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Short communication

Identification and molecular characterization of a novel foveavirus from *Rubus* spp. in Turkey

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ABSTRACT

A novel plant virus was identified by high-throughput sequencing analysis from a raspberry plant showing slight mottling symptom. The complete genome sequence of this virus is 8645 nucleotides long, including the 5′ and 3′ UTRs. Its genome contains five ORFs and is very close to members of the genus Foveavirus (Quinvirinae, Betaflexiviridae) in terms of genome organization, TGB presence and the sizes of the RdRp and CP proteins. The novel virus shares 33.5–51.3 % and 23.3–41.3 % nucleotide identity to other genera of the *Betaflexifiviridae* family based on polymerase (RdRp) and CP genes, respectively. Compared to other foveavirus species, the RdRp protein showed the highest sequence identity (45.3 %) to the RdRp of peach chlorotic mottle virus (PCMV) while the maximal sequence identity for the CP protein was 33.9 % with grapevine rupestris stem pitting-associated virus (GRSPaV). The low nucleotide and amino acid sequence identity with known foveaviruses indicated that it was a novel virus, for which the provisional name "rubus virus 1 (RuV1)" is proposed. The phylogenetic analysis supports the assignment of this virus as a new species of the genus *Foveavirus*. A survey of 537 *Rubus* spp. samples grown in six provinces of Turkey, including some symptomatic samples, showed a RuV1 prevalence of 2.2 %, confirming its presence in both raspberry and blackberry plants in a single province, although no obvious association between virus infection and specific symptoms was found.

Raspberries are very important small fruit crops in the genus *Rubus* of the family *Rosaceae* (Skrovankova et al., 2015) and their global production was 812,735 tons in 2017 (FAOSTAT, 2018). Raspberry has gained considerable importance recently in Turkey and its annual production reached to 5875 tons in 2018 (TUIK, 2018). Red raspberry, *Rubus idaeus* L., is a native berry bush to Turkey and the species name "idaeus" refers to its occurrence on Mount Ida near Troy in northwest Turkey (Huxley, 1992). More than 40 viruses affecting *Rubus* spp. were recently reported by EFSA (2019) but till recently most of the virus diseases were described based on the symptoms on the woody indicator plants by graft inoculation (Martin et al., 2013). After the introduction of high-throughput-sequencing (HTS) technology, the number of novel viruses that have been identified and characterized have increased sharply (Massart et al., 2017; Çağlayan et al., 2019). Therefore, this technology is increasingly being used for the quick identification of viruses replicating in plant tissues (Massart et al., 2014), including for *Rubus* species like black currant (James and Phelan, 2017; Koloniuk et al., 2018).

Foveaviruses (Viruses; Riboviria; Tymovirales; Betaflexiviridae; Quinvirinae; Foveavirus) are characterized by flexuous filaments ($800-1000 \times 12-15$ nm) that contain a non-segmented single-stranded RNA genome and are distinct in having five ORFs and larger CP than most members of the family. Their genome contains five ORFs including RNA-dependent RNA polymerase (RdRp), triple gene block (TGB) proteins, and the coat protein (CP). No vector is known for foveaviruses so far and the natural host range of individual species is restricted to a single or a few hosts (Adams et al., 2012). There has been only one identified member of Foveavirus infecting blackberries, provisionally named as rubus canadensis virus 1 (RuCV-1), from the United States (Sabanadzovic et al., 2013). Regarding the host range, as with other foveaviruses, RuCV-1 infects only woody dicotyledonous species and cannot be mechanically transmitted to herbaceous hosts. Results of a survey in several U.S. states suggested that RuCV-1 is not widespread in the blackberry germplasm.

In order to resolve etiology of mild mottling and vein chlorosis (Fig. 1) of *Rubus idaeus* cv. Rubin, a sample was collected from Hatay

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Fig. 1. Mottling and vein chlorosis on raspberry leaves cv. Rubin, used for HTS analysis.

province of Turkey in April 2018 and used as the source for HTS analysis and further characterization. For field surveys, leaf samples were collected from 123 raspberry and 414 blackberry plants grown in Hatay, Adana, Mersin, Kahramanmaraş (East Mediterranean Region), Rize (Black Sea Region) and Bursa (Marmara Region) provinces where raspberry and blackberry production is economically important in Turkey.

Total RNA extraction, library preparation, high throughput sequencing, RT-PCR and data analysis were carried out as described previously (Çağlayan et al., 2019). The primers were designed such that amplicons shared at least 100 overlapping base pairs to avoid any possible missassembly (Supl. 1). The HTS yielded a total number of 10,428,288 reads ranging from 36 to 151 nucleotides from the raspberry sample. After removing the low quality and duplicated reads, 2,969,162 unique reads were de novo assembled and 63,185 contigs larger than 150 nt were produced. The TBLASTX analysis against a viral refseq database revealed a contig of 8529 nucleotides with an amino acid identity of 62.5 % on 590 a.a. to the peach chlorotic mottle virus (PCMV) of the genus *Foveavirus* in the family *Betaflexiviridae*. Further confirmation done by a BLASTX search on the NCBI non-redundant database resulted in 46 % identity over 75 % coverage of PCMV genome. None of the other contigs were mapped to any other virus or viroid sequences available in GenBank. A single isolate of the virus was identified in the HTS dataset.

The complete genome of the virus, with the proposed name rubus virus 1 (RuV1), was obtained using overlapping RT-PCR and 3' and 5' RACE and was 100 % identical to the assembled contig. This 100 % identity was supported by a complementary analysis on HTS dataset. A SNPs analysis was carried out using the 6155 reads mapped on the RuV-1 contig (geneious mapper, medium low sensitivity, 20 % mismatches and 10 % gaps allowed). This analysis revealed 39 SNPs with a maximum frequency of 15 % which would not interfere with the consensus sequence observed by classical sequencing. The complete genome of the virus was 8645 nt (GenBank accession no. MN944023) including 85 nt 5' UTR and 109 nt 3' UTR. RuV1 comprises 5 open reading frames (ORF) (Fig. 2). ORF1 codes for a replication-related protein (244 kDa) which contains the conserved domains for methyltransferase (MT), 2OG-Fe(II) oxygenase, peptidase (P), helicase (Hel) and RNA-dependent RNA polymerase (RdRp) activity. ORF2 (27 kDa), ORF3 (13 kDa) and ORF4 (7 kDa) form the triple gene blocks (TGBs) (Erhardt et al., 2005) and ORF5 (27 kDa) is the CP gene. The arrangement and structure of these five ORFs are similar to other foveaviruses.

Based on the full genome sequence of the virus, reference genome sequences were retrieved from the GenBank and were used for pairwise sequence alignments and phylogenetic analysis. In order to clarify the taxonomical status of the new foveavirus in the family *Betaflexiviridae*, sequence comparisons were performed for the polymerase and coat protein genes and for the corresponding proteins. Low nucleotide identities were observed between RuV1 and apple stem pitting virus (ASPV) which is the type species of *Foveavirus* genus for RdRp (51.3 %) and CP (30.1 %) genes (Supl. 2). According to the ICTV, viruses of suggested new genera are supposed to be less than 45 % nt identical in those genes with viruses already reported (King et al., 2012). Phylogenetic analysis using full genomic nucleotide sequences of reference sequences in Tymovirales, the RuV1 and apple stem pitting virus (ASPV) were clustered together which confirmed that the new virus belongs to the genus *Foveavirus* (Fig. 3).

Whole genome alignments of the RuV1 and other foveavirus species showed 41.1–50.1 % identity at nucleotide level. The accepted molecular species demarcation criteria for the family *Betaflexiviridae* are more than 28 % nucleotide divergence or 20 % amino acid divergence in the polymerase and coat protein genes (Adams et al., 2012). By these criteria, RuV1 can be considered as a new species in genus *Foveavirus*. Pairwise alignment of protein products of the new virus and foveavirus reference sequences were 39.7–45.3 % for RdRp, 30.6–45.7 % for TGB1, 39.5–52.1 % for TGB2, 26.5–55.2 % for TGB3 and 19.1–33.9 % for CP (Table 1). Recombination plays an important role in evolution of plant RNA viruses and this event has previously been reported to be involved in the evolution of some *Betaflexiviridae* members (Singh et al., 2012; Yoon et al., 2014). However, recombination analysis did not reveal any recombination events in the RuV1 evolution (unpresented results). Subsequently, for better elucidating relationship between RuV1 and other reference sequences belonging to *Foveavirus* genus, their full genome sequences were aligned and the phylogenetic tree was constructed using ML method and GTR + G substitution model. The results showed that RuV1 was closely related to GVT and GRSPaV but it definitely is a distinct species in the foveavirus with robust bootstrap (100 %) (Fig. 4A). Phylogentic trees drawn from RdRp (Fig. 4B) and CP (Fig. 4C) amino acids sequences confirmed previous results and RuV1 was placed in a distinct clade among foveaviruses.

Three primer sets targeting the TGB2-TGB3 (7230 F / 7724R), RdRp (2612 F / 3679R) and CP (7609 F / 8443R) were designed using the RuV1 genome as the reference to confirm the presence of this virus by RT-PCR in both raspberry and blackberry samples collected from major *Rubus* producing provinces in Turkey. In total, 537 *Rubus* samples (123 raspberry, 414 blackberry) were collected from Hatay (222), Bursa (201), Adana (46), Mersin (45), Kahramanmaraş (17), and Rize (6) provinces and tested by RT-PCR. The majority of the samples were collected from Hatay and Bursa where HTS analyzed raspberry plant was collected and *Rubus* production is very important in Turkey, respectively. Among all tested samples, only 12 *Rubus* samples collected from two different orchards in Hatay province (prevalence of 5.4 %) were found infected by RuV1 by using TGB2-TGB3 specific primers, however, among them, only five samples by primers amplifying RdRp and two samples by primers amplifying CP gene were found positive for RuV1. No other sample was positive for RdRP and CP detection. Despite the fact that the originally HTS analyzed raspberry plant was showing slight mottling symptoms, among other RuV1 positive plants (seven raspberries, five blackberries), only one more raspberry plant was

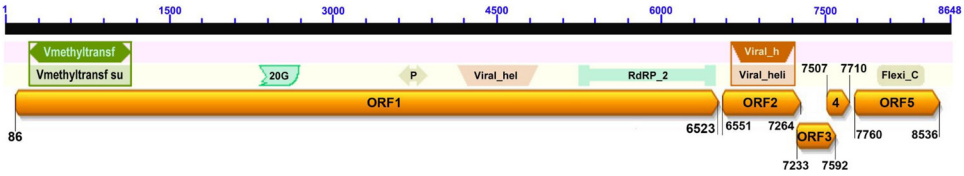

Fig. 2. Genome organization of the new foveavirus and position of conserved domains along the genome. ORF1 codes for a replication-related protein. ORF2, ORF3 and ORF4 constitute the triple gene block which are responsible for virus movement. ORF5 encodes structural coat protein.

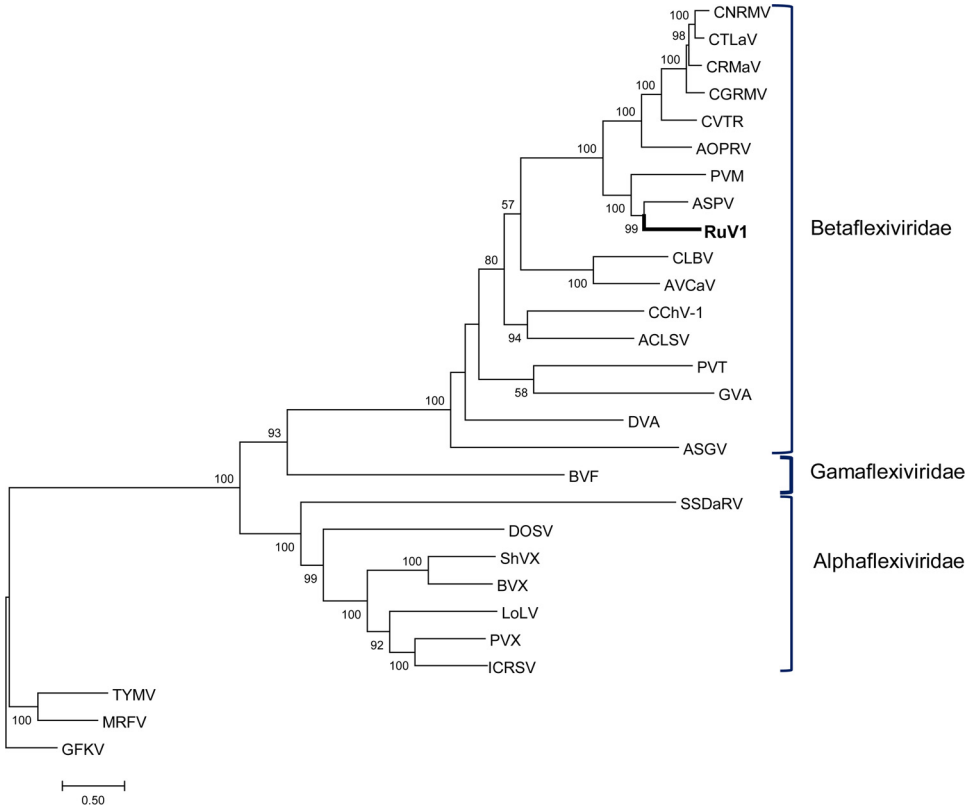

Fig. 3. Maximum likelihood tree based on complete nucleotide sequences of RuV1 (in bold) and reference sequences in the Tymovirales. Bootstrap was done using 1000 replicates and values above 50 % are shown.

showing slight mottling, six of them were showing other virus-like symptoms such as reddening, deformation on the leaves and four of them were symptomless. Beside the whole genome, eleven, four and one amplicons from TGB2 - TGB3 (MN944027, MN944028, MN944029 and MT215227- MT215235), RdRp (MN944024, MN944025, MN944026 and MT215226) and CP (MN944030) regions were sequenced and deposited in GeneBank, respectively. The identity between the genome of the RuV1 isolate sequenced by HTS and these isolates ranged from 82.2–100% at nucleotide level.

The possible association of RuV1 with symptoms will require additional efforts and investigations over time with a focus on infected orchards. The new virus might have been spread locally by vegetative propagation of *Rubus* spp. suckers or stem cuttings. However, its transmission by a vector should be investigated. Koch's postulates have not yet been fulfilled, nor the disease causation studied in depth (Fox, 2020), so we cannot conclude on the virus-symptom correlation or the transmission modes of this novel virus. A scaled biological characterization following a previously proposed framework (Massart et al., 2017) could bring more information on the potential phytosanitary risks.

The genomic sequence of RuV1 obtained in this study enabled the development of a specific RT-PCR for the rapid detection of the virus. Three protocols using 3 different primer pairs targetint RdRp, CP and TGB were developed and applied in parallel on the same samples. Interestingly, the protocol targeting TGB allowed the detection of more isolates of the virus compared to RdRp and CP protocols. This protocol is therefore a good candidate to further study the distribution, transmission and pathogenicity of RuV1 in different geographical conditions. The absence of RT-PCR detection using RdRp and CP protocols raises also questions related to the diversity of RuV1 sequence for these genes, at least on the primer binding site. It is therefore likely that the diversity of RuV1 is only very partially explored and this observation warrants further investigation.

In conclusion, a novel virus in the *Betaflexiviridae*, tentatively named rubus virus 1 (RuV1) was identified in a cultivated *Rubus* spp. and its complete genome determined. Sequence analysis showed that RuV1 is a

Table 1
Pairwise percentage identities of the genomic and gene product sequences between rubus virus 1 (RuV1) and the other members of the *Foveavirus* genus.

Virus	Genome (nt)	Pol (aa)	Pol (nt)	TGB1 (aa)	TGB2 (aa)	TGB3 (aa)	CP (aa)	CP (nt)
Peach chlorotic mottle virus (PCMV)	50.1	45.3	52.6	45.7	43.7	35.8	26.1	36.4
Apple stem pitting virus (ASPV)	48.6	43.2	51.3	44.6	50.4	50	19.1	30.1
Apricot latent virus (ApLV)	48.5	42.9	51.2	43.8	48.8	40.3	21.6	28.6
Asian prunus virus 1 (APV1)	42.2	39.7	49	31.4	50.4	49.3	20.6	29
Asian prunus virus 2 (APV2)	42.8	39.9	49.3	31.4	52.1	38.8	19.2	29.5
Asian prunus virus 3 (APV3)	41.1	40.1	49.3	30.6	51.2	47.8	20.1	28.7
Grapevine rupestris stem pitting-associated virus (GRSPaV)	49.3	41.6	51.1	37.9	39.5	26.5	33.9	42.7
Rubus canadensis virus 1 (RuCV)	48.5	39.8	49.5	36.8	47.5	55.2	28.1	41.1
Grapevine virus T (GVT)	49	40.6	50.3	39.9	46.2	29.9	29	43.3
Apple green crinkle associated virus (AGCaV)	48.7	42.9	51.1	45.5	47.1	50	21	31.9
Cherry Virus B (ChVB)	46.6	39.9	48.8	32.6	48.8	46.4	22.6	31.6

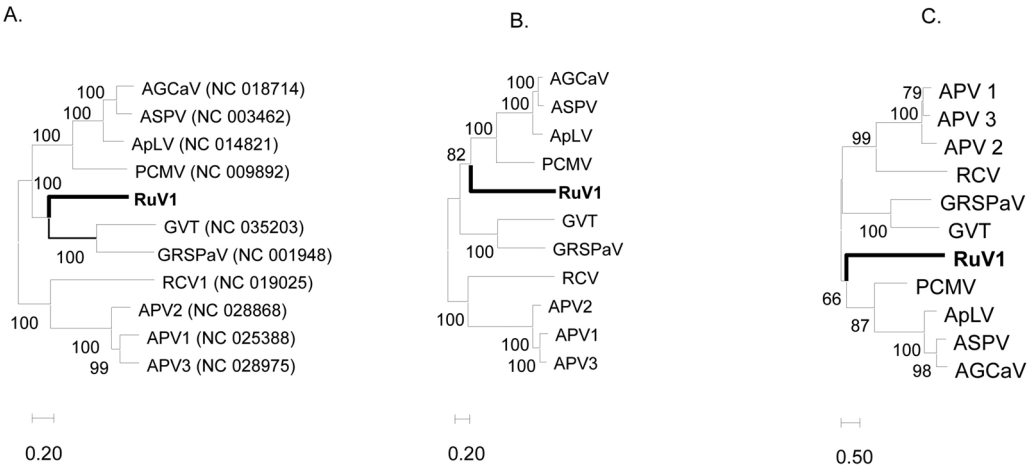

Fig. 4. Maximum likelihood phylogenetic tree reconstructed from RuV1 and other refseq from Foveaviruses. A. complete genome (nt). B. amino acid sequences of RdRp gene. C. amino acid sequences of CP.

new species in the genus *Foveavirus*. An extensive survey of 537 *Rubus* spp. samples from six provinces in Turkey detected RuV1 with a prevalence of 2.2 % and identified two host plants: raspberry and blackberry. These results, combined with the difficulty of detecting some regions of the genome, promote the future application of HTS technologies to provide fast and accurate virome characterization for the monitoring of known and unknown viruses for quarantine and routine diagnosis in certification programs.

Compliance with ethical standards

This article does not contain any investigation involving human or animal participants.

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Declaration of Competing Interest

On behalf of all authors, the corresponding author states that there is no conflict of interest.

CRediT authorship contribution statement

Mona Gazel: Writing - original draft, Methodology, Resources. **Vahid Roumi:** Formal analysis, Data curation. **Kivilcim Ördek:** Investigation, Validation. **Francois Maclot:** Investigation, Validation. **Sebastien Massart:** Software, Supervision, Funding acquisition. **Kadriye Çağlayan:** Conceptualization, Project administration, Funding acquisition.

Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.virusres.2020.198078.

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