

Investigation on Viral Pathogens in *Vitis vinifera* from Four Production Bases in Hangzhou Vicinity of China by sRNAseq and Molecular Validation

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Abstract

This is the first systematic investigation of viral pathogens in *Vitis vinifera* from Hangzhou vicinity of China. About 7 viruses and 5 viroids were annotated from four production bases “Dushicun”, “Wangjiayuan”, “Xiajiangcun”, and “Yangducun” covering 15 cultivars through sRNAseq technique. At least 3 viruses—grapevine leaf roll-associated virus 3 (GLRaV-3), grapevine fleck virus (GFkV) and grapevine geminivirus A (GGVA), and 4 viroids—hop stunt viroid (HSVd), citrus viroid II (CVD-II), grapevine yellow speckle viroid 1 (GYSVd-1) and grapevine yellow speckle viroid 2 (GYSVd-2) infected all four bases. “Yangducun” base showed 11, the most infected pathogens. GYSVd-1 showed the highest accumulation in host of Wangjiayuan base. The main infected pathogens were verified by reverse-transcription polymerase chain reaction (RT-PCR) technique, the detected rate reached to 85% - 100%. The results provide an important basis for effective and precise detection of viral diseases in the area and for the virus-free cultivation in future.

Keywords

Vitis vinifera, Viral Disease, Small RNA Deep Sequencing, RT-PCR

1. Introduction

The Hangzhou vicinity of Zhejiang Province is one of the main table grape-producing

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areas in southern China, which is distributed mainly in “Wangjiayuan”, “Xiajiangcun”, “Dushicun”, and “Yangducun” four production bases covering about 15 cultivars including “Summer Black”, “Shine-Muscat” and stock “Beta” with a total cultivation area of 2000 acres approximately. Nevertheless, viral diseases commonly infect in these bases with the field symptoms of yellowing, mottled, chlorotic, or shrunken leaves, malformed fruit size, and even whole plant death because of the spreading of scion, rootstock, seedlings and agronomic operations. Systematic investigation of viral pathogens in local district has not been performed yet, except characteristics of biology and genome for certain virus such as grapevine fan leaf virus (GFLV) which was once spread widely near two decades ago [1] and viral infections in certain cultivar [2].

Even if more than 80 grapevine viruses have been reported worldwide up to now [3], many of them have mixed infection to the host [2] [4] [5]. Small RNA deep sequencing (sRNA-seq) that has upended the classical detection approaches of plant viruses makes analysis of the whole pathogens in affected host possible [5]. It was applied in virus diagnostic field of various grapevine cultivars and rootstock worldwide recent decade. Besides, the prevalent viral agents, grapevine vein clearing virus [6], grapevine gray norovirus [7], grapevine red leaf-associated virus, grapevine red blotch virus and citrus exocortis viroid [8], grapevine geminivirus A [9], grapevine berry inner necrosis virus and grapevine leaf-roll associated virus 13 [10] [11], grapevine hammerhead viroid [12], citrus cachexia viroid new strains [2], grapevine virus H [13], grapevine asteroid mosaic-associated virus [14], grapevine rupestris vein feathering virus [15], grapevine red globe virus [16], grapevine Kizil Sapak virus [17] and grapevine associated Jivivirus 1 [18] were detected in different grapevine cultivars and vicinities worldwide in succession by sRNA-seq.

In this research, small RNA deep sequencing and molecular validation were applied to systematically study the distribution and types of viral disease in the four grapevine bases above in order to offer valuable information and effective approach of diagnostic and further control for local institution of inspection and quarantine, researchers and farmers.

2. Materials and Methods

2.1. Sample Collection

Chlorosis, yellowing, mottling or shrinking leaves were collected from four grapevine bases separately. Leaf samples from same base were mixed together and ground into a powder in liquid nitrogen, and stored in a freezer at -80°C for further use.

2.2. sRNA-seq

Small RNA extraction, cDNA library construction and deep sequencing were performed on BGISEQ-500 sequencing platform in Huada Gene Co., Ltd. (Shenzhen, China). The quality of sequencing was evaluated using Q20 (the ratio of the number of nucleotides with a quality value greater than 20 to the total number of

nucleotides in the sample). Scalable Vector Graphics (SVG) drawing tool was applied to remove 5' and 3' adapter-linked, polyA-containing reads, less than 18 nt-tags, and the *Vitis vinifera* genome-related sRNAs.

2.3. Data Analysis

The remaining sRNA sequences were compared with the viral genome database (<https://www.ncbi.nlm.nih.gov/genome/viruses>) using Bowtie2 software. All viral genome related sRNA and their antisense reading counts were analyzed using SVG drawing tool. The major virus pathogens were found from the high coverage of that, and TPM (transcripts per million RNA tags) showed the viral proliferation in the infected host.

2.4. Validation by RT-PCR

Primers were designed from genomic information of NCBI, which partially resembled our former designation [2] (Table 1). Total RNA from symptomatic frozen leaf powder of four bases was extracted respectively using OmniPlant RNA Kit (Kangwei Century Bio-Tech. Co., Ltd.). cDNA was synthesized using M-MLV reverse transcriptase (200 U/μl) (Promega). PCR was amplified using PrimeSTAR HS DNA Polymerase (2.5 U/μl) with the reaction condition of initial denaturation at 95°C for 5 min, followed by 40 cycles of 95°C for 30 s, 58°C for 30 s, and 72°C for 30 s, and then final extension at 72°C for 10 min. 3 μl PCR product with 6× loading buffer was loaded in agarose gel electrophoresis at 150 volts and detected under UV light.

Table 1. Designed primer pairs for amplifying main viral pathogens.

Viruses/Viroids	Primer name	Sequence (5' - 3')	T _m (°C)	Position in genome and Size (bp)	Referenced from
grapevine fleck virus	GFkV-CP-F	TGCCGCCTCTCCGTCTGCTGA	58	6435 - 6729 (295)	NC003347.1
	GFkV-CP-R	GGCGGAGGAGGCAGTGGGTTG			
grapevine leaf roll-associated virus 3	GLRaV3-CP-F	CGGCACAAGGAAAATTTAGT	58	13,345 - 13624 (280)	NC004667.1
	GLRaV3-CP-R	ATTCCCTTGCCCATAGTCTTC			
grapevine geminivirus A	GGVA-AC-F	CAGTATCTTCTGAGGCACGA	58	1715 - 2417 (703)	NC031340.1
	GGVA-AC-R	GCTAACGACGCTTATGCTGA			
	GGVA-AV-F	AAGAGGAAATCTTTTCGACC	58	464 - 1031 (568)	NC031340.1
	GGVA-AV-R	AATTGACTCGATTACGACCAG			
hop stunt viroid	HSVd-F	CGAGAGGCGTAGGAGAGAGG	58	105 - 282 (178)	NC001351.1
	HSVd-R	GCGGCAGAGGCTTAGATAGAC			
grapevine yellow speckle viroid 1	GYSVd1-F	CTCCGGATCTTCTTGCTTGTTG	58	2 - 284 (283)	GU170805.1
	GYSVd1-R	GAGACCAAGTCCGCTCGAC			
grapevine yellow speckle viroid 2	GYSVd2-F	AGATGCCTCCGCTAGTCGAG	58	242 - 130 (250)	NC003612.1
	GYSVd2-R	GCCAGCTCTTCGGAATGCACC			

3. Results

A total of 28,670,274 to 29,695,410 small RNAs and 25,945,095 to 27,251,686 clean reads were obtained by small RNA sequencing. The results showed that the Q20 ratio was 98.30%, indicating that the data quality was reliable and the sequencing accuracy was high. It was found that small RNAs detected from 4 bases were mainly between 18 and 32 nt in length. The most abundant category was those 25 nt in length, accounting for 30% - 40% of the total (Figure 1). The proportion of GC in small RNA was 51% in average.

After the genome sequences from *Vitis vinifera* were excluded from clean reads, the left ones were compared to that of viral pathogens in the NCBI database. The copy number of small RNAs matching to that of viral genome was between 680,968 to 1,141,358, accounting for 2.70% to 4.37%, and Wangjiayuan appeared the most. Furthermore, the number of variant small RNAs matching to the viral genome was between 51,041 to 73,939 accounting for 1.08% - 1.85% of the total variant number respectively, while Yangducun showed the most (Table 2).

These sense and antisense small RNAs were mainly related to 12 viral pathogens (including 5 viroids), which share more than 85% genome coverage rate. Among them, GFkV, GLRaV-3, GFV, HSVd, GYSVd-1, GYSVd-2 and CVD-II infected all 4 production bases. Yangdu base has at least 11, the most viral pathogen infection (Table 3). GYSVd-1 appeared the most pathogen accumulation in the host of all 4 bases, and in Wangjiayuan base it showed 17,485.19, the highest TPM (Table 4).

RT-PCR clearly confirmed and verified the co-infection of these viruses and viroids which have high coverage rate with sRNA tags and high pathogen accumulation in their grapevine host from 4 production bases (Figure 2 and Figure 3). The detection rate covers from 85% - 100%.

4. Discussion

China is one of the largest countries for table grapevine production and consumption with the planting area of more than 1.65 million acres. Hangzhou city of Zhejiang province is one of the most important production district in southern China. With the continuous expansion of crop trade, the introduction and

Table 2. Data analysis on mapped sRNAs to that of viral pathogens.

Sample name	Total sRNAs excluding <i>vitis vinifera</i>	Total sRNAs mapping to that of viral genome	Percentage of total sRNAs mapping to virus genome (%)	Unique sRNAs excluding <i>vitis vinifera</i>	Unique sRNAs mapping to virus genome	Percentage of unique sRNAs mapping to virus genome (%)
dushicun	26,213,378	723,601	2.76	3,670,165	57,776	1.57
wangjiayuan	26,088,883	1,141,358	4.37	4,523,954	64,724	1.43
xiajiangcun	25,221,072	680,968	2.70	4,713,028	51,041	1.08
yangducun	25,551,606	711,270	2.78	3,999,810	73,939	1.85

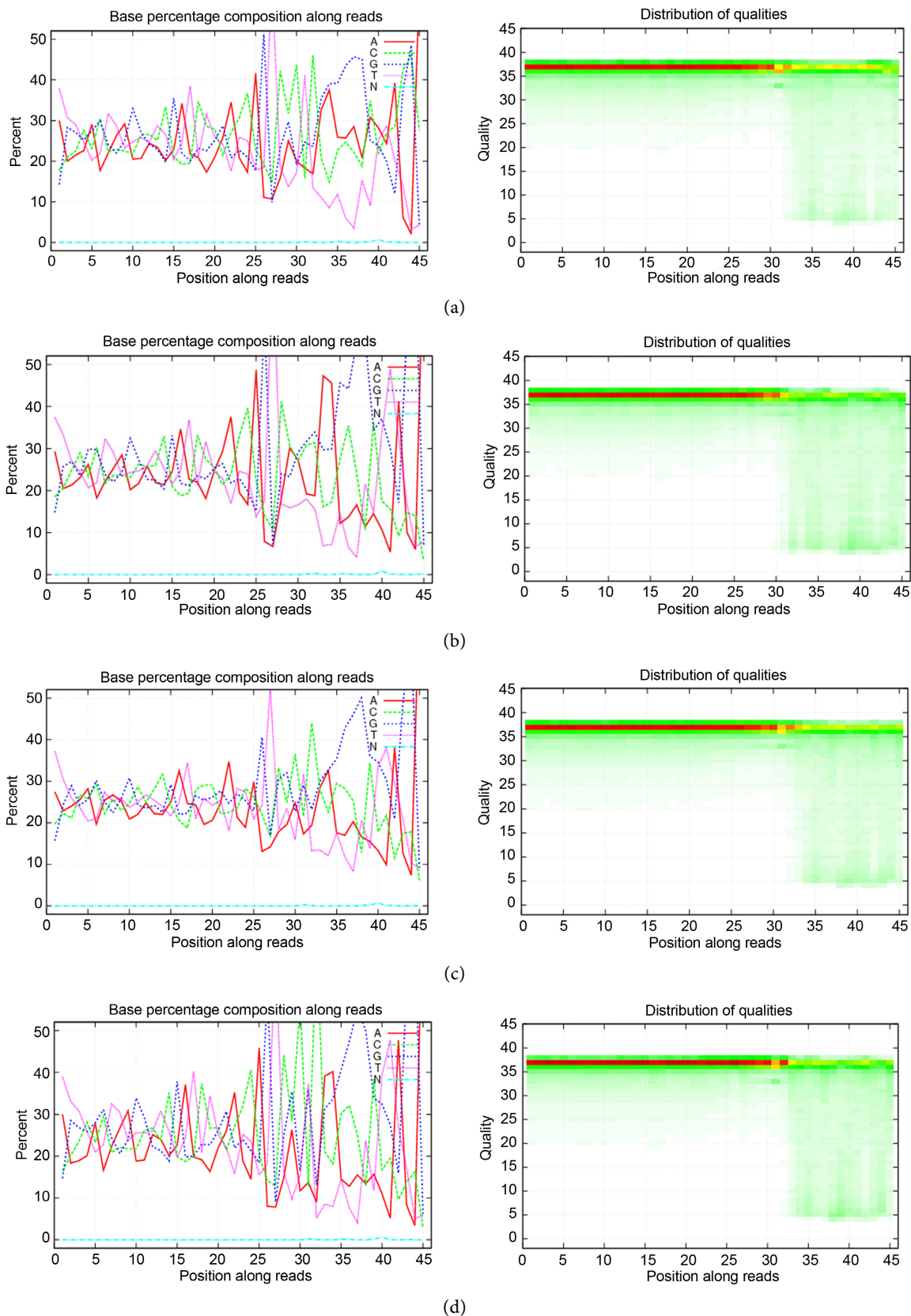


Figure 1. Distribution and frequent percentage of various small RNAs in 4 bases ((a): Dushicun; (b): Wangjiayuan; (c): Xiajiangcun; (d): Yangducun).

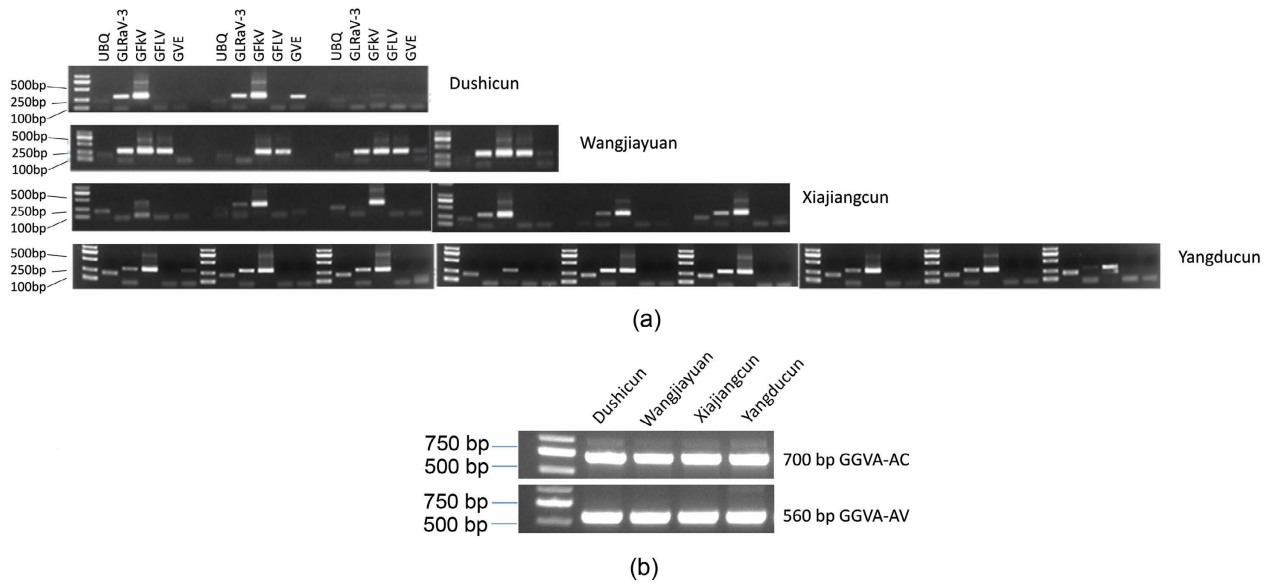


Figure 2. RT-PCR confirmation of partial main co-infected grapevine viruses in four production bases (a, b).

Table 3. 12 Viral pathogens with more than 85% coverage rate of sRNAs through RNaseq from 4 production bases.

NCBI code	Viral pathogens	Coverage rate with sRNA tags (%)	Coverage rate			
			Dushicun	Wangjiayuan	Xiajiangcun	Yangducun
NC_003347.1	Grapevine fleck virus	86.6 - 90.2	+	+	+	+
NC_031340.1	Grapevine geminivirus A	98.2 - 99.6	+	+	+	+
NC_004667.1	Grapevine leafroll-associated virus 3	91.6 - 98.2	+	+	+	+
NC_039072.1	Grapevine fabavirus	94.2 - 96.7	+	-	+	+
NC_011106.1	Grapevine virus E	90.5	-	-	-	+
NC_001948.1	Rupestris stem pitting associated virus	93.2	-	-	-	+
NC_015782.1	Grapevine pinot gris virus	90.2	+	-	-	-
NC_001351.1	Hop stunt viroid	95.7 - 97.0	+	+	+	+
NC_001920.1	Grapevine yellow speckle viroid 1	99.5 - 99.7	+	+	+	+
AF_434679.1	Citrus viroid II	93.0 - 95.0	+	+	+	+
NC_003612.1	Grapevine yellow speckle viroid-2	99.5 - 99.7	+	+	+	+
AY513267.1	Citrus cachexia viroid	85.3 - 94.3	-	+	-	+

Table 4. Viral pathogens with more than 4500 TPM accumulation in 4 bases.

Viruses/Viroids	Dushicun	Wangjiayuan	Xiajiangcun	Yangducun
Grapevine fleck Virus	-	4720.94	-	-
Grapevine geminivirus A	-	-	-	4546.37
Grapevine yellow speckle viroid 1	9480.47	17485.19	13567.54	9442.97
Grapevine yellow speckle viroid-2	5975.08	13375.85	-	5945.81

-.: TPM (transcripts per million RNA tags) < 4500.

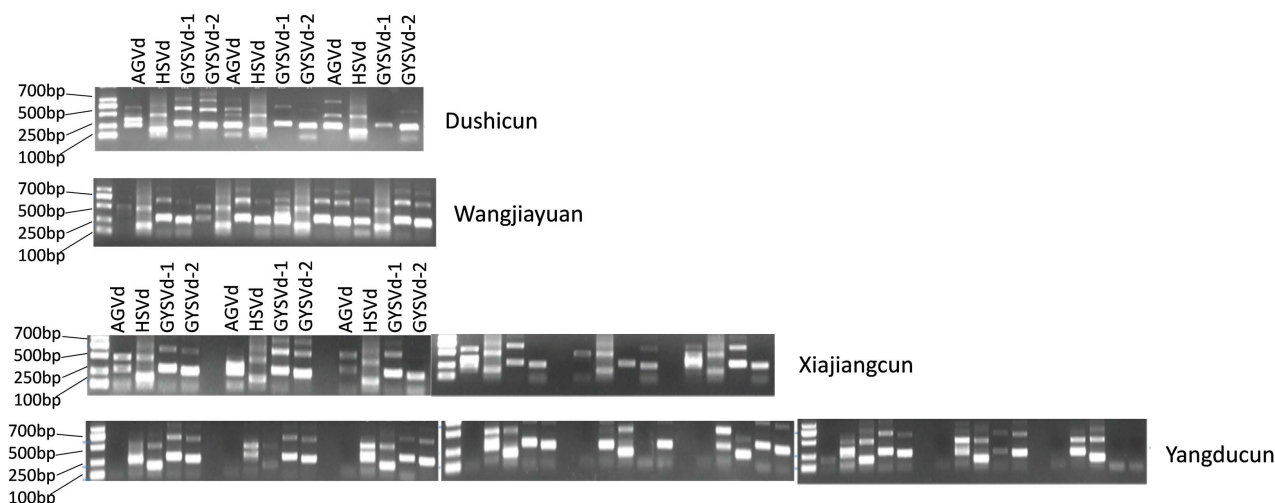


Figure 3. RT-PCR confirmation of partial main co-infected grapevine viroids in four production bases.

transportation of grapevine seedlings, scions and rootstocks required for grafting cultivation, and cultivation approaches have made possible for the transmission and mixed infection of grapevine viral diseases. These detected viral pathogens are also conventional and infect frequently in other grapevine yards worldwide [4] [19] [20].

Yangducun base is one of the regional test stations for grapevine cultivars in national grape production system, where more than 200 idioplasmatic resources from home and abroad are conserved, and more than 10 local new-bred cultivars in “TIANGONG” series are obtained. Frequent and certain scale of breeding and cultivar introduction have increased the risk of infection and transmission of viral pathogens, which also explains the relative largest number of co-infected virus diseases in this base. Therefore, it is particularly important to strengthen the precise detection, isolate affected plants and prevent the spreading of diseases.

GYSVd-1 is one of the most common viroids infecting grapevine worldwide, and often mixed-infect with GYSVd-2 and other viral pathogens. This viroid shows higher activity and proliferation in host of all 4 local bases, especially in Wangjiayuan base, which needs to be paid greater attention to.

5. Conclusion

The research is the first systematic investigation in molecular level for grapevine viral pathogens in local bases. At least 7 viruses and 5 viroids were annotated in four production bases, among them, 3 viruses (GFkV, GGVA, GLRaV-3) and 4 viroids (HSVd, GYSVd-1, GYSVd-2, CVd-II) are the main co-infected viral pathogens distributed evenly in 4 local vicinities at present. The results not only will lay an important foundation for the precise detection of important viral diseases in future seedling exchange process and virus-free tissue culture, and provide preliminary valuable data support for promoting the standardized cultivation technology and grape fruit high-quality production.

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Conflicts of Interest

The authors declare no conflicts of interest regarding the publication of this paper.

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