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ANALYSIS OF VIROME BY HIGH-THROUGHPUT SEQUENCING REVEALED MULTIPLE INFECTION AND INTRA-VIRUS DIVERSITY IN A SINGLE GRAPEVINE PLANT

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The ribosomal-depleted total RNA from white-berry grapevine (*Vitis vinifera*, SK933) plant showing severe chlorosis and downrolling of leaves was used for the high-throughput sequencing (HTS) analysis in order to unravel the potential contribution of the viral pathogens to the symptomatology observed. The combination of *de novo* assembly and mapping of ca. 1.1 millions of HTS reads enabled to identify and characterise a complex viral/viroid infection involving Grapevine leafroll-associated virus-2 (GLRaV-2), Grapevine leafroll-associated virus-3 (GLRaV-3), Grapevine rupestris stem pitting-associated virus (GRSPaV), Grapevine rupestris vein feathering virus (GRVFV), Grapevine Syrah virus-1 (GSyV-1) and Hop stunt viroid (HSVd). The determined nearly complete genomes of GLRaV-2 SK933 showed its high genetic divergence from previously characterised isolates. In case of GRSPaV, two variants representing different evolutionary lineages have been identified in the plant. The results further pinpoint the complexity of grapevine viral diseases and show that mixed virus infection of grapevine is rather a rule than an exception.

Keywords: *Vitis vinifera*, virus, diversity, next generation sequencing

Grapevine (*Vitis vinifera* L.) is one of the most ancient and widely grown crops in the world, used for the production of fresh fruits, wines, juices, and other by-products. On the other hand, grapevine has turned out to be the cultivated plant hosting the highest number of viral pathogens (Martelli, 2017), many of them being associated with economically important diseases, such as leafroll, rugose wood complex, leaf degeneration and flecks (Basso, Fajardo and Saldarelli, 2017).

The study of genetic diversity and the evolutionary mechanisms shaping the virus variability is important to understand virus epidemiology and emergence and is a prerequisite to design effective diagnostic tools and implement effective disease management measures.

Mutations, connected with positive and negative selection and recombination of genome are the main evolutionary forces driving the genetic diversity of viral populations. These processes, leading to the dynamic genetic structure of virus populations, have a significant role in the epidemiology of the grapevine viruses as they constitute the basis of their adaptation to the environment (Almeida et al., 2013; Maliogka et al., 2015).

A rapid, specific and effective diagnose integrated to the certification schemes is one of the most important tools to control grapevine viruses (Zherdev et al., 2018). However, the effectiveness of such measures can be negatively affected by a high virus genetic variability and occurrence of divergent variants escaping the detection (Glasa et al., 2015).

Knowledge on the occurrence of grapevine viruses in Slovakia and their characterisation remains insufficient. First studies aimed to characterize the grapevine viruses spread in Slovakia were based on standard genomic tools (Glasa, Predajňa and Komínek, 2011; Glasa and Predajňa, 2012; Predajňa et al., 2013; Predajňa and Glasa, 2016), possibly not detecting the whole viral complexity present in the grapevines. The recent developments of high-throughput sequencing (HTS) technologies and bioinformatics have drastically improved identification and characterisation of viral pathogens without prior knowledge of their primary structure (Maliogka et al., 2018). HTS has mostly had an impact so far through the identification and characterisation of new grapevine virus species, the study of diseases of unknown aetiology, with the identification of candidate disease-associated agents and, for some viruses, a large improvement of existing diagnostic assays (Saldarelli et al., 2017). In recent years, HTS has provided the possibility to identify and characterize several common or emerging grapevine viruses in Slovakia (Glasa et al., 2014; 2015; 2017; 2018).

The aim of the present work was the unbiased identification and characterization of complete virome present in a grapevine plant showing severe virus-like symptoms.

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Material and method

An approximately 30-year old white-berry grapevine of unknown origin grown in the vineyard in Pezinok (western Slovakia, GPS coordinates: 48° 18' 09.8" N 17° 15' 40.7" E) and showing pronounced chlorosis and leafroll, was selected for the HTS analysis (further referred as the SK933 sample).

Total RNAs from fully developed leaves collected in August 2017 were extracted using the Spectrum Plant Total RNA Kit (Sigma Aldrich, St. Louis, MO, USA) and ribosomal RNA was removed using the Ribo-Zero rRNA Removal Kit (Illumina, San Diego, USA). The sample of ribosomal-depleted total RNA was used for double stranded cDNA synthesis using the SuperScript II kit (Thermo Fisher Scientific, Waltham, USA). The cDNA was then column-purified with the DNA Clean & Concentrator™-5 – DNA kit (Zymo Research, Irvine, USA) and quantified with the Qubit 2.0 Fluorometer (Thermo Fisher Scientific, Waltham, USA). Subsequently, the sample was processed with the transposon-based chemistry library preparation kit (Nextera XT, Illumina, San Diego, USA). Low-cycle PCR and mutual indexing of the fragments was carried out. Fragments were purified with 1.8 × AMPure XP beads (BeckmanCoulter, USA) without size selection. The fragment size structure of the DNA library was assessed using the Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, USA). The equimolar pool of 4nM DNA libraries was denatured, diluted to 13 pM and sequenced (200-bp paired-end sequencing) on the Illumina MiSeq platform (Illumina, San Diego, CA, USA).

High-quality trimmed reads were used for *de novo* assembly and contigs aligned to the viral genomes database (Brister et al., 2015) or to the genome of *V. vinifera* (Velasco et al., 2007) using CLC Genomics Workbench 7.5 and Geneious v.8.1.9 softwares. Alternatively, the reads were mapped against the selected full-length sequences of viruses identified in the previous step to map the *de novo* assembled contigs (retrieved from www.ncbi.nlm.nih.gov).

Phylogenetic analyses and comparisons were performed using the MEGA v.7 (Kumar, Stecher and Tamur, 2016) and DnaSP v.6 (Rozas et al., 2017) softwares.

The nucleotide sequences reported in this paper have been deposited in the GenBank database under accession numbers listed in Table 1.

Results and discussion

The SK933 grapevine plant, exhibiting pronounced chlorosis and downrolling of leaves, was selected for the HTS analysis in order to unravel the potential contribution of the viral pathogens to the symptomatology observed.

The *de novo* assembly of about 1.1 million of high-quality HTS reads (average length of 164.9 bp) from this grapevine sample produced more than 20 000 contigs longer than 280 bp (of which 19 851 contigs mapped to the *V. vinifera* host). Several of the other generated contigs were of viral/viroid origin and led to the identification of a complex co-infection of the SK933 plant by Grapevine leafroll-associated virus-2 (GLRaV-2), Grapevine leafroll-associated virus-3 (GLRaV-3), Grapevine rupestris stem pitting-associated virus (GRSPaV), Grapevine rupestris vein feathering virus (GRVFV), Grapevine Syrah virus-1 (GSyV-1) and Hop stunt viroid (HSVd).

Combination of *de novo* assembly and subsequent mapping of NGS reads against genomes of representative isolates of above-mentioned pathogens retrieved from Genbank enabled to obtain nearly complete genomes for GLRaV-2, GLRaV3, GRSPaV and HSVd, as well as partial sequences of GRVFV and GSyV-1. Interestingly, in case of GRSPaV, two genetically different variants could be assembled from the sequence data.

Multiple alignment of the full-length nucleotide sequence of SK933 GLRaV-2 revealed only 72.1 to 85% identity of Slovak isolate with available GLRaV-2 genomes from different parts of the world. Phylogenetic analysis further confirmed the molecular divergence of SK933 GLRaV-2, clustering in a separate branch, most closely related to the Canadian and Chinese isolates (Fig. 1).

On the contrary, nearly full-length SK933 GLRaV-3 sequence showed low divergence, as compared to other GLRaV-3 isolates, and showed the close phylogenetic relationship with GLRaV-3 isolates from North and South American continent (Fig 2).

Table 1 List of viral/viroid pathogens identified in the SK933 from the HTS dataset (1 163 370 high quality reads, mean length 164,9 bp) and their characteristics

Virus acronym	Virus name	Genus	Family	NGS reads mapped to the reference	Genome coverage	Genbank accession numbers
GLRaV-2	Grapevine leafroll-associated virus-2	Ampelovirus	Closteroviridae	16,902	100%	MN548394
GLRaV-3	Grapevine leafroll-associated virus-3	Closterovirus	Closteroviridae	7,435	99.2%	MN548393
GRSPaV	Grapevine rupestris stem pitting-associated virus	Foveavirus,	Betaflexiviridae	1,335 860	93.3% 49.3%	MN548395a MN548396b
GRVFV	Grapevine rupestris vein feathering virus	Marafivirus	Tymoviridae	288	67.4%	not submitted
GSyV-1	Grapevine Syrah virus-1	Marafivirus	Tymoviridae	432	85.7%	not submitted
HSVd	Hop stunt viroid	Hostuviroid	Pospiviroidae	63	100%	MN548397

a) GRSPaV group 2a variant, b)group 3 variant

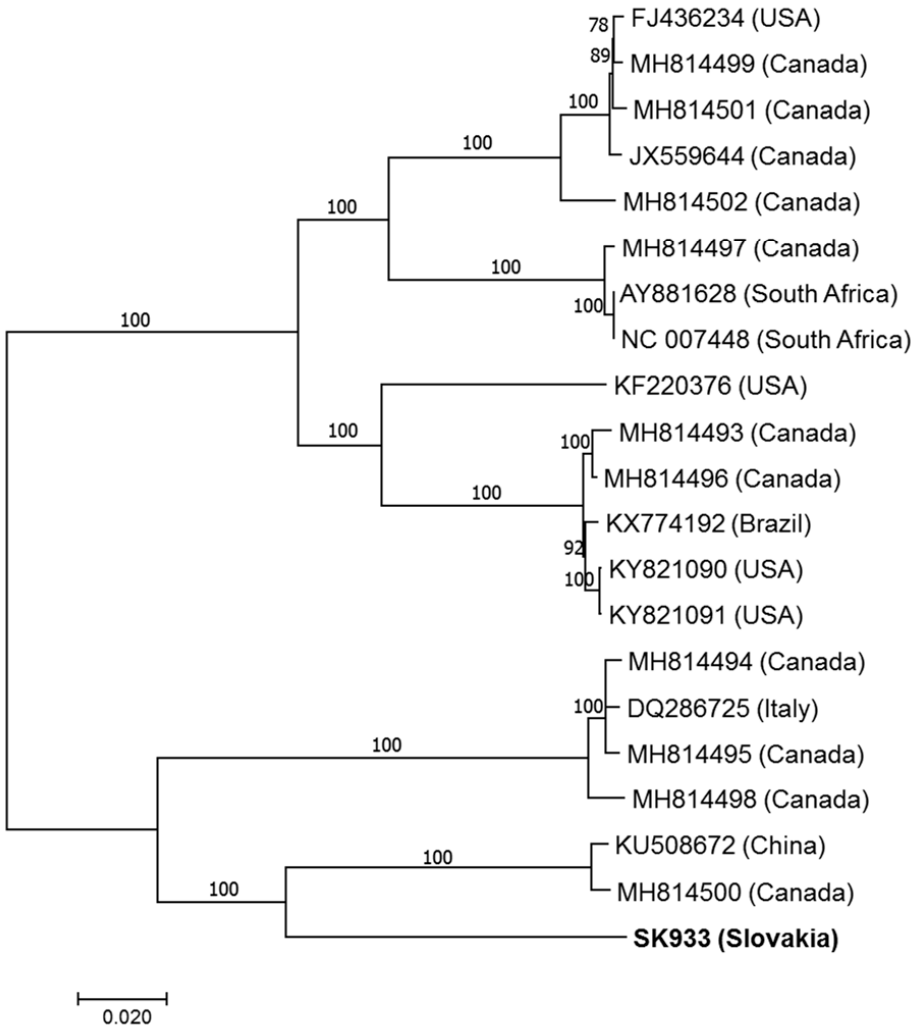

Figure 1 Phylogenetic tree generated on complete nucleotide genome sequences of GLRaV-2 isolates. Isolates are identified by their GenBank accession number and country of their origin. The Slovak isolate sequenced in the present study is highlighted in bold. The scale bar indicates a genetic distance of 0.02. Bootstrap values higher than 70% (1,000 bootstrap resamplings) are indicated

The determined full-length HSVd genome consisted of 297 nt, having the same length and 100% identity with HSVd isolates from Brazil (MF774869, MF774870, MF774873) and China (AB219944).

Two molecularly distinct variants of GRSPaV identified in the grapevine plant differed mutually by 26.8% at the nucleotide level and belonged, respectively, to the phylogenetic groups 2a and 3 (Glasa et al., 2017) and thus to different evolutionary lineages.

In case of two other viruses identified (GRVFV and GSyV-1), their genomes could not be completed because of the lower coverage of the full-length reference sequences (Table 1). However, based on the partial sequence data, both exhibited a close relationship to the respective isolates previously reported from Slovakia (Glasa et al., 2015; Glasa et al., 2019).

HTS-based characterisation of divergent GLRaV-2 variant highlights the need for a continual assessment of the grapevine virus molecular variability (also at the regional level) as a prerequisite to understand the globality of virus variability. Also, the identification of several viruses/viroids and, moreover, different variants of the same virus in a single plant, further emphasizes the complex and heterogeneous nature of grapevine viral diseases (Komínek, Glasa and Komínková, 2009; Glasa et al., 2017), indicating that a complex viral infection of grapevine is rather a rule than an exception.

The close phylogenetic clustering of geographically different isolates from different unrelated countries, or even continents (as observed e.g. for GLRaV-2, GLRaV-3 and HSVd) suggests a long term uncontrolled spread of these pathogens and their widespread dissemination, probably through the exchange and trade of infected propagation material.

The aetiology of the disease observed in the SK933 plant cannot be elucidated properly, as the coexistence of several virus/viroid agents in a single grapevine plant (possibly acting in synergy or antagonism) challenges the establishment of links between the symptoms observed and the presence of a given infectious agent.

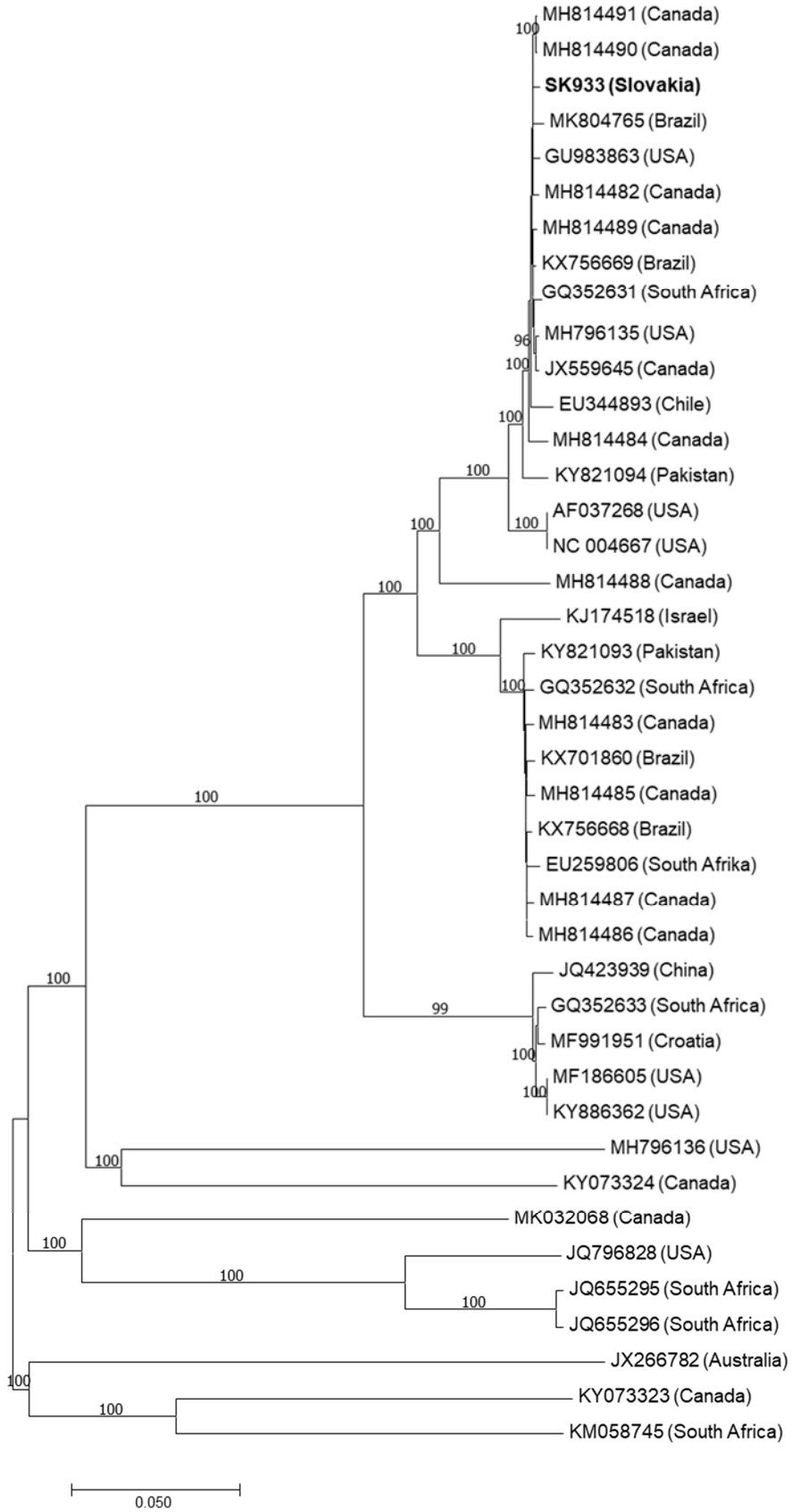

Figure 2 Neighbour-joining phylogenetic tree generated from nearly complete GLRaV-3 sequences. The corresponding sequences of previously characterized isolates are identified by their accession numbers and geographical location. Only bootstrap values ≥70% (1,000 bootstrap resamplings) are indicated. The scale bar indicates a genetic distance of 0.05

HTS technologies have been confirmed to be a powerful diagnostic tool allowing for an exhaustive description of viral species present in many grapevines (Saldarelli et al., 2017). Further information about viromes of grapevines from different production areas or agroecological contexts should help to improve the control and analyses of the different interactions involved in the ecology and pathogenicity of viral agents. This is particularly important for novel viral agents, recently identified by HTS. Moreover, in case of already known viruses, gaining access to the unbiased viral diversity might allow to validate and further improve existing detection assays by fine tuning the detection, e.g. through designing updated primers in order to improve their polyvalence and/or specificity.

Conclusion

In this work, ribosomal-depleted total RNA isolated from leaves of symptomatic grapevine plant grown in a vineyard in western Slovakia was subjected to HTS. Analysis of obtained sequence dataset revealed the presence of complex viral/viroid infection involving members of the Closteroviridae, Betaflexiviridae, Tymoviridae and Pospiviroidae families. Moreover, in case of GRSPaV, two genetically distinct variants were identified. Together, these results further highlight the complex and heterogeneous nature of grapevine virome, hampering a clear-cut establishment of links between the symptoms observed and the respective infectious agent(s) present in the plant.

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