Prevalence of Yellow Leaf Disease (YLD) and its Associated Areca Palm Velarivirus 1 (APV1) in Betel Palm (*Areca catechu*) Plantations in Hainan, China

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Abstract

Yellow leaf disease (YLD) is an economically important disease affecting betel palm in several countries, the cause of which remains unclear despite associations with putative agents, including phytoplasmas. In this study, we screened the potential casual agents associated with YLD in Hainan, China using next-generation sequencing and revealed the association of areca palm velarivirus 1 (APV1) with the YLD-affected palm. The complete genome of the APV1-WNY isolate was determined to be 17,546 nucleotides in length, approximately 1.5 kb longer than the previously reported APV1_HN genome. Transmission electron microscopy showed that APV1 particles are flexuous and filamentous, a typical

morphology of species in the *Clavoviridae* family. Comparison of symptomatic and symptomless tree populations showed a strong association between APV1 and YLD. APV1 was detected in *Pseudococcus* sp. mealybugs sampled from YLD-affected trees in many locations, suggesting that mealybugs are a potential transmission vector for APV1. Although further studies are needed to confirm a causal relationship, these results provide timely information for the prevention and management of YLD associated with APV1.

Keywords: APV1, *Areca catechu* L., RNA-seq, yellow leaf disease

Betel palm (*Areca catechu* L.) is widely cultivated in tropical areas of Asia (Heatubun et al. 2012). Betel palm is affected by numerous pathogens, including fungi (To-anun et al. 2009; Wang et al. 2019), bacteria (Kumar 1983), phytoplasma (Kanatiwela-de Silva et al. 2015; Ramaswamy et al. 2013), viruses (Yang et al. 2018, 2019; Yu et al. 2015), and insects such as *Brontispa longissima* (Li et al. 2007) and *Tirathaba rufivena* (Zhong et al. 2017). Yellow leaf disease (YLD) was first reported in betel palm in India (Raghavan and Baruah 1958). The disease was associated with phytoplasma based on electron microscopy observations and PCR amplification of the 16S ribosomal RNA (rRNA) gene in India (Manimekalai et al. 2010; Nayar and Seliskar 1978; Ramaswamy et al. 2013), China (Che et al. 2010; Luo et al. 2001), and Sri Lanka (Abeyasinghe et al. 2016; Kanatiwela-de Silva et al. 2015). *Proustia moesta* (Westwood) was identified as a putative transmission vector for the phytoplasma in India (Ponnamma et al. 1991, 1997). These investigations support the hypothesis that the causal agent of YLD is a phytoplasma. However, in another study, no phytoplasma was detected in YLD samples from India (Purushothama et al. 2007). Therefore, the causal agent of YLD remains to be verified.

The YLD symptoms described by different authors differ significantly (Manimekalai et al. 2010; Menon 1963; Nayar and Seliskar 1978; Ponnamma 1993), suggesting that the name YLD has been applied to multiple diseases of betel palm. Menon (1963) reported that

the first visible signs of YLD are translucent spots of 1 to 2 mm in the growing spindle, followed by the appearance of brown necrotic streaks running parallel to the lamina of unfolding leaves. This description and symptomatic leaf images by Menon (1963) are highly similar to those described for the recently reported areca palm necrotic spindle-spot virus and areca palm necrotic ringspot disease (Yang et al. 2018, 2019). Nayar and Seliskar (1978) reported that YLD initially manifested as necrotic streaks in the spindle. Foliar yellowing expands from the frond tip to the frond base, although young, middle-aged, or older whorls of leaves may also show the first effects (Nayar and Seliskar 1978). According to our observations, necrotic streaks are not the initial symptoms but the symptoms in the late stage of YLD in Hainan, China, which are often caused by coinfection with fungal pathogens such as *Curvularia pseudobranchyspora* (Wang et al. 2019).

The area of betel palm plantation in Hainan is over 100,000 ha, 40% of which suffers serious losses due to YLD. YLD was first reported in Hainan in 1985 and was attributed to potassium deficiency (Yu et al. 1986). Although YLD was associated with phytoplasma in previous reports from Hainan based on electron microscopy observations and PCR assays (Che et al. 2010; Luo et al. 2001), we were unable to detect phytoplasma in YLD-affected plants sampled from numerous epidemic areas in Hainan. A virus called areca palm velarivirus 1 (APV1) was detected in a YLD sample in Hainan by RNA sequencing (RNA-seq), and its genomic sequence was determined (Yu et al. 2015). To further characterize the potential pathogens that cause YLD in Hainan, a modified RNA-seq method was used that enables the identification of all possible pathogens, including eukaryotes, prokaryotes, and viruses. Digital gene expression (DGE) analysis of diseased and symptomless control samples consistently identified the APV1 (Yu et al. 2015) in the YLD-affected leaf samples. The data collected in this work reveal a close association between APV1 and YLD.

Materials and Methods

Plant materials. The collection sites of samples were indicated in map of Hainan Province (Fig. 1). Samples used for RNA-seq were collected in Wanning City (WNY) and Baoting County (BTY) from betel palm showing typical YLD symptoms, consisting of yellowing leaflet tips in the midcrown (Fig. 2A). Symptomless trees without YLD were similarly sampled within a 50-m distance in any direction

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of YLD-affected palm. Leaf samples for reverse-transcription (RT)-PCR diagnosis were collected from 90 symptomless and 76 YLD-affected trees from different areas (Dingan, Lingshui, Baoting, Tunchang, Ledong, and Qiongzong Counties and Wanning, Qionghai, Haikou, and Danzhou Cities). In all, 68 of the symptomless samples were collected from YLD-epidemic areas and 22 were obtained from the non-epidemic areas of Danzhou and Haikou. All leaves collected from one tree were regarded as one sample. The category and number of samples from each area are shown in Figure 1. Twenty-two YLD leaf samples and corresponding mealybugs from the same leaves were collected for APV1 detection from six locations in Hainan. The samples were stored at -80°C until RNA extraction.

RNA library construction and RNA sequencing. Total RNA was isolated from leaf samples using TRIzol reagent (Invitrogen, China) according to the manufacturer's instructions. The quality and purity of the extracted RNA were examined using agarose electrophoresis, a NanoPhotometer spectrophotometer (Implen, CA, U.S.A.), and the RNA 6000 Nano Kit assay with the Agilent Bioanalyzer 2100 system (Agilent Technologies, CA, U.S.A.). The Ribo-Zero rRNA Removal Kit (Plant Leaf) was used to remove rRNA. Reverse transcription was performed using random hexamers and the second strand was synthesized subsequently. After adaptor ligation, cDNA was purified with the Agencourt AMPure XP system (Beckman Coulter, MA, U.S.A.) to preferentially select 250- to 300-bp fragments prior to generation of the final libraries through PCR. Sequencing was conducted and paired-end reads were generated on the Illumina HiSeq 2500 platform at Novogene Co. (Beijing, China). Raw reads in FASTQ format were filtered to obtain high-quality clean reads. The clean data were submitted to the Sequence Read Archive of NCBI (accession numbers SRX5244021 to SRX5244023). Transcriptome assembly was accomplished using Trinity through combined short reads with a certain overlap length ($k\text{-mer} = 25$) (Grabherr et al. 2011). The assembled unigenes were clustered using default parameters (Davidson and Oshlack 2014). The unigenes were deposited at DNA Data Bank of Japan/European Molecular Biology Laboratory/GenBank under the accession number GHKR00000000.

Gene functional annotation and differential expression analysis. The assembled unigenes were subjected to similarity searches against the NCBI nonredundant nucleotide and protein databases (Altschul et al. 1997; Buchfink et al. 2015). The clean data were aligned to the assembled transcriptome (mismatch for bowtie2 = 0), and the read count and the expected number of fragments per kilobase of transcript sequence per millions base pairs sequenced for each gene were obtained. Differential expression

analysis was conducted using the DEGseq R package using a P value < 0.05 cut off and a minimum 2.5-fold change (Wang et al. 2010).

Determination of the complete APV1 genomic sequence from the WNY sample. To verify the genomic sequence of APV1 from the WNY sample, 11 primer sets covering the near complete virus genome (Supplementary Table S1) were designed based on the differentially expressed gene (DEG) sequences (Supplementary Table S2) aligned with a previously reported APV1_HN genome (KR349464). Rapid amplification of cDNA ends (RACE) was carried out to confirm the 5' and 3' terminal sequences using the 5'/3' RACE Kit, second generation (Roche, Indianapolis, IN, U.S.A.), according to the manufacturer's instructions. For 3' RACE, total RNA was polyadenylated prior to reverse transcription using *Escherichia coli* Poly (A) Polymerase (New England Biolabs, Ipswich, MA, U.S.A.). PCR was carried out using PrimeSTAR GXL DNA Polymerase (TaKaRa, Dalian, China), and the resulting products were incubated with Taq polymerase at 72°C for 10 min, then ligated into the pMD-19T vector (TaKaRa). Three independent positive clones of each fragment were subjected to Sanger sequencing (Sangon Biotech, Guangzhou, China). Overlapping triplicate sequences were assembled into the complete genome using SeqMan Pro 7.1.0 (Lasergene; GATC Biotech) and the final consensus sequence was determined by comparison of the triplicates with the original DEG sequences. Open reading frames (ORFs) were predicted using ORF Finder with default settings (<https://www.ncbi.nlm.nih.gov/orffinder/>).

Fig. 1. Location of sample collection sites in Hainan. Stars and gray circles indicate the sampling locations. Numerals indicate the number of samples collected at each site.

Fig. 2. Symptoms progression of yellow leaf disease (YLD) in Hainan, China. **A**, Discoloration of leaflet tips of mid-crown leaves. **B**, Yellowing symptoms begin in the lowermost leaves. **C**, Abrupt demarcation of yellow and green areas in an affected leaf. **D**, Chlorosis spreads along vascular tissue with a sharp green-yellow border. **E**, Panoramic view of YLD symptoms in a betel palm plantation.

Virus purification and electron microscopy. Virus purification was performed according to a previous description (Hull 2001). YLD-WNY leaf tissue (60 g) was ground in five volumes of extraction buffer (0.2 M sodium acetate, pH 5.0, and 0.4% β -mercaptoethanol). The homogenate was filtered through four layers of cheesecloth, held on ice for 30 min, and centrifuged at $17,200 \times g$ for 10 min. PEG6000 was added to the supernatant, along with NaCl and Triton X-100, to final concentrations of 8%, 0.2 M, and 0.5%, respectively. The mixture was stirred at 4°C for 2 h, then centrifuged at $18,275 \times g$ for 15 min. The pellet was suspended in 30 ml of 32 mM Tris-citrate buffer (pH 6.5) containing 0.5% Triton X-100. The suspension was then centrifuged at $23,000 \times g$ for 10 min to pellet impurities. The clear liquid was further centrifuged at $140,000 \times g$ and 4°C for 2 h through a 5-ml cushion of 30% sucrose. The supernatant was discarded, and the pellet was resuspended in Tris-citrate buffer. The viral suspension was subjected to sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). Additionally, leaf tissue was mixed with sterilized distilled water at a ratio of 1:10 (g/ml) and ground with a mortar and pestle. The mixture was placed on ice for 5 min, and the resulting supernatant and purified viral suspension were immediately dropped separately onto precoated copper grids (200 mesh). After adsorption for 2 min, the loaded grids were stained with 1% phosphotungstic acid for 2 min. The stained grids were dried under a tungsten lamp for 30 min, then examined with a transmission electron microscope (Hitachi HT-7700; Japan).

RT-PCR. Total RNA was extracted from each leaf sample using the Universal Plant Total RNA Extraction Kit (BioTeke, China) and from mealybugs using TRIzol reagent (Invitrogen), following the manufacturer's instructions. The extracted RNA was subjected to reverse transcription using TransScript One-Step gDNA Removal and cDNA Synthesis SuperMix (TransGen Biotech, China) with random primers, following the manufacturer's instructions. PCR assays were carried out in 20- μ l reaction volumes containing 1 μ l of cDNA, 8 μ l of double-distilled H₂O, 10 μ l of 2 $\times$ EcoTaq PCR SuperMix (TransGen Biotech), and 0.5 μ l of each 10 μ M primer. In all, 27 primers were designed for amplification of genome of APV1-WNY isolate (primer numbers 1 to 27), five primer

pairs for diagnostic assays (primer numbers 28 to 37), one pair (YLDV-CP-F/YLDV-CP-R) for phylogenetic analysis, and one pair (ITS2-M-F/ITS2-M-R) for mealybug classification (Supplementary Table S1).

Genetic diversity analysis of APV1 isolates. The complete coat protein (CP) gene of APV1 was amplified using the YLDV-CP-F/YLDV-CP-R primer pair (Supplementary Table S1) with cDNA from YLD-positive plant samples and mealybug samples collected from various epidemic sites. RT-PCR was performed as described above. Multiple alignment and construction of phylogenetic trees were performed as previously described (Li et al. 2020).

Results

Symptoms of yellow leaf disease in Hainan. Yu et al. (1986) first reported the betel palm YLD symptoms in Hainan: yellowing was initiated in the lowermost leaves and expanded upward to younger leaves. The yellowing leaves became gray-brown due to necrosis and fell off at the later stages. No green-yellow border was evident, and no infection with any known pathogen was observed. There was no apparent infection center and, therefore, this YLD was proposed to be caused by potassium deficiency (Yu et al. 1986). The symptoms of YLD that we observed on betel palm in Hainan begin with yellowing of leaflet tips of the midcrown (Fig. 2A) or the lowest leaves (Fig. 2B). At the early stage, clear demarcation of affected yellow and normal green areas could be observed on the infected leaves (Fig. 2C), which distinguishes this disease from physiological yellowing. Chlorosis spread along the direction of vascular tissue while the midribs remained green, forming a green-yellow border (Fig. 2D). At the later stage, the yellowing expanded to younger and lower leaves, while the green-yellow border on older leaves became unclear. Newly emerging leaves were stunted and the crown size was markedly reduced, resulting in the “bunchy top” symptom. Additionally, the YLD-affected betel palm were more sensitive to drought and cold stresses based on anecdotal observations. In winter or the dry season, YLD-affected betel palm orchards showed more serious yellowing symptoms, and the whole plantation appeared burnt (Fig. 2E). The yellowing symptom became milder and could be nearly invisible in the rainy and warm seasons.

RNA-seq revealed the potential causal agent of YLD. To identify the potential causal agent of YLD in Hainan, two leaf samples with typical YLD symptoms and no known fungal or bacterial diseases were collected from YLD epidemic areas in BTY and WNY. Leaf tissues from one symptomless betel palm served as the negative control sample. RNA-seq generated a total of 68,564,262 (BTY), 97,421,918 (WNY), and 92,208,844 (symptomless) raw reads from the three sequenced libraries (Supplementary Table S3). After quality check and filtering, 252,275,270 clean reads were obtained from all three libraries (Supplementary Table S3). Following de novo assembly, DGE analysis, and BLASTx against nonredundant protein, nonredundant nucleotide, Swissprot, protein domains or families (PFAM), Gene Ontology, clusters of eukaryotic orthologous groups, and other databanks, six DEGs assembled by 88,750 (BTY) and 13,639 (WNY) clean reads were determined to be homologous to sequences of APV1_HN (KR349464) (Supplementary Table S2). The remaining nonhost DEGs were detected in both symptomatic and symptomless samples and belonged to several fungal, bacterial, and arthropod species and panax notoginseng virus A (genus *Totivirus*) (Supplementary Table S2), indicating that they are likely not involved in YLD. Taken together, these results indicate that APV1 is the likely putative agent of YLD in Hainan, further confirming the results of a previous study (Yu et al. 2015).

Viral agent purified from YLD-affected samples. Virus purification was attempted for a YLD-positive WNY sample and a symptomless sample from a nonepidemic area. The putative viral suspension was first subjected to SDS-PAGE analysis, revealing a weak band between 50 and 75 kDa in the WNY sample and no band in the symptomless sample (Fig. 3A). To further determine whether the viral suspension contained APV1, total RNA was

Fig. 3. Viral purification results and transmission electron micrographs. **A**, Sodium dodecyl sulfate polyacrylamide gel electrophoresis analysis of the purified viral suspension: CK = symptomless sample, YLD = yellow leaf disease sample, protein marker = prestained Blue Plus (TransGen Biotech, China), and kDa = kilodaltons; **B**, Reverse-transcription PCR detection of areca palm velarivirus 1 WNY isolate in the purified viral suspension. DNA ladder = D2000 plus (Monad Biotech, China) and bp = base pair. **C** and **D**, Electron micrograph of purified virion.

isolated from the virus suspension for RT-PCR using TRIzol reagent. APV1 was detected in the WNY sample but not in the control (Fig. 3B). The virus suspension was further subjected to transmission electron microscopy. Flexuous and filamentous particles, the typical morphology of the family *Closteroviridae*, were

observed. Interestingly, the length of virion particles ranged from about 2,000 nm (Fig. 3C) to 3,500 nm (Fig. 3D). The differing lengths of virion particles might be due to immature virions undergoing assembly, broken virions after purification, or virions with end-to-end aggregation.

Fig. 4. A, Schematic comparison of the genome organization of areca palm virus 1 (APV1) isolates APV1_HN (KR349464) and APV1-WNY (MK956940). UTR = untranslated region, L-PRO = papain-like protease, MTR = RNA methyltransferase, HEL = RNA helicase, RdRp = RNA-dependent RNA polymerase, p4 = 4-kDa protein, Hsp70h = heat shock protein 70 homolog, p21 = 21-kDa protein, p60 = 60-kDa protein, CP = coat protein, CPm = minor coat protein, p26 = 26-kDa protein, p18 = 18-kDa protein, p19 = 19-kDa protein, and p25 = 25-kDa protein. **B**, Phylogenetic analysis based on amino acid sequences of the HSP70 gene of members of the family *Closteroviridae*.

Determination of the complete genome of the APV1-WNY isolate.

To sequence the entire genome of APV1 isolated from sample WNY, 11 fragments covering the nearly complete virus genome were amplified using RT-PCR, and the two terminal fragments were obtained through 5' and 3' RACE using primers 1 to 27 (Supplementary Table S1). Thirteen fragments were used to generate the 17,546-nucleotide (nt) complete genome of APV1-WNY isolate from Hainan (MK956940), which is longer than the previously deposited APV1_HN genome (16,080 nt; KR349464). The genome of the WNY isolate possesses 11 ORFs with a 48-nt 5' untranslated region (UTR) and a 225-nt 3' UTR. The 5' terminal region, including ORF1a, and the 3' terminal region, including ORF10, of the WNY isolate are approximately 1,000 and 500 nt longer, respectively, than those of the previously reported APV1_HN genome (Fig. 4A), suggesting that this isolate might be a novel genetic variant of APV1 that is significantly different from the previously reported APV1_HN isolate (Yu et al. 2015). Although the 5' RACE has not been finished, 17,419 nt of the BTY isolate have been amplified by PCR and sequenced. BTY and WNY genomes share identical 3' UTR length (225 nt) and 99.6% nucleotide identity. The 5' UTR and 3' UTR of APV1_HN reported by Yu et al. (2015) show codon continuity and sequence homology with ORF1a and ORF10, respectively, of YLD-WNY, suggesting that APV1_HN is incomplete at both 5' and 3' ends.

Both APV1-WNY (MN956940) and APV1_HN (KR349464) share the same genome organization but ORF1a showed notable sequence variation (83.0% nucleotide and 84.8% amino acid identities). Both isolates shared higher sequence similarity at >90% nucleotide and amino acid identities in seven ORFs (ORF3, ORF4, ORF5, ORF6, ORF7, ORF8, and ORF9). APV1-BTY also shared similar levels of sequence similarity in these seven ORFs, suggesting that these regions could be selected for primer design for diagnosis of APV1. Due to its possible sequence incompleteness, ORF10 of APV1_HN encodes a 19-kDa protein, which is evidently shorter than the 25-kDa protein encoded by ORF10 of APV1-WNY. ORF10 of both isolates shared 84.4 and 94.7% nucleotide and amino acid identities, respectively.

Phylogenetic analysis of amino acid sequences of the heat shock protein 70 homolog (HSP70h) of 44 sequences of various members of the *Closteroviridae* family showed that both APV1 isolates clustered within the *Velarivirus* clade along with six other species; that is, little cherry virus 1 (LChV-1), grapevine leafroll-associated virus 7 (GLRaV-7), and cordyline virus 1- to -4 (CoV-1 to -4) (Al Rwahnih et al. 2012; Jelkmann et al. 2012; Lim et al. 2015; Melzer et al. 2011). The four *Closteroviridae* genera were separated, and the clade formed by most *Velarivirus* spp. was close to the *Crinivirus* clade (Fig. 4B), consistent with previous reports (Jelkmann et al. 2012; Lim et al. 2015). However, some putative *Velarivirus* spp. such as

blueberry virus A, olive leaf yellowing-associated virus, persimmon virus B, and actinidia virus 1 formed a separate clade in close proximity to the genus *Closterovirus* (Fig. 4B). Another putative velarivirus, mint vein banding-associated virus, formed an independent cluster between the genera *Closterovirus* and *Ampelovirus* (Fig. 4B).

APV1 is associated with YLD. In the investigation of association between APV1 and YLD, five specific primer sets (YLDV1, YLDV2, YLDV3, YLDV4, and YLDV5) were designed according to the consensus sequences of conserved ORFs of APV1-WNY and APV1_HN to minimize potential false-negative results in the diagnosis (Supplementary Table S1). RT-PCR amplified bands of the expected sizes from sample WNY. Sequencing showed that each PCR-obtained band shared 98% identity with corresponding sequences of APV1_HN (data not shown), revealing conserved sequences in APV1. APV1 was detected by RT-PCR in all 76 YLD-affected symptomatic samples collected from eight locations (Table 1), with at least two of five primer sets tested. YLDV4 were recommended for routine diagnosis because of the sequence consensus in all known APV1 isolates and higher detection ratio in YLD samples collected in Hainan. In addition, 8 of the 90 symptomless samples, 4 each from the YLD epidemic areas of Qionghai and Wanning, tested positive for APV1 by RT-PCR (Table 1). None of the samples collected from the nonepidemic areas of Haikou and Danzhou was positive for APV1 (Table 1). Thus, the results showed a strong and consistent association between APV1 and YLD.

Detection of APV1 in mealybugs. As a first step in the identification of potential vectors of APV1, we first searched for known insect vectors of the family *Closteroviridae*, including aphids, whiteflies, and mealybugs, in betel palm plantations. The field survey revealed that mealybugs (Fig. 5A) were widespread in Hainan, seriously infesting betel palm; aphids and whiteflies were seldom observed. To determine the taxon of the mealybugs, specific DNA markers were used in PCR, as previously described (Malausa et al. 2011). The phylogenetic analysis of the obtained ITS2 gene sequence fragment revealed that the mealybug clustered with *Pseudococcus comstocki*, with 98% nucleotide identity (Supplementary Fig. S1). The morphological characteristics of the mealybug appear similar to *P. comstocki*. However, the mealybug species collected in Hainan is tentatively named as *Pseudococcus* sp. until additional, more definitive molecular and morphological data become available.

Twenty-two mealybug samples and the corresponding plant tissue samples were collected from YLD-affected betel palm from six locations in Hainan. In RT-PCR, APV1 was detected in all of the plant samples and in 20 of 22 mealybug samples (Fig. 5B). Sequencing results revealed that the amplified DNA fragments from the mealybugs were virus specific and shared >98% identities with the corresponding sequences obtained from the plants (data not

Table 1. Detection of areca palm velarivirus 1 by reverse-transcription PCR in betel palm leaf samples collected in different sites

Sites	Area	Sample types ^a	Collected samples (n)	Detected samples (n)	Ratio of detection (%)
Baoting	Epidemic	YLD symptom	4	4	100
Dingan	Epidemic	YLD symptom	18	18	100
Qionghong	Epidemic	YLD symptom	7	7	100
Tunchang	Epidemic	YLD symptom	3	3	100
Wanning	Epidemic	YLD symptom	14	14	100
Qionghai	Epidemic	YLD symptom	18	18	100
Lingshui	Epidemic	YLD symptom	3	3	100
Ledong	Epidemic	YLD symptom	9	9	100
Dingan	Epidemic	Symptomless	21	0	0
Tunchang	Epidemic	Symptomless	20	0	0
Qionghai	Epidemic	Symptomless	8	4	50
Wanning	Epidemic	Symptomless	19	4	21
Haikou	Nonepidemic	Symptomless	12	0	0
Danzhou	Nonepidemic	Symptomless	10	0	0

^a YLD = yellow leaf disease.

shown). These results showed that *Pseudococcus* sp. mealybugs can ingest APV1, and this species could be a candidate for vector transmission assays.

Genetic diversity analysis of APV1 isolates. To evaluate the genetic diversity of APV1 isolates, phylogenetic analysis was carried out using the CP sequences obtained from leaf and mealybug samples in this study and corresponding sequences of APV1 retrieved from GenBank (MN296251 to MN296270). The sequences derived from mealybugs and their corresponding plant sources were marked with the suffixes “-M” and “-P”, respectively. The results showed very close clustering of the mealybug- and host-plant-derived sequences, indicating close genetic relationships of virus isolates sampled from mealybugs and the betel palm trees from which the insects were collected (Supplementary Fig. S2). Additionally, phylogenetic analysis also revealed geography-specific clustering patterns. All three samples collected in Northern Hainan (Dingan) were clustered together and three samples collected in Midland Hainan (Qiongzong) were separated from other samples. Samples collected in Southern Hainan (Ledong, Lingshui, and Baoting) and Eastern Hainan (Qionghai and Wanning) grouped together. An additional primer (YLDV-CP-F/R) was designed based on alignment of the CP gene sequences of the different isolates for routine PCR diagnosis.

Discussion

Although the YLD symptoms described by different authors are not identical, the common symptom is similar to what we observed in Hainan (Fig. 2). Luo et al. (2001) classified YLD into two types: “yellowing type” and “bunchy top type”. In our opinion, these two types of YLD may be different stages of symptom development. Phytoplasmas have long been associated with typical YLD symptoms (Kanatiwela-de Silva et al. 2015; Luo et al. 2001; Manimekalai et al. 2010; Nayar and Seliskar 1978; Ponnammam et al. 1991). However, in another study, no phytoplasma was detected in YLD-symptomatic betel palm in India (Purushothama et al. 2007). We collected numerous samples from YLD-affected betel palm and analyzed them for the presence of phytoplasmas using PCR. None of the samples were positive for phytoplasmas. In this work, RNA-seq was employed to identify the potential causal agent of YLD. No phytoplasma genes were identified. RNA-seq revealed a consistent association of APV1 with YLD in the study areas. Yu et al. (2015) used Illumina small RNA sequencing to molecularly characterize APV1 from a diseased areca palm showing YLD symptoms in Hainan. However, the field distribution of the virus and its biological properties were not determined. Also, an independent verification of the virus genome sequence by RT-PCR and Sanger sequencing was not performed. The results presented in this work confirm the association between APV1 and YLD, in agreement with Yu et al. (2015). Furthermore, our results

contributed to bridging the knowledge gaps in the Yu et al. (2015) study via a comprehensive description of the disease phenotype, the determination of the particle morphology of the associated APV1, Sanger sequencing of its complete genome, and the extensive analyses of several field-collected samples from multiple YLD endemic and nonendemic areas. We also showed that *Pseudococcus* sp. mealybugs are capable of ingesting APV1; however, the possible role of this species in virus transmission needs to be investigated.

The family *Closteroviridae* comprises more than 30 plant viruses that infect a wide range of economically important crops. Closteroviruses are monopartite or bipartite positive-sense single-stranded RNA plant viruses with flexuous, filamentous virions (Dolja et al. 2006; Karasev 2000; Rubio et al. 2013). Currently, the family consists of four genera, including *Ampelovirus*, *Closterovirus*, *Crinivirus*, and the recently approved *Velarivirus* (Martelli 2019). Viruses belonging to the same genus can be distinguished from others based on several or all of the following criteria; their particle size, size of CP (using the deduced amino acid sequence data), serological specificity using discriminatory monoclonal or polyclonal antibodies (if available), genome structure and organization (number and relative location of the ORFs), amino acid sequence of relevant gene products (polymerase, CP, and HSP70) differing by more than 25%, vector species and specificity, magnitude and specificity of natural and experimental host range, and cytopathological features (i.e., aspect of inclusion bodies and origin of cytoplasmic vesicles) (Lefkowitz et al. 2018). Current members of genus *Velarivirus* include APV1 (KR349464), LChV-1 (Y10237), GLRaV-7 (HE588185), CoV-1 (HM588723), CoV-2 (JQ599282), CoV-3 (JQ599283), and CoV-4 (JQ599284) (Al Rwahnih et al. 2012; Jelkmann et al. 2012; Lim et al. 2015; Melzer et al. 2011). Our results provide further support for the assignment of APV1 to the genus *Velarivirus*. The genome organization and phylogenetic analysis showed that APV1_HN and APV1-WNY are genetic variants of the same *Velarivirus* spp. (Fig. 4). No known vector has been identified for members of the genus *Velarivirus* (Martelli 2019); hence, further studies are needed to investigate the competency of *Pseudococcus* sp. mealybugs to vector APV1.

Although the present work provides additional evidence to support the association of APV1 with YLD, further studies are needed to fulfill Koch’s postulate and establish causation. Although mechanical transmission is possible for a few closteroviruses such as beet yellows virus, citrus tristeza virus, and grapevine leafroll-associated virus 2 (Goszczynski et al. 1996), none of the currently described velariviruses are known to be mechanically transmissible. Our attempts to mechanically inoculate APV1 virion suspension to betel palm failed to induce YLD symptoms (data not

Fig. 5. A, Mealybugs infesting leaves of areca palm. B, Areca palm virus 1 (APV1) was detected by reverse-transcription PCR in yellow leaf disease leaf samples (top) and corresponding mealybug samples from the same leaves (bottom). Lanes 1 to 22 = samples collected from different locations, including Wanning, Ledong, Lingshui, Qionghai, and Tunchang in Hainan. The Wanning sample and symptomless sample served as positive (+) and negative (–) controls, respectively. Lane M = DNA marker D2000 plus (Monad Biotech, China) and bp = base pair.

shown). Therefore, future efforts will focus on the construction of an APV1 infectious clone and the identification of its arthropod vector.

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