were extracted and reverse transcribed using random primers and the Maxima® reverse transcriptase essentially as described in Poudel et al. [14]. Reverse transcription was performed to increase the detection limit by also targeting the RNA intermediate form and mRNA transcripts of the virus. cDNA quality was evaluated using the NADH dehydrogenase ND-2 subunit (ndhB gene) transcript as an internal control [15]. Samples were screened with primers RYNV6F/RYNV6R [16] and DDF/DDR (this study) (Supplementary Table 1) that amplify a 463 bp and 203 bp of the genome, respectively. Two samples, K20 and K26, were tested positive and were further assayed to determine whether they were infected by the episomal or endogenous forms of the virus. To remove host genome and any integrated viral forms, TNA samples were digested by Turbo™ DNase (ThermoFisher Scientific, USA) for 30 min at 37°. Successful digestion was assessed using the ndhB control. The primers can amplify the genomic copy that incorporates an intron and give a band of ~1300 bp [15] that easily differentiates from the transcripts of ~720 bp, with the latter being the only amplified after digestion (data not shown). Both samples were retested with RT-PCR (Fig. 1). As K20 was positive for only one primer set, possibly due to the sequence divergence at the primer region, K26 was chosen for further analysis, including rolling circle amplification (RCA), which preferentially amplifies circular DNA using the Illustra TempliPhi 500 Amplification Kit (GE Healthcare, Buckinghamshire, UK) [17]. Back-to-back primers RYNVB2BF/RYNVBR2BR were designed within the DDF/DDR screening region were used on the RCA product, yielding a product of approximately 8000 bp (Fig. 1). Using RYNV-BS as a reference, primers were designed across ~1 kb overlapping regions to amplify the full genome (Fig. 1; Supplementary Table 1). Each section was amplified and sequenced at least three times, and the consensus genome was deposited in GenBank under accession MZ358192.

The blackberry isolate was compared against the only two other, fully sequenced isolates, both from raspberry. Sequences were aligned using MUSCLE in MEGA 5.2 [18]. ORFinder was used to identify ORFs (http://www.ncbi.nlm.nih.gov/projects/gorf) and the Sequence Demarcation Tool Version 1.2 (SDTv1.2) was applied to assess pairwise identity [19]. Each ORF was analyzed individually to determine the selection pressure using FUBAR [20] within Selection Map version1 [21].

The RYNV-BiH is 7816 nucleotides (nt) long and shares 82% and 97% full-genome identity with the RYNV-Ca and RYNV-BS, respectively (Table 1). For this analysis, we follow the RYNV-Ca ORF designation as was the first
published. A single-nucleotide insertion in RYNV-BiH disrupts the presumed beginning of ORF1, resulting in a premature stop codon. Badnaviruses may utilize alternative start codons [22–24]; therefore, RYNV-BiH was reanalyzed with ORFinder allowing for alternative start codons and a UUG was identified. Evidence for the alternative start codon is the fact that the presumed ORF shares 92–98% pairwise amino acid (aa) identity to its raspberry counterparts. The functions of ORF 1 and downstream ORF 2 have not been characterized in depth and their function is unknown. All isolates contain a large polyprotein (ORF 3) [2, 13] with all essential replication-associated motifs including ribonuclease H and reverse transcriptase, pepsin-like aspartate protease, and a zinc-binding motif. Both ORF 4 and 6 codes for putative proteins with nuclear export signals [13]. The genome organization of RYNV-BiH and RYNV-BS are similar with five ORFs whereas RYNV-CA encodes seven (additional ORFs 5 and 7) (Table 1).

Table 1 Percentage pairwise identity of the genome and ORFs shared by all fully sequenced isolates of rubus yellow net virus

| Compared RYNV isolates | Percentage pairwise identity |
|------------------------|-----------------------------|
|                        | ORF 1 nt/aa | ORF 2 nt/aa | ORF 3 nt/aa | ORF 4 nt/aa | ORF 6 nt/aa | Full genome |
| RYNV-BiH RYNV-BS       | 96%/98%     | 96%/87%     | 97%/98%     | 98%/99%     | 96%/90%     | 97%         |
| RYNV-BiH RYNV-Ca       | 87%/92%     | 82%/84%     | 82%/90%     | 75%/67%     | 79%/59%     | 82%         |
| RYNV-BS RYNV-Ca        | 87%/90%     | 81%/76%     | 82%/90%     | 74%/67%     | 78%/57%     | 82%         |

Herein, we present the first RYNV episomal genome from an asymptomatic blackberry and also the first report of the virus in Bosnia and Herzegovina. This isolate is most similar to RYNV-BS, an isolate of possible European origin, whereas it is quite divergent when compared to the RYNV-Ca. This may be because of the geographic isolation of the isolates or because as indicated by Ho et al. [11] RYNV-Ca may be an endogenous form which integrated into the raspberry genome over 150 years ago. While sequence availability is scarce and more genome data are needed to fully explore this virus, we hypothesize that the host (raspberry vs blackberry) does not significantly affects virus fitness. This is particularly interesting if we take into account that the RYNV-BS isolate is likely at a genetic equilibrium, as it is infecting the plant assayed positive for the virus for more than 20 years, whereas the blackberries sampled in BiH have been in the ground for 5 years at the time of sampling. This work further expands our understanding of RYNV diversity and host range.

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Author contributions DS, DD, and IET contributed to study conception and design. Material preparation, data collection, and analysis were performed by MV, DS, and DD. The first draft of the manuscript was written by all authors; and all authors commented on previous versions of the manuscript. All authors read and approved the final manuscript.

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Data availability The nucleotide sequence of the virus reported in this work has been deposited in the GenBank database under accession number MZ358192.

Declarations

Conflict of interest The authors declare no conflict of interest.

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