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APPLICATION OF NEXT-GENERATION SEQUENCING FOR SIMULTANEOUS DETECTION OF VIRUSES, VIROIDS AND PHYTOPLASMAS IN GRAPEVINE AND FRUIT TREES

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Introduction

Viral, viroidal and phytoplasma diseases cause severe crop losses in viticulture and orchards (Basso et al., 2017). Blackwood disease of grapevine (bois noir (BN), a grapevine yellows disease associated with the phytoplasma species '*Candidatus* Phytoplasma solani'), European stone fruit yellows (ESFY, '*Ca.* Phytoplasma prunorum'), apple proliferation (AP, '*Ca.* Phytoplasma mali') and pear decline (PD, '*Ca.* Phytoplasma pyri') belong to the most prevalent and economically important phytoplasma diseases of grapevine (*Vitis vinifera* L.), respectively of fruit trees in Europe (Seemüller & Schneider, 2004). The emergent symptoms of Grapevine enation disease (GED) have been reported in Germany in 2006. The etiology of GED, causing formation of enations on the underside of basal leaves and growth depression of infected plants, still remains unknown (Martelli, 2014).
RNA sequencing combined with metagenomic analysis enables an unbiased analysis of infected plant samples. Therefore NGS (Illumina MiSeq) was applied in this study for detection of viral and phytoplasmic infections in grapevine and fruit tree samples.

Materials and Methods

Two grapevine samples (from BN or GED diseased grapevine) were collected in vineyards in Rhineland-Palatinate. Leaf material from three fruit trees (*Prunus* rootstock, apple and pear tree showing ESFY, AP and PD symptoms, resp.) were sampled in the greenhouse and orchard at the JKI (OW, Dossenheim, BW). Total nucleic acids were extracted from 1 g leaf tissue of symptomatic or asymptomatic samples using a CTAB extraction method (Jakovljevic et al., 2016). Total RNA was subjected to a NGS pipeline (Knierim et al., 2017), conducted at the DSMZ. Paired-end 2x301 sequencing was performed on Illumina MiSeq platform. NGS data were analyzed using the bioinformatic software Geneious (Fig. 1).

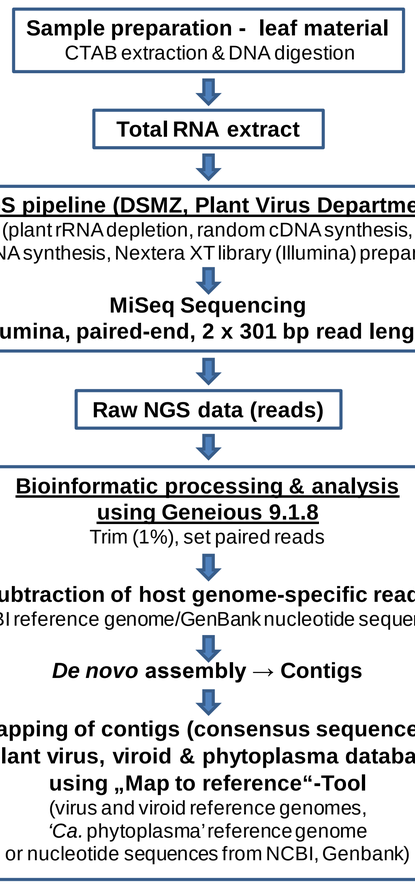

Fig. 1. NGS sample preparation methods followed by NGS pipeline at DSMZ and bio-informatic analysis of untargeted metagenome dataset.

	Sample disease/ origin/ etiology	raw NGS data: # reads (Ø length)	Sub-traction of host genome	*de novo* assembly: # contigs (# assembled reads)	Identified sequences from virus, viroid & phytoplasma databases	NCBI accession number	# contigs mapped to reference	reference sequence coverage (range) (%)	pairwise identity (range) (%)	PCR
Bois noir	**BN** diseased **_V. vinifera_ L. cv. Riesling** (Bernkastel-Kues, RP) (asymptomatic shoot) **_Ca._ Phytoplasma solani**	2 x 1,279,589 reads (266 nt)	91.2% of reads mapped to host genome - 68,423 unaligned reads	9,444 contigs (27,840 reads)	'Bois noir' phytoplasma strain CH-1 (16S & 23S ribosomal RNA genes), strain 284/09 Stolbur phytoplasmadraft, strain 231/09 Stolbur phytoplasma draft	HQ589193 FO393427 FO393428	11	0.1 - 16.7	96.9 - 100.0	-
					GLRaV-1	NC_016509	6	10.3	83.4	-
					GRSPaV	NC_001948	8	39.9	79.9	+
					Grapevine Pinot gris virus	NC_015782	1	7.5	95.3	n. d.
					Grapevine satellite virus	NC_021480	1	32.8	98.9	n. d.
					Hop stunt viroid	NC_001351	1	90.1	87.2	n. d.
Grapevine enation disease	**GED** **_V. vinifera_ L. cv. Dornfelder exhibiting growth depression** (Worms-Herrnsheim, RP) **unknown etiology**	2 x 738,170 reads (243 nt)	97.5% of reads mapped to host genome - 15,455 unaligned reads	2,421 contigs (8,349 reads)	GRSPaV	NC_001948	14	85.6	75.7	+
					GVA	NC_003604	7	24.2	74.5	+
					Grapevine yellow speckle viroid-1	NC_001920	1	77.9	88.2	n. d.
					Hop stunt viroid	NC_001351	2	100.0	80.3	n. d.
Apple proliferation	**AP** diseased **_Malus domestica_ cv. Golden Delicious** (greenhouse at JKI, OW, BW) (symptomatic shoot) **_Ca._ Phytoplasma mali**	2 x 730,424 reads (270 nt)	80.9% of reads mapped to host genome - 278,703 unaligned reads	68,080 contigs (227,744 reads)	*Candidatus* Phytoplasma mali strain AT complete chromosome	NC_011047	382	26.2	90.4	+
European stone fruit yellows	**ESFY** diseased **_Prunus_ rootstock GF655/2** (greenhouse at JKI, OW, BW) (symptomatic shoot) **_Ca._ Phytoplasma prunorum**	2 x 1,034,899 reads (265 nt)	55.0% of reads mapped to host-specific sequences - 931,177 unaligned reads	151,100 contigs (879,075 reads)	*Candidatus* Phytoplasma prunorum - NCBI nucleotide sequence entries (250 contigs mapped to 53 of 161 available sequences)	detailed examination of 16 entries	31	9.6 - 100.0	65.2 - 99.6	+
					Prunus necrotic ringspot virus RNA 1	NC_004362	22	100.0	94.6	+
					Prunus necrotic ringspot virus RNA 2	NC_004363	2	100.0	95.2	
					Prunus necrotic ringspot virus RNA 3	NC_004364	21	100.0	95.0	
					Apple chlorotic leaf spot virus complete genome	NC_001409	19	94.4	89.3	+
Pear decline	**PD** diseased **_Pyrus communis_ cv. Williams Christ** (orchard at JKI, OW, BW) (symptomatic shoot) **_Ca._ Phytoplasma pyri**	2 x 817,371 reads (236 nt)	35.2% of reads mapped to host-specific sequences - 1,058,684 unaligned reads	210,895 contigs (935,732 reads)	*Candidatus* Phytoplasma pyri - NCBI nucleotide sequence entries (1,809 contigs mapped to 66 of 166 available sequences)	detailed examination of 14 entries	125	7.7 - 100.0	57.0 - 96.2	-

Table 1. Summary of analyzed grapevine and fruit tree samples including sample features, results of NGS data processing and analysis of processed NGS data: *de novo* assembled contigs were mapped to (reference) sequences from virus, viroid and phytoplasma databases. Identified genomes/sequences with respective coverage, pairwise identity and number of mapped contigs are displayed, contrasted with specific PCR results. GLRaV-1, *Grapevine leafroll-associated virus* 1; GRSPaV, *Grapevine rupestris stem pitting associated virus*; GVA, Grapevine virus A; PNRSV, *Prunus necrotic ringspot virus*; ACLSV, *Apple chlorotic leaf spot virus*; n. d., not determined.

Results and Conclusion

The applied NGS protocol enabled the detection of prevalent multiple infections, thus viruses, viroids & phytoplasmas were identified in bois noir diseased grapevine (Table 1). Grape-vine viruses and viroids were found in GED sample. In addition to *Ca.* Phyto-plasma prunorum, two viruses (PNRSV, ACLSV) were found in ESFY diseased *Prunus* rootstock. Low titer infections in tissues are detectable, since phyto-plasmas (negatively tested by normal PCR assay) were identified in bois noir and pear decline samples. *Ca.* Phyto-plasma mali was identified in AP sample. Besides viruses & phytoplasmas detec-ted by PCR, viruses & viroids were found in addition. This NGS technique as a sensitive universal, unbiased analysis method, enabled the parallel detection of phytoplasmas, viruses & viroids in a single grapevine or fruit tree sample.

References
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