

Communication

A Putative Ormycovirus That Possibly Contributes to the Yellow Leaf Disease of Areca Palm

Xiaoqing Niu ¹, Zhongtian Xu ², Yujing Tian ², Siyun Xiao ², Yuan Xie ², Zhenguo Du ², Weiquan Qin ^{1,*} and Fangluan Gao ^{2,*} 

¹ Coconut Research Institute, Chinese Academy of Tropical Agricultural Science, Wenchang 571339, China; xqniu@catas.cn

² Institute of Plant Virology, Fujian Agriculture and Forestry University, Fuzhou 350002, China; xuzhongtian@nbn.edu.cn (Z.X.); yujingtian@fafu.edu.cn (Y.T.); siyunxiao@fafu.edu.cn (S.X.); xieyuan@fafu.edu.cn (Y.X.); duzhenguo@fafu.edu.cn (Z.D.)

* Correspondence: wqqin@catas.cn (W.Q.); rainy@fafu.edu.cn (F.G.)

Abstract: Yellow leaf disease (YLD) poses a significant challenge to areca palm cultivation, yet its etiology remains uncertain. During our investigation of YLD-affected areca palm plants, transcriptome sequencing revealed an RNA contig exhibiting striking similarities to the RNA-dependent RNA polymerase (RdRp) of ormycoviruses. Subsequent gene cloning techniques yielded the full-length sequence of this RNA, potentially representing either the complete or partial genome of a hitherto unidentified ormycovirus, tentatively named areca palm yellow leaf-associated ormycovirus (APY-LaOMV). RT-PCR detection found that APYLaOMV is present in over 30% of YLD-affected areca palm samples but is absent in healthy ones, suggesting a potential link between APYLaOMV and YLD. In summary, these data could be valuable in understanding the etiology of YLD in areca palms.

Keywords: areca palm; ormycovirus; ORFan; RNA-dependent RNA polymerase



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1. Introduction

Areca palm (*Areca catechu* L.) is a palm species indigenous to tropical regions of Asia and Oceania [1]. In addition to being harvested for chewing purposes, the areca palm has been traditionally used in various cultures for its potential medicinal properties. Presently, areca palm cultivation is widespread throughout tropical regions of South and Southeast Asia, serving as an important agricultural commodity for many local economies [1]. In China, areca palm cultivation is predominantly concentrated in Hainan Province, where the climate is conducive to its growth. As of 2018, the total cultivated area of areca palm in Hainan had expanded to approximately 0.11 million hectares, substantially enhancing the income and livelihoods of numerous farmers in the province [2].

The cultivation of areca palm faces several threats, with one of the most significant being yellow leaf disease (YLD). YLD affects the foliage of areca palm, causing the leaves to turn yellow and eventually leading to reduced photosynthesis and overall plant health [3]. The disease was first noted in India in 1914 [4] but has since become widespread in various areca palm-growing regions across Asia and Oceania, particularly in areas where areca palm cultivation is extensive [5]. The etiology of YLD remains a subject of ongoing debate. While earlier studies implicated phytoplasmas as the causative agent [6], recent investigations have proposed a closterovirus named areca palm velarivirus 1 (APV1) as a primary candidate [5,7,8]. Additionally, a totivirus named areca palm latent totivirus 1 (APLTV1) was identified, although APLTV1 seems to be more prevalent in healthy areca palm rather than diseased ones [9]. Beyond biotic factors, certain abiotic elements like nutritional imbalances and environmental stressors also appear to contribute to YLD [1]. Therefore, the disease is likely multifactorial, arising from a combination of biological agents and environmental conditions rather than a singular cause.

Ormycoviruses represent a recently proposed category of RNA viruses [10]. Unlike conventional RNA viruses, ormycoviruses feature an RNA-dependent RNA polymerase (RdRp) lacking the traditional GDD catalytic triad [11]. Instead, they possess alternative configurations such as NDD, GDQ, or, less commonly, SDD, HDD, or ADD [10]. In this short communication, we present our findings on the detection of a putative ormycovirus in areca palm, tentatively named areca palm yellow leaf-associated ormycovirus (APYLaOMV), and discuss its potential implications for YLD in this plant species.

2. Materials and Methods

2.1. Transcriptome Sequencing Experiments

Four areca palm leaf samples displaying characteristic YLD symptoms (Figure S1) were collected in Baoting County of Hainan Province in July 2022. Total RNA was extracted from a mixed pool of these samples using the CTAB method [12]. Subsequently, a cDNA library was prepared using the “Illumina TruSeq Total RNA with rRNA Sample Preparation Kit” and sent to Novogene (Beijing, China) for paired-end transcriptome sequencing on the Illumina Novaseq™ 6000 (Illumina, San Diego, CA, USA) instrument (150 bp × 2). Raw RNA-Seq datasets were processed to eliminate low-quality reads and adapter sequences, accomplished through fastp [13]. Reads shorter than 36 nt were systematically discarded. De novo assembly of the resulting 45,869,010 clean reads was conducted using the Trinity assembler 2.8.5 [14] and the assembled contigs were subjected to BLASTx 2.13 searches against the NCBI nonredundant nucleotide database (accessed on 14 September 2022).

2.2. Molecular Characterization of APYLaOMV

The 5′ and 3′ sequences of APYLaOMV were obtained by rapid amplification of cDNA ends (RACE) experiments. Potential open reading frames (ORFs) within the APYLaOMV genome were identified using the ORF Finder program available at NCBI. The RdRp of APYLaOMV was aligned with homologous sequences from related ormycoviruses using the MAFFT algorithm (v7.310) [15] implemented in PhyloSuite 1.23 [16]. Pairwise identity matrixes were generated based on the MAFFT alignments using SDT 1.2 [17]. A maximum likelihood (ML) phylogenetic tree was reconstructed to infer the relationships between APYLaOMV and other ormycoviruses. The tree was generated using IQ-TREE 1.68 [18] under the Q.pfam + F + I + G4 evolutionary model, which was determined by ModelFinder [19]. The reliability of the ML tree was assessed through ultrafast bootstrapping with 10,000 replicates.

2.3. Investigating the Relationship between APYLaOMV and YLD

To explore the association between APYLaOMV and YLD, a total of 544 areca palm samples exhibiting typical YLD symptoms and 20 healthy samples were collected from various locations in Hainan. The presence of APYLaOMV in these samples was determined by RT-PCR, utilizing APYLaOMV-R1 as the reverse-transcription primer and APYLaOMV-F1 and APYLaOMV-R1 as the PCR primers. Concurrently, the presence of APV1 and APLTV1 in these samples was investigated using primers designed from conserved regions of the two viruses (Table S2).

3. Results

3.1. Detection of a Putative Ormycovirus in Areca Palm Samples Showing YLD

In our quest to understand the etiology of YLD, we conducted transcriptome sequencing experiments. Consistent with previous reports, contigs corresponding to APV1 or APLTV1 were easily identifiable in our dataset [5,7,8]. However, in addition to these contigs, we observed the presence of a contig sharing significant similarities with the RdRp of eight ormycoviruses. This contig spans 3085 nucleotides in length and was represented by 555,480 reads in the sequencing library, strongly suggesting the presence of a putative ormycovirus in our samples. For simplicity, we refer to this putative ormycovirus as areca palm yellow leaf-associated ormycovirus (APYLaOMV). The final sequence of APYLaOMV,

which spans 3063 nucleotides (nts) excluding the 3' poly(A) tail, was deposited in GenBank under the accession number OR204700.

Most ormycoviruses possess a second genome segment that potentially encodes the capsid protein (CP) of the virus in addition to a segment encoding RdRp [10]. However, despite extensive analysis of our deep sequencing data, we were unable to identify any contigs corresponding to this second segment of APYLaOMV. Additionally, all our attempts to detect this segment using conventional PCR proved unsuccessful. As a result, we presume that APYLaOMV is a monopartite virus in this study, although we acknowledge the possibility that its second segment may have eluded detection.

3.2. APYLaOMV as a Novel Member of the Genus Alphaormycovirus

A single ORF was identified from the genome of APYLaOMV. This ORF extends from nt 17 to 3007 (Figure 1A) and encodes a putative RdRp consisting of 997 amino acid residues. As the RdRp of prototypic RNA viruses [11], the RdRp of APYLaOMV has the typical A-B-C motifs (Figure 1B). However, in line with the observations made from other ormycoviruses, the motif C of the APYLaOMV RdRp is characterized by an NDD triplet instead of the conventional GDD (Figure 1B). As shown in Figure 1C, the APYLaOMV RdRp shares 30.3% amino acid sequence identity with the RdRp of *Plasmopara viticola* Lesion-Associated Ormycovirus 7 (PvLaOMV7) and exhibits amino acid identities ranging from 20.3% to 25% with the RdRps of other ormycoviruses.

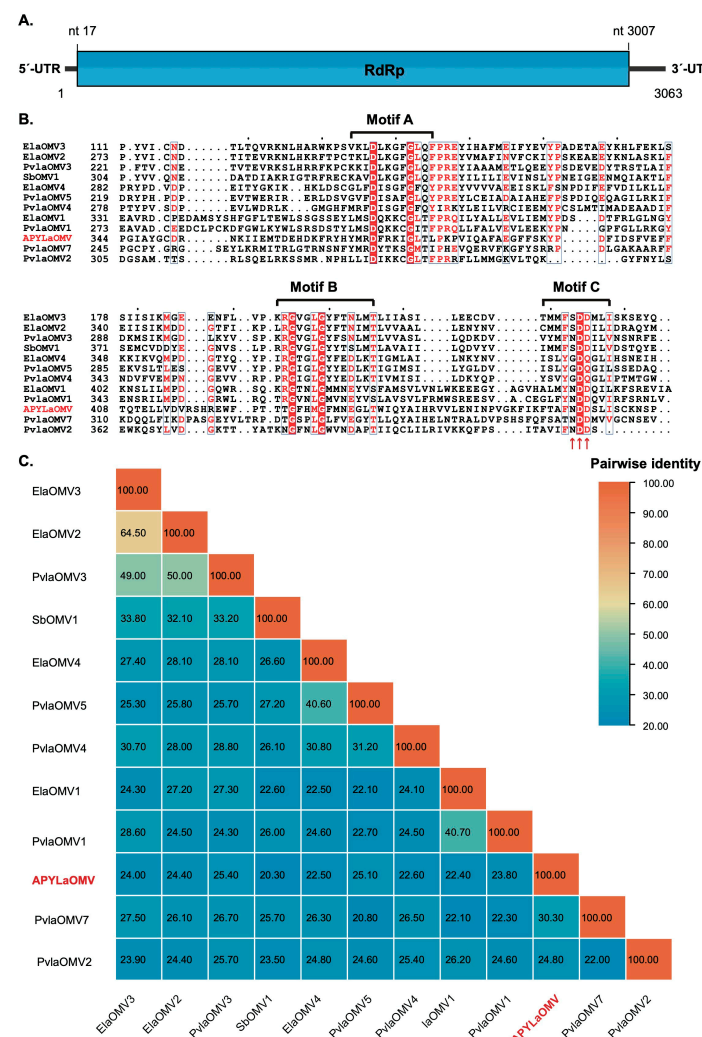


Figure 1. (A) Genomic structure of APYLaOMV. (B) Multiple sequence alignment of the typical A-B-C motifs from RdRp palm domain among ormycoviruses. (C) Pairwise comparisons between amino acid

sequence identity of putative RdRP compared with those of known ormycoviruses. APYLaOMV is indicated in red bold font. The NDD triad in the motif C of the APYLaOMV RdRp is indicated by red arrows. ElaOMV1 (erysiphe lesion associated ormycovirus 1, USW07195), PvlaOMV1 (*Plasmopara viticola* lesion associated ormycovirus 1, USW07200), PvlaOMV7 (*Plasmopara viticola* lesion associated ormycovirus 7, USW07199), PvlaOMV2 (*Plasmopara viticola* lesion associated ormycovirus 2, USW07197), PvlaOMV3 (*Plasmopara viticola* lesion associated ormycovirus 3, USW07202), ElaOMV3 (erysiphe lesion associated ormycovirus 3, USW07204), ElaOMV2 (erysiphe lesion associated ormycovirus 2, USW07207), SbOMV1 (*starmerella bacillaris* ormycovirus 1, USW07206), PvlaOMV4 (*Plasmopara viticola* lesion associated ormycovirus 4, USW07209), PvlaOMV5 (*Plasmopara viticola* lesion associated ormycovirus 5, USW07210), ElaOMV4 (erysiphe lesion associated ormycovirus 4, USW07208).

Ormycoviruses have been categorized into three genera—alphaormycovirus, betaormycovirus, and gammaormycovirus—based on sequence identity and phylogenetic analyses [10]. Our phylogenetic analysis supports this classification, revealing three major clades. APYLaOMV was located within Clade III, which comprises only alphaormycoviruses, suggesting that APYLaOMV belongs to this genus. However, it is important to note that Clade III can be further divided into two distinct subclades, IIIa and IIIb, as shown in Figure 2. Moreover, while Clade Ia and Clade II are exclusively composed of betaormycoviruses and gammaormycoviruses, respectively, Clade Ib includes viruses that have been classified as alphaormycoviruses, betaormycoviruses, or gammaormycoviruses. This suggests that further refinements may be needed for the classification of ormycoviruses.

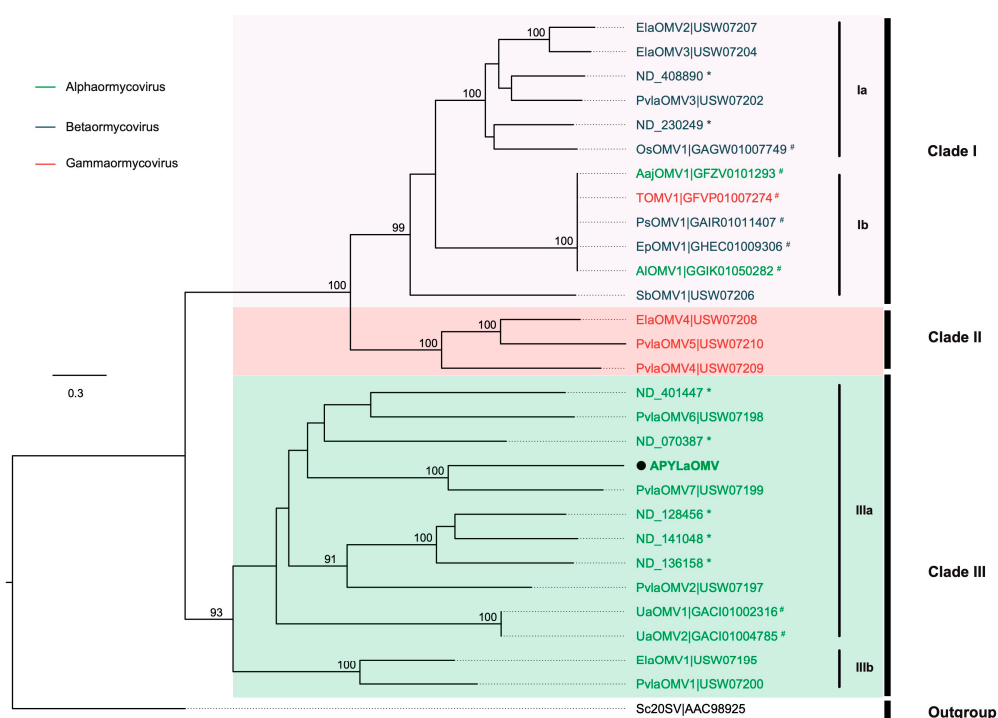


Figure 2. Maximum likelihood phylogenetic tree based on RdRP amino acid sequences of ormycoviruses. For each node, the bootstrap values (only >90%) are above branches. *Saccharomyces cerevisiae* 20S narnavirus was used as an outgroup. APYLaOMV identified from this study is marked with a black dot. See Figure 1 for details of the abbreviations of the viruses shown in the tree. The reference ID of Transcriptome Shotgun Assembly (TSA) Database and SeqID identified from the RVMT database, as presented by Forgia et al. [10], are marked in * and #, respectively.

3.3. Prevalence of APYLaOMV in Areca Palm Samples Showing YLD

To investigate the relationship between APYLaOMV and YLD, we detected its presence in 544 areca palm samples displaying typical YLD symptoms and 20 healthy areca palm

samples, as described in Section 2.3. For comparison, the presence of APV1 and APLTV1 in these samples was also assessed.

In line with existing data [8], APV1 displayed a robust association with YLD, with a remarkably high average detection rate of 71% in symptomatic areca palm samples (Table 1). Importantly, APV1 was entirely absent in all 20 healthy areca palm samples (Table S1). APLTV1 was also detected in symptomatic areca palm samples, albeit at a lower detection rate of approximately 30% (see Table 1). However, APLTV1 was also found in 5 out of the 20 healthy areca palm samples (Table S1). The detection rate of APYLaOMV in symptomatic areca palm samples was comparable to that of APLTV1 (Table 1). However, it was not detected from any of the 20 healthy areca palm samples (Table S1).

Table 1. Detection rate (%) of three viruses in areca palm plants collected from 18 different cities in Hainan Province.

Sampling Location	Tested Sample	Positive for			Double or Triple Infections			
		①	②	③	① + ②	② + ③	① + ③	① + ② + ③
Baoting	27	44.44	74.07	25.93	22.22	7.41	11.11	7.41
Qionghai	31	35.48	64.52	35.48	6.45	6.45	3.23	0.00
Lingao	26	42.31	80.77	34.62	15.38	26.92	0.00	11.54
Wanning	44	34.09	59.09	22.73	0.00	11.36	4.55	0.00
Ledong	25	24.00	88.00	36.00	8.00	12.00	0.00	12.00
Ding'an	41	43.90	73.17	41.46	12.20	12.20	4.88	17.07
Danzhou	27	59.26	51.85	29.63	0.00	0.00	29.63	0.00
Wuzhishan	54	44.44	44.44	42.59	0.00	7.41	24.07	0.00
Tunchang	33	33.33	69.70	27.27	6.06	9.09	3.03	6.06
Baisha	27	40.74	77.78	37.04	11.11	3.70	3.70	14.81
Changjiang	22	13.64	100.00	13.64	9.09	9.09	9.09	4.55
Haikou	27	25.93	77.78	25.93	7.41	3.70	11.11	3.70
Sanya	33	36.36	60.61	27.27	6.06	3.03	15.15	0.00
Chengmai	26	34.62	76.92	15.38	11.54	0.00	15.38	0.00
Qiongzhang	29	6.90	75.86	20.69	0.00	3.45	0.00	0.00
Dongfang	17	17.65	88.24	41.18	0.00	35.29	0.00	5.88
Wenchang	30	23.33	76.67	16.67	6.67	0.00	10.00	0.00
Lingshui	25	20.00	84.00	20.00	4.00	12.00	8.00	0.00
Total	544	33.64	70.77	29.23	6.62	8.46	9.19	4.41

① APYLaOMV; ② APV1; ③ APLTV1.

It is worth noting that for all three viruses, especially APLTV1 and APYLaOMV, their detection rates in symptomatic areca palm samples exhibited substantial variation depending on the sample collection locations. Remarkably, over 87% of the samples from Danzhou and Wuzhishan tested positive for APYLaOMV and APLTV1. In contrast, despite its high average detection rate, APV1 was only detected in 24 out of the 54 (44%) symptomatic areca palm samples from Wuzhishan (see Table 1). Furthermore, 8 out of these 24 (33%) APV1-positive samples from Wuzhishan also tested positive for APLTV1 or APLTV1 and APYLaOMV.

4. Discussion

In this study, we identified a putative ormycovirus named APYLaOMV in areca palm. Given the challenge of establishing a direct link between APYLaOMV and YLD, we examined its prevalence in YLD-affected areca palm plants to gain insights into its potential involvement. The inconsistent presence of APYLaOMV in all YLD-affected samples suggests that it may not be the sole causal agent of YLD. However, its presence in diseased samples and absence in healthy ones imply that it could contribute to disease development, possibly in conjunction with other factors. Notably, APYLaOMV is the most prevalent virus in YLD samples from Danzhou and Wuzhishan. This suggests that APYLaOMV may play a particularly important role in YLD in these regions. Alternatively,

the weather, areca palm cultivars, or cultivation methods in these regions may be more conducive to the prevalence of APYLaOMV.

Among the three viruses detected in over 500 YLD samples, none were found in all samples. Even APV1, which has a well-established association with YLD, was detected in only 71% of YLD-affected plants, with lower detection rates in certain regions. This underscores the multifactorial etiology of YLD, where various agents may contribute to disease manifestation. Notably, two potyviruses were recently reported from areca palm [20,21]. As these potyviruses were reported to induce symptoms distinctive to YLD, we did not assess their presence in our YLD samples. However, considering the complexity of YLD, further experiments are warranted before excluding their potential contribution to YLD.

All known hosts of ormycoviruses have thus far been fungi [10,22]. Considering the close relationship between plants and fungi, it is conceivable that APYLaOMV might infect a fungus associated with areca palm. However, the absence of visible fungal infection signs in our samples does not support this hypothesis. Moreover, the abundance of APYLaOMV reads in our deep sequencing data argues against the possibility of an endophytic fungus being the source, given their typically low abundance. Therefore, we lean towards the belief that APYLaOMV is a plant virus. This interpretation aligns with findings in virology, where various virus groups, once thought exclusive to fungi, such as totiviruses, have been found to infect plants as well [9]. Nevertheless, further investigations are required to confirm this hypothesis.

Typically, ormycoviruses have two genomic components, with one encoding RdRp and the other encoding the CP of the virus [10]. However, in the case of APYLaOMV, only the genomic component encoding RdRp was identified. Several explanations might account for this observation. Firstly, the CP-encoding segment of APYLaOMV might be highly divergent from any nucleotide sequences available in GenBank, thus eluding our homology-dependent detection strategies. Secondly, APYLaOMV may be a virus that does not encode a CP, as reported for the viruses within the family *Endornaviridae* [23]. Lastly, APYLaOMV could depend on a second virus for encapsidation, suggesting that it may be a satellite virus. Further research into these possibilities will be invaluable for understanding the ecology and evolution of ormycoviruses, a novel and underexplored group of viruses.

5. Conclusions

In conclusion, this report represents the first documentation of APYLaOMV in areca palm. It introduces a new candidate for consideration in the ongoing exploration of YLD mechanisms in areca palm.

Supplementary Materials: The following supporting information can be downloaded at: <https://www.mdpi.com/article/10.3390/f15061025/s1>, Figure S1: The YLD symptoms on areca palm; Table S1: Detection of three viruses in 20 healthy areca palm plants; Table S2: Primers used in this study.

Author Contributions: W.Q. and F.G.: conceptualization and design; F.G.: methodology; X.N., Y.T., S.X. and Y.X.: data curation; Z.X. and F.G.: formal analysis; W.Q., Z.D. and F.G.: interpretation of results; W.Q., Z.D. and F.G.: writing original draft. All authors have read and agreed to the published version of the manuscript.

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Data Availability Statement: All data used in this study are publicly available on NCBI. The complete genome sequence obtained in this study has been deposited in GenBank with accession numbers OR204700.

Conflicts of Interest: The authors declare no conflicts of interest. The funders had no role in the design of the study; in the collection, analyses, or interpretation of data; in the writing of the manuscript; or in the decision to publish the results.

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