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Small RNA sequencing studies of populations of plant virus families in tropical rainforest of Xishuangbanna

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ABSTRACT

The tropical rainforest of Xishuangbanna has the most abundant and cryptic plant species biodiversity in China. To explore the populations of plant virus families in Xishuangbanna tropical rainforests, fifteen pools of plant samples from different forest locations were collected and used for small RNA analyses by high throughput sequencing. All contigs were classified and annotated with the NCBI Nt database to determine the species distributions, and comparisons were conducted using the Blast algorithm. The viral sequences in the Clean Reads were compared with the relevant sequences, using the Kraken software system, to infer their possible classification and to analyze the abundance of each species statistically. The number of Clean reads of every pool sample was from 1573897 to 26878598, and the average number of Clean reads was 20322116. The average number of clean reads is taken as 0.0036% from the average number of Raw reads. The results from a total of 3703 viral sequences were annotated, and these represented a total of 16 plant virus families. Among these, 1952 Geminiviridae sequences comprised the dominant annotated virus proportion. 732 sequences belonged to the Potyviridae, and 192 sequences were characteristic of members of the Caulimoviridae. The viral sequences primarily originated from dicots and monocotyledonous plants, including herbaceous and woody plants. Generally, the total viral sequences from herbaceous plants represented 66.7 %, and 33.3 % were from woody plants, and amongst these, 57.1% dicots represented the plant hosts, whereas 42.9 % were derived from monocotyledonous families. The presumptive Geminiviridae and Potyviridae viruses have broad host ranges, and different families were annotated from each sample site in Xishuangbanna. This study lays a foundation for future research on the evolution and utilization of viruses within tropical rainforests and those of cultivated agricultural areas.

Keywords: virus, family, population, tropic forest, Xishuangbanna

INTRODUCTION

Plant viruses affecting agriculture production have been investigated extensively, but aside from cultivated plants, little is known about virus distribution in other plant ecosystems. According to the 9th report of the International Committee on Taxonomy of Viruses (ICTV) (Roossinck 2011; Owens et al., 2012) about 900 species of plant viruses were listed, and almost all of these are plant pathogens (Roossinck, 2011), and these accounted for 77% of the viruses that were recognized in 2012 by the ICTV. However, due to host ambiguities and the difficulties of virus isolation using traditional virology research methods, it has been difficult to carry out research on plant virus distribution in alternative habitats. Hence, most plant virus research has focused on agriculturally important viruses, but it is slowly being recognized that some viruses contribute to the survival of the host (Marquez et al., 2007). These studies suggest that some viruses are actually beneficial to the host in various ways, often by helping the host take advantage of the fierce competition in the natural environment. Studies such as those of Marquez et al. (2007) have found that symbiosis with fungi can play an important role in helping plants survive drought and other adverse environmental conditions (Marquez et al., 2007; Roossinck, 2010; Roossinck et al., 2010; Roossinck, 2011). These studies provide strong evidence that viruses and their symbiosis play an important role in the evolution of life on earth (Roossinck, 2011; Roossinck, 2005). Nevertheless, little is currently known about the role and

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significance of plant viruses in non-agricultural ecosystems. Next-generation sequencing technology provides new and rapid analyses to identify known or unknown viruses that are difficult to isolate and characterize in different ecosystems, such as lakes, oceans, plants, plant virus, and even infected animals (Wei, 2012; Yang et al., 2013; Ge et al., 2013; Grover et al., 2010; Roossinck, 2012; Roossinck, 2010; Ma et al., 2016). Muthukumar et al. (2009) studied the presence of plant viruses in the Tallgrass Prairie Preserve in Oklahoma, USA, and identified genome sequences from different organisms, including plants, bacteria, fungi, and viruses. The results indicated that the viruses in the Highland Grassland Reserve were different from those in adjacent agricultural systems. Scheets et al. (2011) detected the members of Tombusviridae, and Min et al. (2012) studied the molecular characteristics, ecology, and epidemiology of Tymovirus infections of *Asclepius viridis*. Vaskar et al. (2015) also studied the taxonomic composition of viruses in Oklahoma Highland Grassland Reserve to explore the role and significance of plant viruses in this ecological niche. However, until the present time, there have been no reports about plant virus distributions in tropical rainforests.

Xishuangbanna is located in southwest central China at the northernmost end of the Southeast Asia tropical rainforests which represent one of three major tropical rainforest areas in the world. The Xishuangbanna Tropical Rainforest is a member of the biodiversity conservation circle of the United Nations, and a Chinese ecological demonstration zone, and contains a wealth of plant, animal, and microbial resources (Yang, 2011; Zhu, 2015; Zhu, 1994). The rich ecological diversity of these regions consists of a wide variety of plant species, but essentially nothing is known about plant virus populations or their composition within this region or any other tropical rainforest area in the world. Therefore, we have initiated a study of plant virus populations in the Xishuangbanna region to provide preliminary information about this important ecosystem. Our study provides a preamble for more extensive systematic investigations of plant and other viruses using small RNA analyses to clarify the distribution of plant virus families and their diversity within the region and their role in the ecological stability of the region. The study also provides a foundation to compare the distribution and evolution of crop viruses in rejoins adjacent to the tropical rain forest.

MATERIALS AND METHODS

Plant samples

From 2015 to 2016, 15 pools of mixed plants were collected from 15 random sites in Xihsuangbanna, as shown in Figure 1 and Table 1. The leaves from each sampling site were frozen in liquid nitrogen wrapped in aluminum foil and stored at -80 °C.

Main Reagent

The EASYspin plant microRNA extraction kit (Aidlab Biotechnologies Co.) was used for mRNA isolation and protein identification.

Small RNA extraction and library construction for sequencing

A total 15 pools of mixed plants from Xishuangbanna were used as samples, and their small RNAs were extracted using the EASY spin methodology according to the kit instructions, which were immediately used to construct cDNA libraries. For cDNA library constructions, total RNAs from the plant were used as templates for polymerize chain reaction (PCR) analyses. A joint sequence was linked at the small RNA 3' end before reverse transcription to avoid self-interactions between the 3'and 5' termini. Subsequently, PCR amplification using random primers were conducted, and the products were separated in PAGE gels for fragment purification. In preliminary tests, the sequences were quantified with Qubit 2.0, and the sizes of synthesized cDNAs were determined with Agilent 2100. Then, the effective cDNA concentrations (>2nM) were determined with qPCR, and sequenced with the Illumina HiSeq 2500 sequencing platform (Biomarker Technologies Co. LTD).

Data Processing and Virus Annotation

The original image data files sequenced by Illumina HiSeq2500 platform were transformed into Raw Data or Raw Reads by base calling, and then snRNAs, Sc RNA repeat sequences, rRNA, and tRNAs were filtered and annotated for RNA classification. The small RNA contigs were further spliced using the Velvet de novo Assembler for short-read sequencing technologies with velvet software (Zerbino, 2010). The resulting contigs were classified and annotated with the NCBI Nt (NCBI non-redundant nucleotide sequences; ftp://ftp.ncbi.nlm.nih.gov/blast/db/) database to determine the species distributions, and comparisons were conducted using the Blast algorithm (Korf, 2003), in which the parameter limitation is 1e-5 for BLASTN. The viral sequences in the Clean Reads were compared with the relevant sequences, respectively, using the Kraken software system

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(Wood and Kraken, 2014), to infer their possible classification and to analyze the abundance of each species statistically. The viral sequences were annotated and classified in the Xishuangbanna Tropical Forest.

RESULTS

Sample distribution

A total of 15 pool Leaf samples was collected from 13 sites (A to O) throughout the Xishuangbanna Tropical Rainforest in Yunnan Province of China (Figure 1 and Table 1). The pool samples distributed at Mengyang Town Nature Reserve in Jinghong City, the nature reserve of Menglun Town, Mengla County, Menglun Town Nature Reserve, Mengla County, and other sites where were abundant plant resources in Xishuangbanna tropical Rainforest in Yunnan, from elevation was from 795 m to 1381 m, the plants resourced from herb to wood, and from Dicots and Monocotyledons, representative the plant distribution in tropic rainforest Xishuangbanna.

Data output of small RNA sequencing

In these experiments, the quality of the sequences from each plant sample was managed, and after the construction of the cDNA library, we used Q-PCR to quantify the effective library sequence concentration to ensure the quality of the library. High-quality sequences (Clean Reads) were obtained by removing low-quality sequences. Clean Reads were filtered NCRNAs and repetitive sequences, such as ribosomal RNA (rRNA), transfer RNA (tRNA), intranuclear small RNA (snRNA), and nucleolar small RNA (snoRNA) to ensure the quality of the experimental samples and the accuracy of the analysis results in each step of the study. The statistics data of small RNA sequencing show in Table 2. The results that the number of original Raw reads sequence was from 18618044 to 30056704, with an average of 2485974. The number of small RNA sequences whose length was less than 18 bp after removing joints ranged from 480406 to 3026389, with an average of 1368999. The number of sequences whose length was greater than 30 bp after removing joints ranged from 654641 to 6254772, with an average of 254326. Because the content of N exceeds 10%, the number of reads filtered out ranged from 89010 to 18005, with an average of 95025. Finally, the number of Clean reads was gotten from 1573897 to 26878598, with an average of 20322116. These manipulations resulted in 304.83 M Clean Reads, no less than 15.74 M Clean Reads, was used to classify and annotate 320.5 contigs obtained by stitching and assembling to ensure the accuracy of the information of 3703 virus-related sequences and 16 plant virus families (Figure 2). Throughout our experiments, the annotated sequences were submitted to NCBI (SRA accession: SRP158336, Temporary Submission-ID: SUB43936 22).

Plant virus families annotated in the tropical rainforest of Xishuangbanna

All contigs were classified and annotated with the NCBI Nt database to determine the species distributions, and comparisons were conducted using the Blast algorithm. The viral sequences in the Clean Reads were compared with the relevant sequences, respectively, using the Kraken software system, to infer their possible classification and to analyze the abundance of each species statistically. Among the viruses annotated in the A to O sampling sites are shown in Figure 1. The families Caulimoviridae, Geminiviridae, and Potyviridae were the most widely distributed. The families Alphaflexiviridae, Bunyaviridae, Luteoviridae, and Tymoviridae had lower distributions in the sites and were annotated at three or fewer locations, whereas the Pospiviroidae and Rhabdoviridae samples were each detected at only one sampling site. The primary sequences annotated in this study consisted of more than 90% homology for each family listed in Figure 2. A total of 3703 viral sequences was annotated to 16 plant virus families, and among these, nine primary families were annotated and detected > 10 times. These included the families Alphaflexiviridae, Bunyaviridae, Caulimoviridae, Geminiviridae, Luteoviridae, Pospiviroidae, Potyviridae, Rhabdoviridae, and Tymoviridae (Table 3). Among these, 1952 Geminiviridae sequences were annotated and represented the dominant family. The 732 Potyviridae sequences represented the second most abundant family, and 192 Caulimoviridae sequences were annotated, followed by decreasing amounts of 14 other families. The remaining families shown in Figure 2 were detected < 10 times and hence are not included in Table 3. These included the families Bromoviridae, Nanoviridae, Nyamiviridae, Partitiviridae, Secoviridae, and Tombusviridae. Many viruses in these families cause major losses to crop production and rely on insects (aphids, whiteflys, thrips etc.) for local and long-distance transmission.

Description of plant families from which the virus sequences were derived

The pool samples from which the annotated families originated came primarily from herbaceous and woody dicots with a smaller proportion from monocotyledonous plants as well as more primitive families (Table 3). The Dicot representatives

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included the widely distributed Compositae and Euphorbiaceae, Malvaceae, Rosaceae, and Solanaceae families, each of which included a large number of herbs, shrubs, and succulents of agricultural importance. The Caricaceae family includes papayas, and the Thymelaeceae are a cosmopolitan family of flowering Dicots that are mostly of tropical origin that include Daphne, and other ornamental species were also represented. Monocots were not prevalent but did include the Zingiberaceae, which contains a number of aromatic perennial shrubs that are prevalent in tropical forests. Potyvirus sequences were also detected in the Pepper family (Piperaceae), a primitive family with a large number of flowering shrubs belonging to basal Angeosperms that were prevalent prior to the evolution of the Dicots and Monocots. The broad geminivirus host range also included the Taxaceae (Yew Family), a primitive coniferous family of small trees and shrubs. This diversity of hosts and the limited relationship of several of the families to crop plants grown in southern China suggests that many of the virus species in the Xishuangbanna Tropical Rainforest are endogenous, rather than having arisen from contiguous agricultural areas. Hence, it is entirely likely that the Xishuangbanna Tropical Rainforest could serve as a virus reservoir for crop species that are introduced near the rainforest in the future.

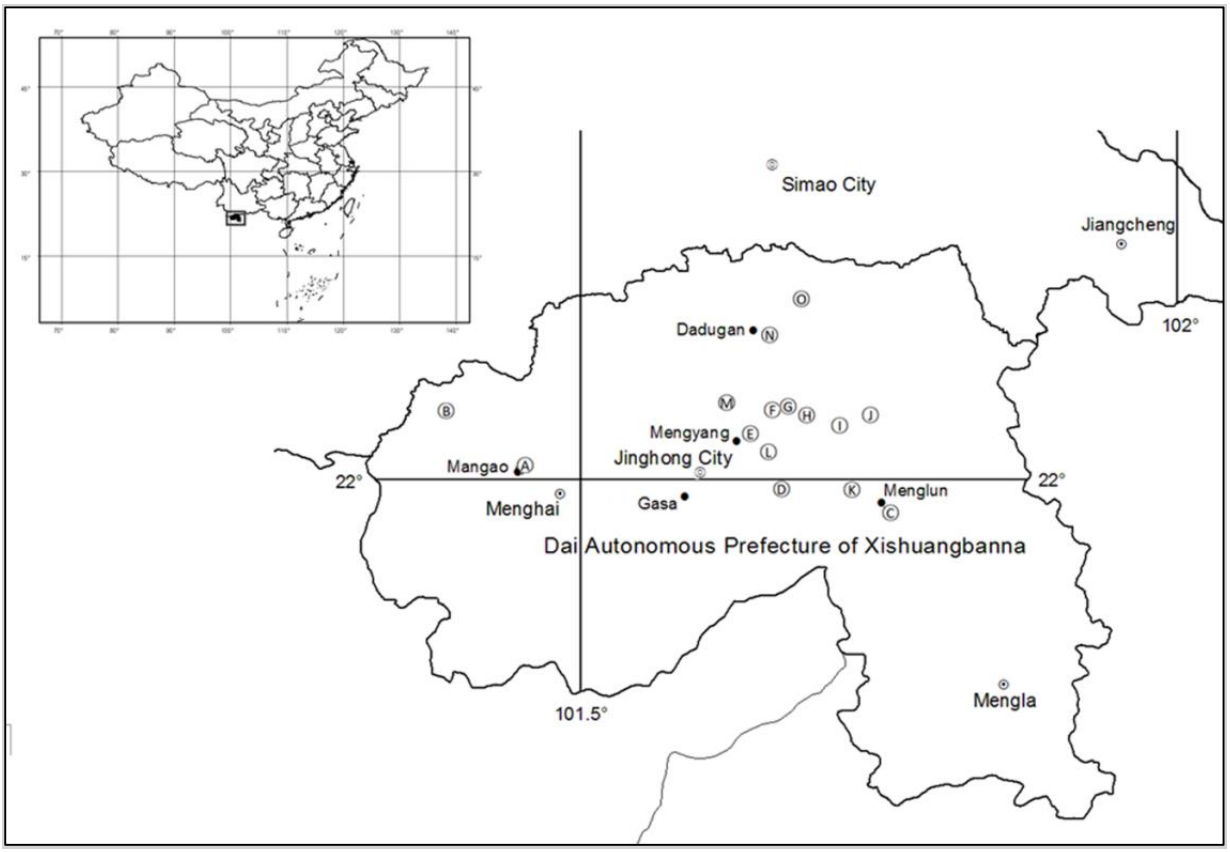

Figure 1. Distribution of virus sampling sites at the tropic forest in Xishuangbanna, Yunnan Province of China
Notes：city; town； county；Ⓐ to Ⓞ：15 sample collection sites from A to O

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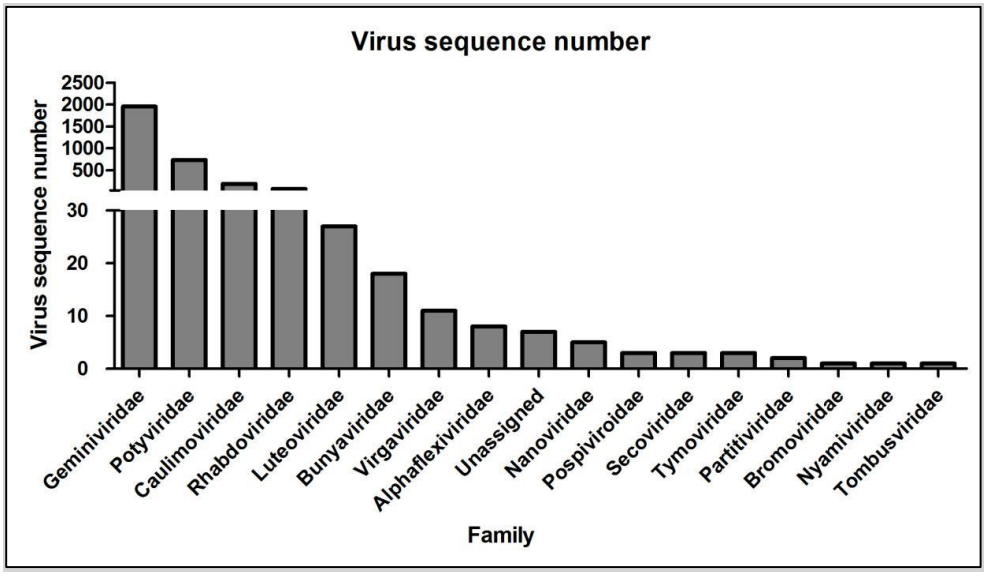

Figure 2. Numbers of virus sequences of virus families

DISCUSSION

In total, the viral sequences of nine plant virus families consisting of the Alphaflexiviridae, Bunyaviridae, Caulimoviridae, Geminiviridae, Luteoviridae, Pospiviroidae, Potyviridae, Rhabdoviridae, and Tymoviridae were annotated according to the viral sequencing identity (>90%) and detection number (>10). Among those families, the Geminiviridae sequences dominated and had broad distribution. Potyviridae and Caulimoviridae were common at most sample sites; however, fewer viral sequences were annotated to other families. The Tomato leaf curl Joydebpur virus and Tomato leaf curl Karnataka virus species in the Begomovirus genus in the Geminiviridae, had the highest detection rates of all sequences. However, Alphaflexiviridae, Bunyaviridae, Luteoviridae, and Tymoviridae sequences were detected at only 2 and 3 sampling points, respectively. Specific viruses annotated were tomato spotted wilt Tospovirus in the Bunyaviridae, Pepper vein yellows Polerovirus in the Luteoviridae, Tobacco vein distorting Potyvirus in the Potyviridae. Wild tomato mosaic virus, a possible of Potyvirus species in the Potyviridae, also had very high detection rates, and these are worthy of further identification and verification. Pospiviroidae and Rhabdoviridae sequences were detected at only one sample site each. The of nine main virus family sequences were annotated both from herbaceous and woody plants, and more sequences were detected in dicots than monocotyledons. Generally, the virus sequences were more prevalent in herbaceous than woody plants and in more dicots than monocotyledons, and these hosts collectively account for hosting the vast majority of the annotated viral sequences. Therefore, we infer that herbaceous dicots derived are more likely to be virus-infected in the tropical rain forest than monocotyledons and woody plants. Plant virus diseases of Solanaceae, Gramineae, Leguminosae, Cruciferae, and Cucurbitaceae plants are predominant in Yunnan, and the most harmful are tobacco mosaic diseases, potato virus diseases, lily virus disease, maize dwarf mosaic disease, etc. (Zhang and Li, 2001 ; Hong et al., 2001), so there are more the host plants of farmland plant viruses are herbs and dicots. Therefore, the plant types of herbs and dicots are more likely to be the source of farmland plant viruses. It is of great significance to study their evolution and the interaction between viruses and hosts comparing the tropic forests and farmlands.

The viruses of Geminiviridae are the important pathogen affecting crops in Yunnan and southern China (Yan et al., 2002 ; Yang et al., 2011 ; Zhang et al., 2002). The viruses of Potyviridae have a wide host range, causing serious economic losses to food crops, cash crops, and horticultural crops（Hong et al., 2001; Zhang, 2010）while the host range of Caulioviridae is narrow (Owens, 2012). They were indeed the main pathogens of crop diseases in agricultural cultivation areas. The interesting thing is that our study shows that Geminiviridae, Potyviridae, and Caulioviridae are dominant populations in the tropical rain forests of Xishuangbanna. Are these viruses of Geminiviridae, Potyviridae, and Caulioviridae from tropical rainforests related to the viruses of ones from agricultural areas? The pathogenic evolution of plant viruses in the tropical rainforest deserves further study in the future.

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Table 1. Pool sample geography and plant resources

Pool sample number	Place	Longitude and latitude	Elevation /m	Herb or wood	Plant type (M:Monocotyledons D: Dicotyledons)
S01	Nature reserve of Mangao at Menghai County	N22°01'12.59" E100°19'937.61"	1212	H	D
S02	Nature reserve of Xishuangbanna River Basin, Gasa Town, Jinghong City	N22°10'03.45" E100°9'36.92"	712	H	D
S03	Huludao, Menglun Town, Mengla County	N21°55'12.47" E101°16'06.65"	545	W	D
S04	Basha Laozi Village, Jino Mountain Jino Nationality Township, Jinghong City	N21°59'28.47" E101°00'01.19"	1034	H	D
S05	Basha Laozi Village, Jino Mountain Jino Nationality Township, Jinghong City	N21°59'28.47" E101°00'01.19"	1034	H	D
S06	Jinghong City nearby	N22°06'29.32" E100°55'11.33"	806	H/W	D
S07	Tiaoba River Village, Mengyang Town, Jinghong City	N22°10'15.84" E100°59'16.89"	849	H/W	D
S08	Mengyang Town Nature Reserve in Jinghong City	N22°09'22.83" E101°03'30.67"	749	H	D/M
S09	Mengyang Town Nature Reserve in Jinghong City	N22°07'55.32" E101°08'45.09"	1381	H/W	D
S10	Nature reserve of Menglun Town, Mengla County	N21°58'38.77" E101°10'33.33"	940	H	D
S11	Menglun Town Nature Reserve, Mengla County	N22°03'49.54" E100°58'01.06"	1032	H	D
S12	Wild Elephant Valley, Mengyang Town, Jinghong City	N22°11'11.97" E100°51'48.41"	795	H/W	D
S13	Dadugang Township, Jinghong City	N22°21'23.16" E100°58'16.14"	1328	H	D/M
S14	Dadugang Township, Jinghong City	N22°27'01.55" E101°02'57.87"	886	W	D
S15	Dadugang Township, Jinghong City	N22°09'22.78" E101°13'23.97"	1129	H	D/M

Note: H: Herb; W: Wood; D: Dicotyledons; M: Monocotyledons

Table 2. The statistics data of small RNA sequencing

Number of Pool Samples	Raw reads	Length<18	Length>30	N%>10%	Clean reads
S01	26262871	813765	4611684	18005	20819417
S02	23478580	1023143	4337951	16109	18101377
S03	23258049	956255	6254772	15793	16031229
S04	23457569	852400	4847823	16171	17741175
S05	22099253	1719675	3650579	14847	16714152
S06	25558415	1051545	2397810	116019	20733120
S07	26032741	2013554	1415993	143493	21683556
S08	23893777	3026389	1104308	102656	18914701
S09	23835377	1422754	1888847	133992	19648756
S10	24621159	616569	1410155	137951	21677193
S11	26934996	2622017	1381004	146751	21847629
S12	18618044	2224506	654641	89010	15738897
S13	27455472	1000918	1458737	154457	23963604
S14	27336604	711092	1184827	149788	24338340
S15	30056704	480406	1559762	170322	26878598
Average	24859974	1368999	2543926	95025	20322116

Note: Raw reads: Sequencing raw data; <18nt reads: reads less than 18 nucleotides after removing the junction; >30nt reads: reads with a mass value greater than 30 nucleotides; N>10%: reads with at least unknown base N>10%; Clean reads: Reads with a mass value greater than or equal to 30 nucleotides.

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Table 3. The main 9 plant virus families annotated in tropic rainforest of Xishuangbanna

No	Plant Virus Family in Alphabetical Order	Nucleic Acid Type	Average % Identity	Average E-value	Average Sequence Length	Numbers of times Detected	Source plants			
							Plant type			
							H: Herbaceous or W :Woody	M: Monocotyledonous or D: dicotyledonous	Plant Family	Sampling Site
1	*Alphaflexiviridae*	Plus Strand RNA	98.15	2.11×10^{-7}	54	11	H/W	D	*Compositae*	C, D
2	*Bunyaviridae*	Minus Strand RNA	94.36	3.02×10^{-7}	57.85	30	H	M/D	*Compositae*、*Malvaceae*	A, B, G
3	*Caulimoviridae*	Retrovirus DNA	91.35	2.16×10^{-7}	23	20	H/W	M/D	*Taxaceae*、*Compositae*	E, H, I, M, O
4	*Geminiviridae*	Single Strand DNA	93.27	1.71×10^{-7}	76.11	257	H/W	M/D	*Taxaceae*、*Compositae*、*Malvaceae*、*Solanaceae*	A, B, D, E, K, O
5	*Luteoviridae*	Plus Strand RNA	93.77	2.70×10^{-7}	58.85	250	H/W	M/D	*Caricaceae*、*Thymelaeceae*	F, G, J
6	*Pospiviroidae*	Circular Viroid RNA	94.67	2.19×10^{-7}	72	20	H/W	D	*Solanaceae*	E
7	*Potyviridae*	Plus Strand RNA	93.4	2.41×10^{-7}	86.31	350	H/W	M/D	*Zingiberaceae*、*Piperaceae* *Euphorbiaceae*、*Rosaceae*、*Solanaceae*	B, C, D, E, F, G, J, K, M,
8	*Rhabdoviridae*	Minus strand RNA	100	2.42×10^{-7}	67	11	H	D	*Compositae*	A
9	*Tymoviridae*	Plus Strand RNA	90	1.66×10^{-6}	20	11	H/W	M/D	*Solanaceae*、*Euphorbiaceae*	H, I, M

Note: H: herb; W:wood; M: monocotyledons ; D: dicotyledons

CONCLUSIONS

This study clarifies the plant virus family population in the tropical rainforest of Xishuangbanna. The viral sequences from herbaceous plants were obviously greater than from woody plants, and virus sequences isolated from dicots were obviously more extensive than from monocotyledons. This work lays a foundation for further research on the pathogenesis and evolution of plant viruses in the tropical rainforest virus and those in agricultural areas.

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