DETECTION AND CHARACTERIZATION OF PLANT PATHOGENIC VIRUSES IN HAWAII

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Abstract

Historically and currently virus diseases have caused significant loss to agricultural stakeholders in HI (HI) and worldwide. Pineapple was one of HI's most important agricultural crops for many years though recently production has declined. Mealybug wilt of pineapple (MWP) is one of the most destructive diseases of pineapple worldwide and the causal agents are single-stranded RNA (ssRNA) viruses know as *Pineapple mealybug wilt-associated viruses* (PMWaVs) of the *Ampelovirus* genus of the *Closteroviridae* family. Though the disease etiology of MWP is not completely understood much of the genomes of the various PMWaVs have been characterized previously; this study furthers the characterization of several PMWaVs. To clarify the mysterious etiology of MWP it is important that the function of the genes of the various PMWaVs are well understood, underlying the importance of genome characterization.

The genomes of PMWaV-1, PMWaV-2 and PMWaV-3 were first characterized in HI prior to this study, but only PMWaV-1 has been completely characterized. Two putative PMWaV member species: PMWaV-4 and PMWaV-5 had been reported from HI and Australia respectively. The putative PMWaV-4 was first reported in 2005. The PMWaV-4 heat shock protein 70 (HSP70) was the only gene reported. Prior to 2011, the criteria for species demarcation in the *Ampelovirus* genus was based off a 10% divergence of the amino acid (aa) sequence of the HSP70, RNA dependent RNA polymerase (RdRp) and coat protein (CP) genes; in 2011, the criteria was changed to a 25% divergence of the same three genes. In this study we characterize the remainder of the PMWaV-4 genome. Total RNA was used as the template for cDNA library construction and subjected to high-throughput sequencing (HTS), on an Illumina TruSeq 500 platform, multiple assembly pipelines were utilized for contig assembly and a 13,150 nucleotide (nt) scaffold was assembled that shares a high level of sequence homology with the 13,070 nt PMWaV-1 reference genome (AF414119). The aa similarity of the three species demarcation genes of PMWaV-4 compared to PMWaV-1 is 89, 87 and 85% for the RdRp, HSP70 and CP respectively. Based off the sequence similarity to PMWaV-1 we have determined PMWaV-4 to be a strain of PMWaV-1 rather than a distinct species. Additionally, HTS assemblies of PMWaV-1, PMWaV-2 and PMWaV -3 genomes shared a 99, 99 and 97%, respectively, nt similarity to their originally published genomes and supplemented the

preexisting PMWaV-2 and PMWaV-3 sequences with 166 and 641 nt, respectively, in the 5' termini of the viruses.

The potyvirus *Banana bract mosaic virus* (BBrMV) and badnavirus *Canna yellow mottle virus* (CaYMV) were previously identified infecting *Alpinia purpurata* (flowering ginger) in HI. Recently, farmers growing flowering ginger have reported severe virus-like disease symptoms from multiple farms on Oahu, HI. Surveys were conducted in September 2016 and April 2018 molecular and serological techniques were used to assay symptomatic flowering ginger for the presence of the previously identified viruses. CaYMV was found to be widespread at all but one location however no BBrMV was detected in any samples from either survey. Characteristic symptoms of CaYMV and BBrMV were not observed, however streaking symptoms in CaYMV and BBrMV negative leaves, stems and bracts were observed. The lack of conclusive evidence linking CaYMV or BBrMV infection with the symptomatic flowering ginger assayed during this study may indicate the presence of another pathogen's involvement. Further study is necessary to conclude the casual organism of the disease found on flowering ginger.

Honolulu, HI is home to many community gardens which function to provide many urban dwellers the privilege of access to land for growing of edible and horticultural plants. Unfortunately, these areas are often 'hotbeds' of disease and pathogen dispersal. Recently, we identified several different plants infected with viruses from diverse crop and horticultural species. Potyvirus-specific ELISA detected potyviruses in symptomatic *Passiflora* spp. (passionfruit) and *Phaseolus lunatus* (lima bean). RT-PCR assay using degenerate potyvirus-specific primers then confirmed the ELISA results. Amplicons from the RT-PCR assay were cloned and sequenced using Sanger sequencing. Sequencing results revealed the potyviruses to be *Watermelon mosaic virus* (WMV) and *Bean common mosaic virus* (BCMV) infecting and passionfruit and lima bean respectively. WMV had previously been reported infecting passionfruit in HI. This was the first report of BCMV in HI and the sequence homology showed a high degree, 92% nt and 100% aa similarity to a BCMV isolate from China. A BCMV-specific ELISA was used to reconfirm the sequencing results. The unusually diverse amount of plants grown in a community garden as well as the high volume of individuals with access to the area provide the ideal circumstances for dissemination of plant pathogens.

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List of Abbreviations

Acronym	Definition
BBrMV	*Banana bract mosaic virus*
BCMV	*Bean common mosaic virus*
CaYMV	*Canna yellow mottle virus*
MWP	Mealybug wilt of pineapple
PMWaVs	*Pineapple mealy bug wilt associated viruses*
PMWaV-1	*Pineapple mealy bug wilt associate virus-1*
PMWaV-1 HN	*Pineapple mealy bug wilt associate virus-1* isolate Hainan
PMWaV-2	*Pineapple mealy bug wilt associate virus-2*
PMWaV-3	*Pineapple mealy bug wilt associate virus-3*
PC or PCV	*Pineapple Closterovirus*
GLRaV-1	*Grapevine leaf roll-associated virus-1*
GLRaV-3	*Grapevine leaf roll-associated virus-3*
GLRaV-4	*Grapevine leaf roll-associated virus-4*
LChV-2	*Little cherry virus-2*
PBNSPaV	*Pea bark necrosis stem pitting-associated virus*
WMV	*Watermelon mosaic virus*
RNA	ribonucleic acid
DNA	deoxyribonucleic acid
ssRNA	single-stranded RNA
dsRNA	double-stranded RNA
cDNA	complementary DNA
aa	amino acid
nt	nucleotide
CP	coat protein
CPd	coat protein duplicate
HEL	helicase
MET	methyltransferase
HSP	heat shock protein
RdRp	RNA dependent RNA polymerase
SHP	small hydrophobic protein
ORF	open reading frame
PCR	polymerase chain reaction
RT-PCR	reverse transcriptase PCR
qPCR	quantitative PCR
STDP-PCR	single tube dual primer PCR
IMC-RT-PCR	immunomagnetic capture RT-PCR
ELISA	enzyme linked immunosorbent assay
ISEM	immunosorbent electron microscopy
TBIA	tissue blot immunoassay
MAb	monoclonal antibody
USDA-ARS	United State Department of Agriculture -Agricultural Research Service
NCGR	National Clonal Germplasm Repository
PBARC	Pacific Basin Agricultural Research Center
NCBI	National Center for Biotechnology Information
BLAST	Basic Local Alignment Search Tool
HTS	high-throughput sequencing
contig	contiguous consensus sequence
mM	millimolar
µl	microliter
ng	nanogram

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Chapter 1

Literature Review

1.1 History of Mealybug Wilt of Pineapple

Mealybug wilt of pineapple (MWP) disease was first described in Hawaii (HI) in 1910[1]. The unusual disease etiology that involves the association of wilt, ants and mealybugs was first reported in 1925 when a relationship between ants, which protect the mealybugs and the reduction of disease incidence was noticed[2-5]. Studies revealed a direct relationship between mealybugs and MWP disease[6]. Observations that mealybug exposure did not always lead to symptoms and mealybugs transferred from symptomatic plants to healthy plants causing symptoms to developed suggested a transmissible factor was involved in MWP etiology)[7, 8]. Based on these findings a hypothesis was put forth that MWP involved a virus that was involved in the disease etiology; possibly transmitted by mealybugs[9, 10].

1.2 Viruses, Vector and Etiology of MWP Disease

Virus particles were isolated from MWP symptomatic plants in HI, Australia and Cuba confirming the virus hypothesis[11-15]. The pineapple virus was originally referred to as pineapple closterovirus (PC or PCV) because of its inclusion in the *Closteroviridae* family but was later renamed as *Pineapple mealybug wilt associated virus* (PMWaV) and classified as a member species of the *Ampelovirus* genus of the *Closteroviridae* family[16-18]. The virions of PMWaVs are flexuous rods are about 1200 nm in length, 10x12 nm in diameter (**Figure 1.1**), and have linear, positive single stranded RNA (ssRNA) genomes range from between 13 to 15 kb in size. PMWaVs are systemic, phloem limited viruses found throughout the plant in the roots, leaves, stems, fruits, crowns and ratoons of pineapple[17]. The genome of PMWaV-2 was characterized in HI[19] and was the first PMWaV genome to be characterized, followed by PMWaV-1[20] and PMWaV-3[21]. Interestingly, only pineapple plants subjected to the mealybug vectors: *Dysmicoccus brevipes* or *D. neobrevipes* (**Figure 1.1**) presence and infected with PMWaV-2

exhibit MWP symptoms (**Figure 1.1**); pineapple plants with the presence or absence of mealybugs and infections of PMWaV-1 or PMWaV-3 were not correlated with MWP symptom development[18, 22-25]. Two other pineapple viruses have been reported and proposed as tentative PMWaV type members: PMWaV-4 and PMWaV-5 reported in HI and Australia respectively[26, 27].

1.3 Detection of PMWaV

Monoclonal antibodies (MAbs) for PMWaV-1 and PMWaV-2 were developed and used in tissue blot immunoassays (TBIAs) and immunosorbent electron microscopy (ISEM)[17, 23]. Later, development of a reliable reverse-transcription PCR (RT-PCR) assays provided more reliable detection of PMWaVs[23, 26]. An immunomagnetic capture-reverse transcriptase-PCR (IMC-RT-PCR) assay was developed for the detection of PMWaV-1, -2 and -3[28]. A reliable, rapid and sensitive real-time RT-PCR (qRT-PCR) assay was developed to detect and quantify PMWaV-2[29]. A highly sensitive single-tube nested PCR assay was developed for detection of PMWaV-2[30].

1.4 Distribution of PMWaV

PMWaV-1 has been reported in Australia, Brazil, China, Costa Rica, Cuba, Guyana, Honduras, India, Indonesia, Kenya, Martinique, Philippines, Sri Lanka, Taiwan Thailand and HI[21, 23, 27, 31]. PMWaV-2 has been reported in Australia, Brazil, Costa Rica, Cuba, Honduras, Indonesia, Kenya, Malaysia, Philippines, Sri Lanka, Taiwan and HI[21, 23, 31, 32]. PMWaV-3 has been reported in Australia, Cuba, Taiwan and HI[21, 25, 31, 33, 34]. PMWaV-4 has only been reported in HI[26]. PMWaV-5 has only been reported in Australia[27].

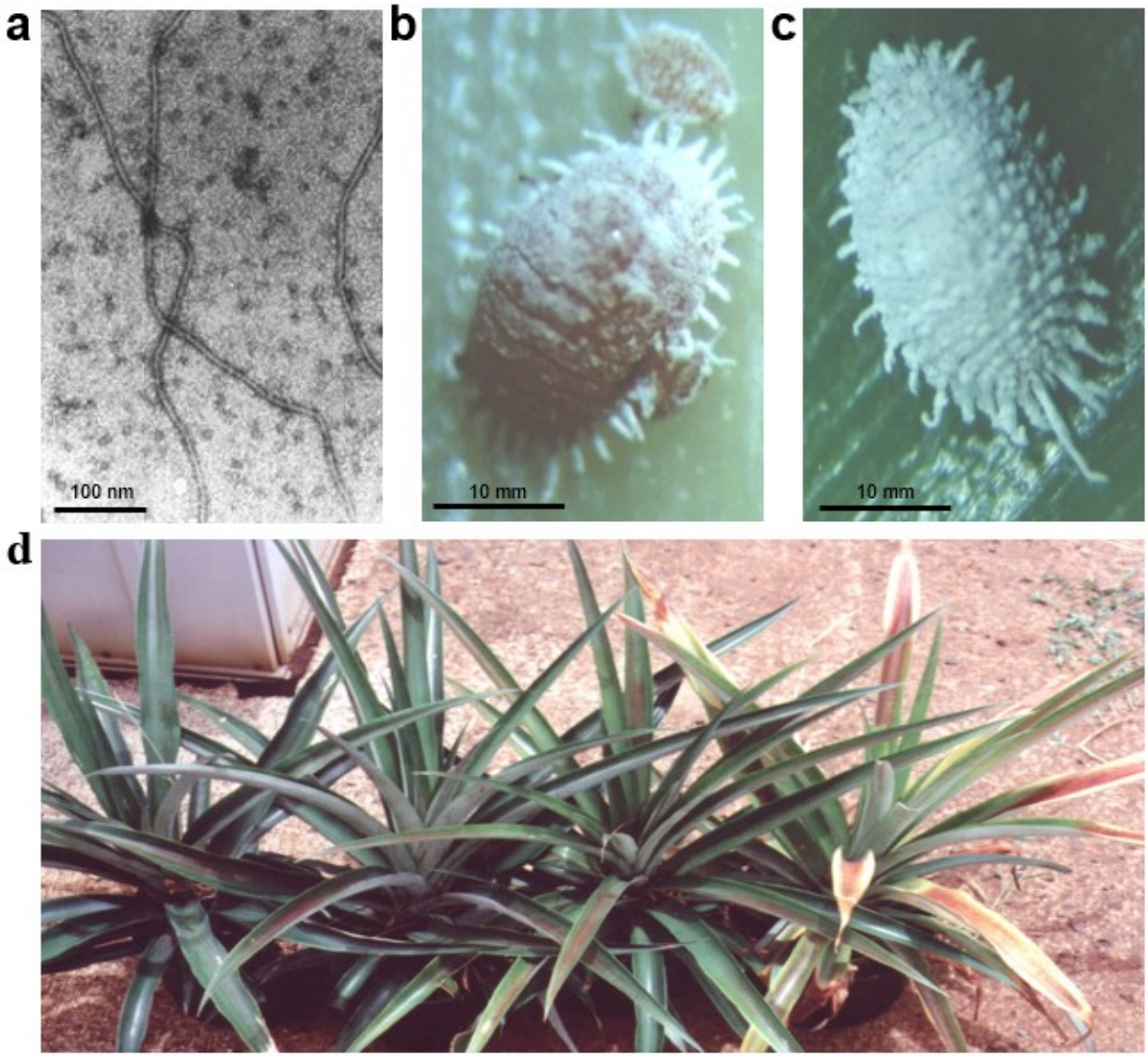

Figure 1.1 Mealybug vectors, virus particles and etiology of MWP disease
In Hawaii, the complex etiology of MWP disease includes: (**a**) *Pineapple mealybug wilt-associated virus*-2 (PMWaV-2) and mealybug vectors, either (**b**) *Dysmicoccus brevipes* or (**c**) *D. neobrevipes*. For MWP disease symptom development the presence of PMWaV-2 and mealybugs is required as shown in (**d**): from left to right: mealybug and PMWaV-2 free pineapple; mealybug free, PMWaV-2 infected pineapple; mealybug infected, PMWaV-2 free pineapple; mealybug and PMWaV-2 infected pineapple with symptoms of MWP disease

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Chapter 2

Further Genome Characterization of Pineapple mealybug wilt-associated viruses

2.1 Abstract

The genomes of *Pineapple mealybug wilt associated-virus*-1 (PMWaV-1), PMWaV-2 and PMWaV-3 were first characterized in Hawaii (HI) prior to this study, but only PMWaV-1 has been completely characterized. Two putative PMWaV member species, PMWaV-4 and PMWaV-5, have also been reported from HI and Australia respectively. The putative PMWaV-4 was first reported in 2005. The PMWaV-4 heat shock protein 70 (HSP70) was the only gene reported. Prior to 2011, the criteria for species demarcation in the *Ampelovirus* genus was based off a 10% divergence of the amino acid (aa) sequence of the RNA dependent RNA polymerase (RdRp), HSP70 and coat protein (CP) genes; in 2011, the criteria was changed to a 25% divergence of the same three genes. In this study we characterize the remainder of the PMWaV-4 genome. Total RNA was used as the template for cDNA library construction and subjected to high-throughput sequencing (HTS), on an Illumina TruSeq 500 platform, multiple assembly pipelines were utilized for contig assembly and a 13,150 nucleotide (nt) scaffold was assembled that shares a high level of sequence homology with the 13,070 nt PMWaV-1 (AF414119) reference genome. The aa similarity of the three species demarcation genes of PMWaV-4 compared to PMWaV-1 is 89, 87 and 85% for the RdRp, HSP70 and CP respectively. Based off the sequence similarity to PMWaV-1 we have determined PMWaV-4 to be a strain of PMWaV-1 rather than a distinct species. Additionally, HTS assemblies of PMWaV-1, PMWaV-2 and PMWaV -3 genomes shared a 99, 99 and 97%, respectively, nt similarity to their originally published genomes and supplemented the preexisting PMWaV-2 and PMWaV-3 sequences with 166 and 641 nt, respectively, in the 5’ termini of the viruses.

2.2 Introduction

Pineapple mealybug wilt-associated virus-1 (PMWaV-1), PMWaV-2 and PMWaV-3 are recognized member species of the *Ampelovirus* genus of the *Closteroviridae* family. The *Ampelovirus* genus is split into two subgroups based on genome length and organization, subgroup I and subgroup II. Subgroup I is comprised of viral species with large and complex genomes greater than 17,000 nucleotide (nt) with nine to twelve open reading frames (ORFs). Subgroup II is comprised of viral species with smaller less complex genomes about 13,000–14,000 nt in length with six ORFs[35]. It is important to note that PMWaV-2 belongs to subgroup I while PMWaV-1 and PMWaV-3 belong to subgroup II. In 2011, the International Committee on Taxonomy of Viruses (ICTV) recognized updated inclusion criteria for member species demarcation of ampeloviruses to require a divergence of at least 25% (previously 10%) of the amino acid (aa) sequence for three taxonomically relevant genes: RNA-dependent RNA polymerase (RdRp), heat shock protein 70 (HSP70) and coat protein (CP) genes[35].

The ampelovirus subgroup I member, PMWaV-2 (AF283103) genome was first characterized in Hawaii (HI), published in 2001,[19] (**Figure 2.1**) and only lacks characterization of its 5' terminus. PMWaV-2 is comprised of 10 open reading frames (ORFs) flanked by untranslated regions (UTR) on its 5' and 3' termini (though the 5' UTR has yet to be characterized)[19]. From the 5' terminus of PMWaV-2 ORFs include: ORF1a encodes the protease (PRO), methyltransferase (MTR) and helicase (HEL); ORF1b encodes the RdRp; ORF2 encodes p5, a small hydrophobic protein (SHP); ORF3 encodes the HSP70, ORF4 encodes p46, a protein of unknown function; ORF5 encodes the CP; ORF6 encodes a coat protein duplicate (CPd); ORF7 encodes p20, a silencing suppressor; ORF8 encodes p22 a silencing suppressor; ORF9 encodes p6, a protein of unknown function[19].

The complete genome of PMWaV-1 (AF414119) was published in 2008[20] and the partial genome of PMWaV-3 (DQ399259) in 2009[21] (lacking characterization of the 5' end); both of these ampeloviruses are members of subgroup II and their genomes were also first characterized in HI (**Figure 2.1**). PMWaV-1 and PMWaV-3 are similar compared to PMWaV-2 and share a similar genomic organization to each other, distinct from PMWaV-2. From the 5' end of PMWaV-1 and PMWaV-3, following a UTR (though the UTR of PMWaV-3 remains uncharacterized), ORFs include: ORF1a encodes MTR and HEL; ORF1b encodes a RdRp,

ORF2 encodes a SHP (p6); ORF3 encodes a HSP70; ORF4 encodes p61, a protein of unknown function; ORF5 encodes a CP; ORF6 encodes p24, a protein of unknown function[19, 21].

The putative PMWaV-4 (EU372003) reported in 2005[26] in HI (**Figure 2.1**). Prior to this study, only a 1,599 nt sequence of the HSP70 gene of PMWaV-4 was characterized ; the PMWaV-4 HSP70 gene shared a 74% nt and 87% aa similarity to PMWaV-1 (AF414119) indicating the potential of a new ampelovirus member species based off of the species demarcation criteria at the time[26]. Interestingly, it was reported in China that the PMWaV-1 isolate Hainan (PMWaV-1 HN) has an additional 72 bp in the 3' of the HSP70 gene compared to other PMWaV-1 isolates[36]. The additional sequence on PMWaV-1 HN encodes the aa residue: "ETGLLTLGRQQREIIYKRHGFESN" and interestingly has a 65% similarity to that of the 3' end the PMWaV-4 (EU372003) HSP70 gene which has the same increased length of coding region[36]. The putative PMWaV-5 (EF488753) was reported in 2008[33] in Australia (**Figure 2.1**). PMWaV-5 has partial sequences for the HEL, RdRp, p6 and HSP70 genes characterized[33]. What little sequence is available for PMWaV-4 and PMWaV-5 shares greater similarity to PMWaV-1 and PMWaV-3 than to PMWaV-2. However, the ICTV does not consider PMWaV-4 or PMWaV-5 official type members of the *Ampelovirus* genus of the *Closteroviridae* family because of the lack of characterization of the three taxonomically relevant genes to ampeloviruses and therefore unknown similarity to existing member species.

The purpose of this study is to use high-throughput sequencing (HTS) tools to further characterization the genomes of the PMWaVs in HI. The lack of a clearly understood etiology of MWP disease could potentially be due to genome length and organization differences of the ampelovirus subgroups to which the various PMWaV are divided. The longer, more complex genome of PMWaV-2 which has been shown to encode RNA silencing suppressors[37] which could be factors in PMWaV-2's exclusive ability, albeit in concert with mealybugs, to develop symptoms of MWP disease in infected plants in HI. Further complicating this PMWaV-2 was found not clearly associated with development of disease symptoms in Australia[33], Ecuador[38] or Cuba[31]. Clarity in genomic organization and gene function of all type members of the PMWaV complex is necessary to unravel the poorly understood etiology of MWP disease. In this study we use HTS tools to assemble the genomes of PMWaVs for the first time and characterize the genome of the putative PMWaV-4. The additional data generated from this study indicated the putative PMWaV-4 is a distinct strain of PMWaV-1, but not a separate ampelovirus species. In

the future, as more PMWaV species and/or strains are identified, it may be prudent to follow the same nomenclature adopted to clarify different strains of *Grapevine leafroll-associated virus*-4 (GLRaV-4), where GLRaV-5, GLRaV-6 and GLRaV-9 were renamed GLRaV-4 strain 5, 6 or 9[39]; therefore, the designation of PMWaV-4 suggested is PMWaV-1 strain 4. However, as to not confuse the reader, we will refer to PMWaV-1 strain 4 as PMWaV-4 throughout the course of this manuscript.

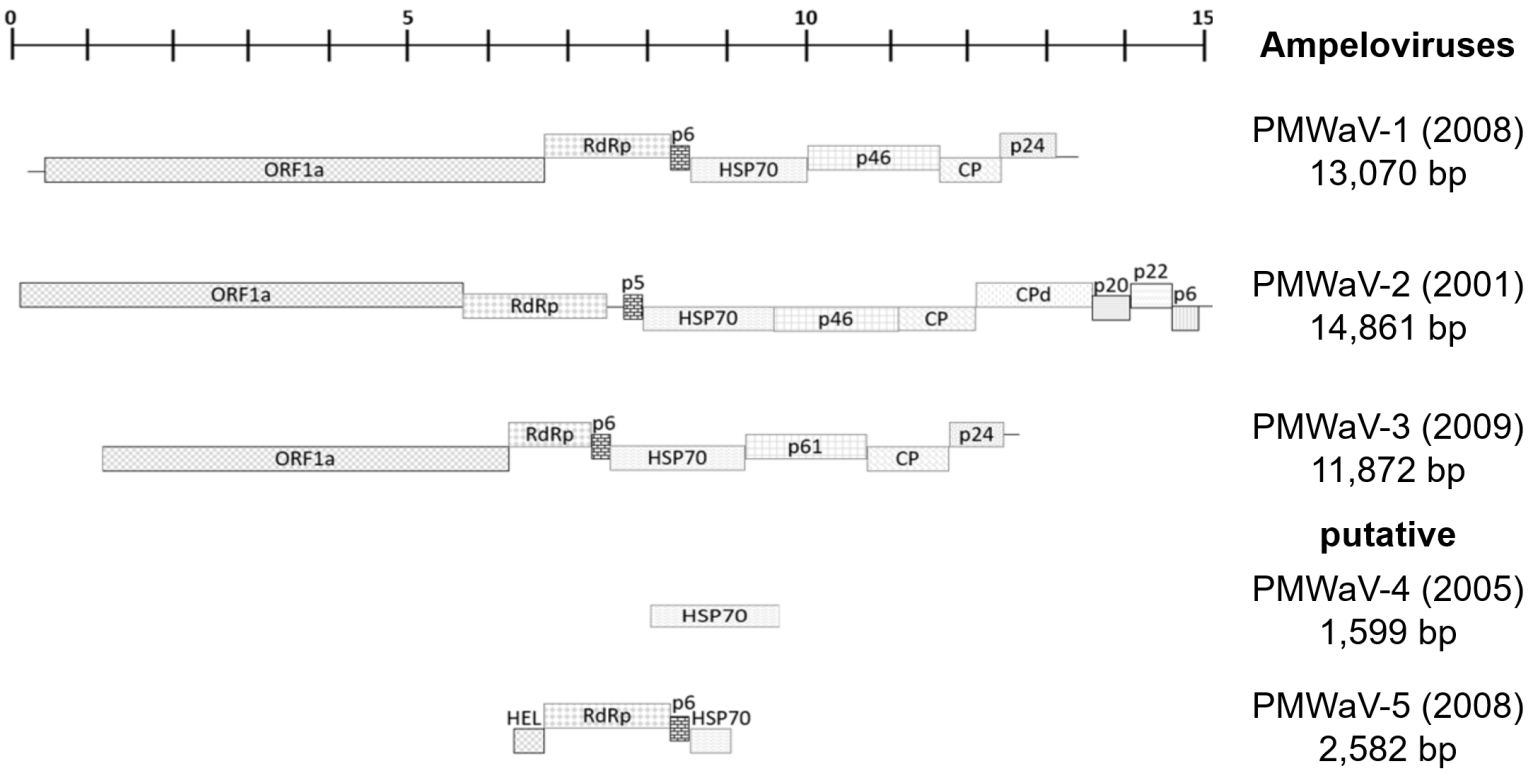

Figure 2.1 Genome organization of PMWaVs prior to this study
Genome organization and length (prior to this study) of *Pineapple mealybug wilt-associated virus*-1 (PMWaV-1, AF414119), PMWaV-2 (AF283103), PMWaV-3 (DQ399259) and the putative PMWaV-4, EU372003) and putative PMWaV-5 (EF467920, EF467921, EF467922, EF488753)

2.3 Materials and Methods

2.3.1 Plant Material

In 2017, small leaf tissue from the crowns of pineapple fruit still attached to the mother plant were collected from the United States Department of Agriculture Agricultural Research Service National Clonal Germplasm Repository (USDA-ARS NCGR) Daniel K Inouye U.S. Pacific Basin Agricultural Research Center (PBARC) in Hilo HI, USA and mailed on ice for analysis at the Plant Virology Lab, Plant and Environmental Protection Sciences Department (PEPS), College of Tropical Agriculture and Human Resources (CTAHR), University of HI at Manoa (UHM) on Oahu, HI, USA. USDA-ARS NCGR PBARC maintains germplasm repositories for many important agricultural crops. Pineapple accessions previously identified to be infected with PMWaVs were chosen for source material for total RNA extraction and subsequent downstream applications.

2.3.2 Total Nucleic Acid Isolation

Approximately 0.1g of pineapple basal leaf tissue was used per total nucleic acid (TNA) extraction. The RNeasy Plant Mini Kit (QIAGEN, Redwood City, CA, USA) or Spectrum Plant Total RNA Kit (Sigma Aldrich, Milwaukee, Wis., USA) were used following the manufacturers protocols for the extraction of TNA. TNA extractions were immediately stored at -80°C following isolation. TNA extractions were not treated with DNase underlying the classification as TNA as samples contain both RNA and DNA.

2.3.4 RT-PCR and Sanger Sequencing

For complementary DNA (cDNA) synthesis: 2 µl of RNA extract and 1 µl of random primers (50 ng/µl, Promega, WI, USA) were heated at 70°C for 5 min before immediately quenching on ice; then 5 µl M-MLV 5X reaction buffer (Promega. USA), 5 µl dNTP's (2.5 µM), 0.5 µl recombinant RNasin ribonuclease inhibitor (40 U/µl, Promega, USA) and 1.0 µl M-MLV reverse transcriptase (200 U/µl. Promega, USA) were added and the reaction was brought to a final volume of 20µl with nanopure H2O and incubated for 60 min at 37°C. The PCR reagents used were:10 µl 2X GoTaq Green master mix (Promega, USA), 1 µl forward/reverse primers (10

µM; **Table 2.1**), 1.0 µl DNA, 7 µl nanopure H_2O and 1 µl cDNA for a total volume of 20 µl. The cycle conditions used were: 94°C for 4min; 30 cycles at 94°C for 1min, 54°C for 1min, 72°C for 1min; and then 72°C for 10 min. PCR amplicons were cloned into the pGEM-T Easy vector (Promega, Madison, WI, USA), heat shock transformed in *E. coli* DH5α, red/white screened using MacConkey agar and sequenced using Sanger sequencing.

2.3.5 Illumina Sequencing

TNA extractions from the pineapple accessions Hana 158, 160 and 187 were mailed on dry ice to the University of California at Davis (UC-Davis) Plant Foundation Services (PFS) for Illumina sequencing. Aliquots of the TNA samples were subjected to ribosomal RNA (rRNA) depletion and cDNA library construction using the TruSeq Stranded Total RNA with Ribo-Zero Plant kit (Illumina, San Diego, CA, USA). Sequencing was performed on the Illumina NextSeq 500 platform and yielded approximately 37 million raw HTS reads per pineapple accession (Hana 158, 160 and 187) sequenced.

2.3.6 De Novo Assembly and Mapping

PFS Pipeline

Illumina reads were adapter trimmed and subsequently *de novo* assembled into contiguous consensus sequences (contigs) of at least 200 nt in length using CLC Bio Genomic Workstation v8.5.1 (Qiagen, Hilden Germany)[40]. Assembled contigs were compared against the database of viruses using the Basic Local Alignment Search Tool (BLAST)[41].

Galaxy Pipeline

Illumina reads were also uploaded onto the online Galaxy platform (usegalaxy.org)[42] in fastq.gz format. For a quality control check reads were analyzed with the FastQC tool[43]. Reads were next *de novo* assembled using the Trinity tool[44]; the reads from the Hana 160 pineapple accession assembled into 94,561 contigs from the ~37 million reads. The 94,561 contigs were downloaded from the Galaxy platform in fasta format and imported into Geneious v7.1[45] and contigs were mapped against the reference genome of PMWaV-1 (AF414119).

2.3.7 Multi-loci Phylogenetic Analysis

The multi-loci analysis resulted in a phylogenetic tree constructed using the Maximum Likelihood method based on the Le Gascuel model[46]. The tree with the highest log likelihood (-25491.91) is shown. The percentage of trees in which the associated taxa clustered together is shown next to the branches. Initial trees for the heuristic search were obtained automatically by applying the Neighbor-Join and BioNJ algorithms to a matrix of pairwise distances estimated using a JTT model, and then selecting the topology with superior log likelihood value. A discrete Gamma distribution was used to model evolutionary rate differences among sites (+G, parameter = 1.8850). The rate variation model allowed for some sites to be evolutionarily invariable ([+I], 9.16% sites). The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. The analysis included concatenated amino acid sequences of the RdRp, HSP70 and CP genes (in the order of their genomic organization) of 10 ampeloviruses, the PMWaV-4 HTS genome assembly from this study and the *Beet yellows virus* (BYV) the closterovirus type member as an outgroup (12 sequences total). All positions with less than 95% site coverage were eliminated, that is, fewer than 5% alignment gaps, missing data, and ambiguous bases were allowed at any position. There were a total of 1159 positions in the final dataset. Analyses were conducted in MEGA7[47].

2.3.8 Tissue Blot Immunoassay

Pineapple leaves of accessions Hana 158, Hana 160 and Hana 187 previously confirmed through molecular testing to be infected with PMWaV-2, PMWaV-4 and PMWaV-1 respectively, from USDA-ARS NCGR PBARC were collected and used for a TBIA. The same protocol reported in [17, 18] was used for this assay.

Table 2.1 Primers used to amplify the PMWaVs

Virus	Primer	Sequence (5'→3')	Amplicon (nt)	Target Gene
PMWaV-1	225	ACAGGAAGGACAACACTCAC	600	HSP70
	226	CGCACAAACTTCAAGCAATC		
PMWaV-2	223	CATACGAACTAGACTCATACG	600	HSP70
	224	CCATCCACCAATTTTACTAC		
PMWaV-3	263	AGTTCACTGTAGATTTCGGA	495	HSP70
	264	ATTGATGGATGTGTATCG		
PMWaV-4	267	GGTACAGGCCGGATAAA	450	HSP70
	268	AACTTGGGCGTCGTA		
PMWaV-4	1932	AAGTCCGCCACAAACTTGGA	474	ORF1a
	1933	TTCCCTGCCGAAACAAGGTT		
PMWaV-4	1934	GAGTTTTTCCCGCGGTCCTA	460	ORF1a
	1935	TGTGATTCAAGGCGGGAGAC		
PMWaV-4	1936	TTGTCTGCGATCGCCTTCTT	629	ORF1a
	1937	TCCCGGAAAGTCTTCCTCCA		
PMWaV-4	1944	TCGAGGAAACTGAAAAGTTCCG	949	RdRp
	1945	CAAATCTGTGGACGCGCAAG		
PMWaV-4	1946	TCGGGTAAAGGTAATTGGTCGT	1627	HSP70
	1947	ACCGACACTGAGCAAAGAACA		
PMWaV-4	1948	AGCGCAGATAACAATGAAGATCA	725	CP
	1949	AGGAGTTCGCCGATCAGTTG		
PMWaV-4	1951	TCTTCCGTGGAGGATGGG	750	RdRp
	1952	CACATATGGCGCGTTCATGG		
PMWaV-4	1953	TGAATGGTTAGACCGTGAAGGA	1500	ORF1a
	1954	GCTTTGCAATTCAGACTGGCT		
Degenerate ampelovirus	249	GGARGTNGGNWWHGAMTTYGGNACNAC	650	HSP70
	250	GANVHRTCRAAMGTSCCTCCNCCRAARTC		

2.4 Results

2.4.1 RT-PCR

RT-PCR was used to identify candidate starting material for HTS. We assayed pineapple accessions from the USDA-ARS NCGR PBARC for presence of PMWaVs. Virus infected pineapple accessions were identified for the four PMWaVs found in HI. Pineapple accession Hana 187 was positive for PMWaV-1, accession Hana 158 for PMWaV-2 and PMWaV-3, accession Hana 160 for PMWaV-4 (**Figure 2.2**).

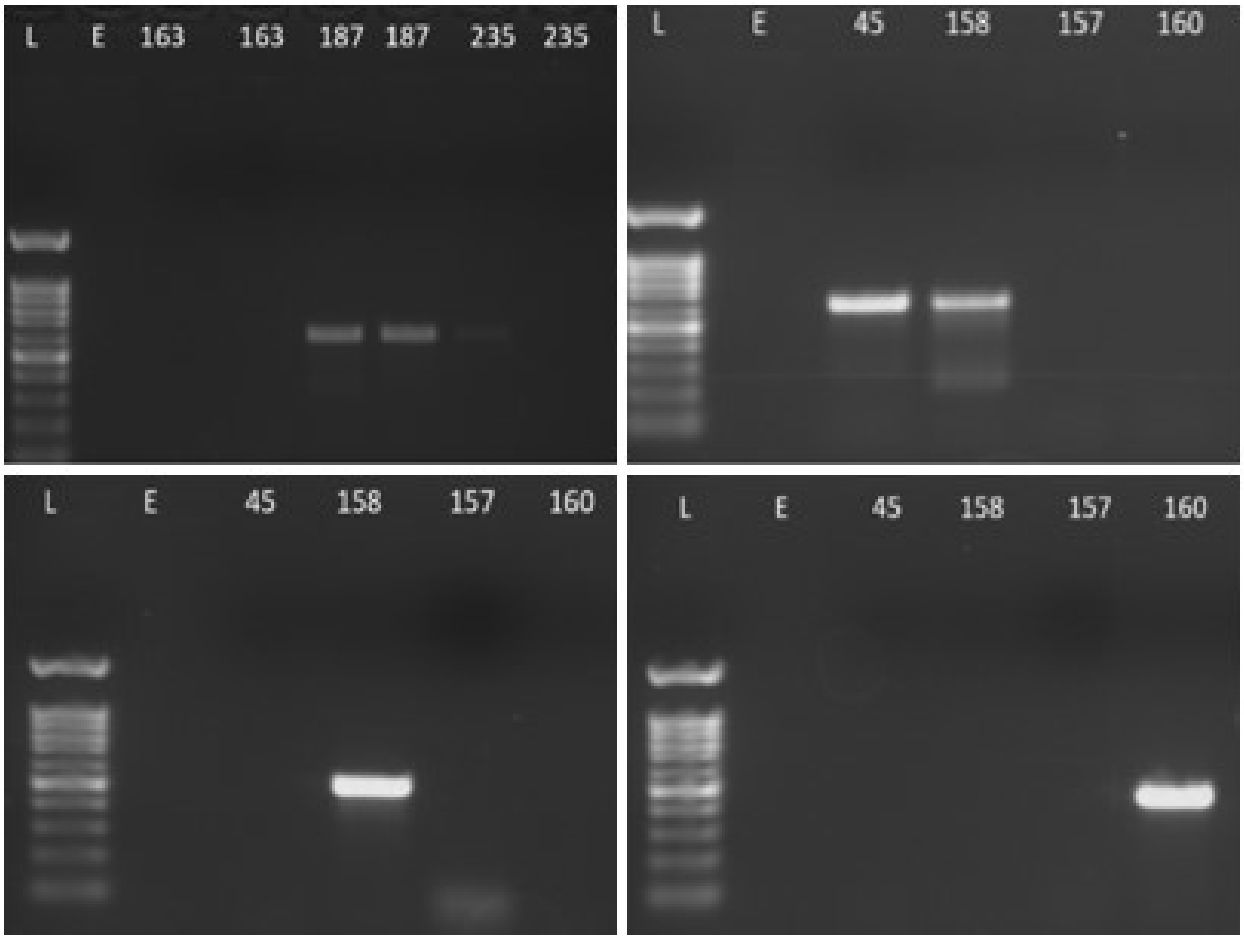

Figure 2.2 RT-PCR of PMWaVs HSP70
RT-PCR of accessions from USDA-ARS NCGR PBARC: Hana 45, 157, 158, 160, 163, 187 and 235. *Pineapple mealybug wilt-associated virus*-1 (PMWaV-1), 600 nt expected product size (top left); PMWaV-2, 600 nt expected product size (top right); PMWaV-3, 495 nt expected product size (bottom left); and PMWaV-4, 450 nt expected product size (bottom right); primer sets used (*Table 2.1*) amplify a partial HSP70 gene

2.4.2 Further Genome Characterization of PMWaV-4

HTS Dataset 1 PFS Pipeline

TNA extractions from pineapple accession Hana 160 were sent to UC-Davis PFS, for sequencing. UC-Davis PFS returned HTS dataset that contained two contigs from Hana 160 (**Table 2.2**). The BLAST tool was used to analyze the contigs assembled by the PFS pipeline. Consistent with expectations, BLASTx results for the 5,325 nt contig (**Table 2.2**) returned a 99% aa similarity to the HSP70 gene of PMWaV-4 (EU372003) as the only PMWaV-4 sequence reported to GenBank prior to this study was from the HSP70 gene[26]. The initial characterization of PMWaV-4 (EU372003) was limited to a 1,599 nt sequence, the complete CDS of the HSP70 gene[26].

PMWaV-1 (AF414119) shares the highest sequence similarity to PMWaV-4, so we aligned the 5,325 nt contig to PMWaV-1 as only the HSP70 sequence[26] of PMWaV-4 was available for alignment[48]; the contig aligned to the PMWaV-1 HSP70 ORF from positions 8,271-9,804 and extended 1,089 nt (positions 7,182-8,270) towards the 5' terminus and 2,710 nt (position 9,805 to 12,515) towards to 3' terminus. The HSP70 gene of PMWaV-1 is flanked by p6 and RdRp genes on the 5' end and p46, CP and p24 genes on the 3' end; we therefore assumed PMWaV-4 sequences aligned to positions 7,182-8,270 and 9,805-12,515 of PMWaV-1 would contain the other typical ampelovirus genes. To confirm our hypothesis, we used the ORF Finder tool[48] and identified RdRp, HSP70, p46, CP and p24 ORFs in the 5,325 nt contig. The 5,325 nt contig aa and nt similarity to PMWaV-1 (AF414119) was very high (**Figure 2.3, Table 2.3**) suggesting the putative PMWaV-4 is not a distinct PMWaV species. We designed PMWaV-4 specific primer pairs (**Table 2.1**) using the 5,325 nt contig sequence from HTS dataset 1, PFS pipeline and the Primer-BLAST tool[49] to amplify the RdRp, HSP70 and CP genes of PMWaV-4.

The PMWaV-4 RdRp, HSP70 and CP primers amplified the expected product size (

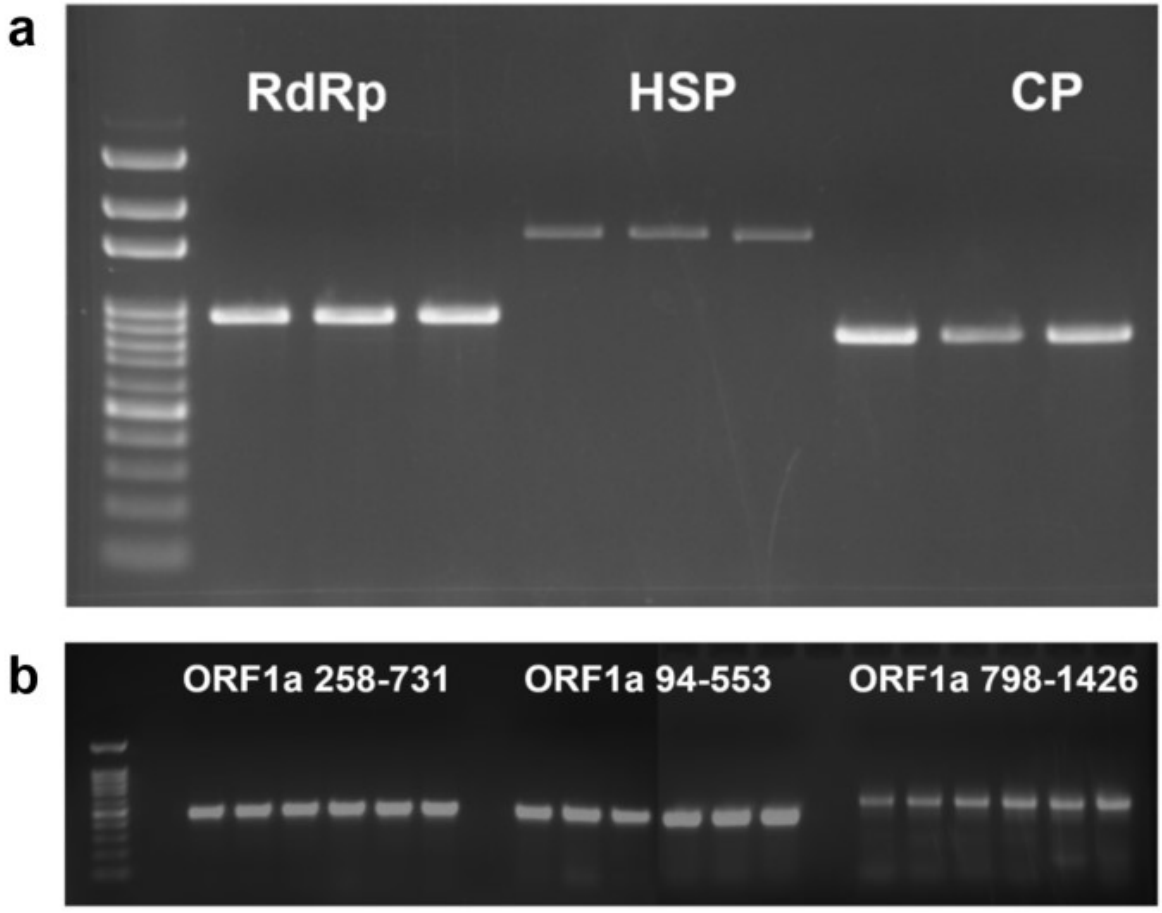

Figure 2.4), and Sanger sequencing results validated the HTS dataset 1, PFS pipeline assembly sequence of the 5,325 nt contig.

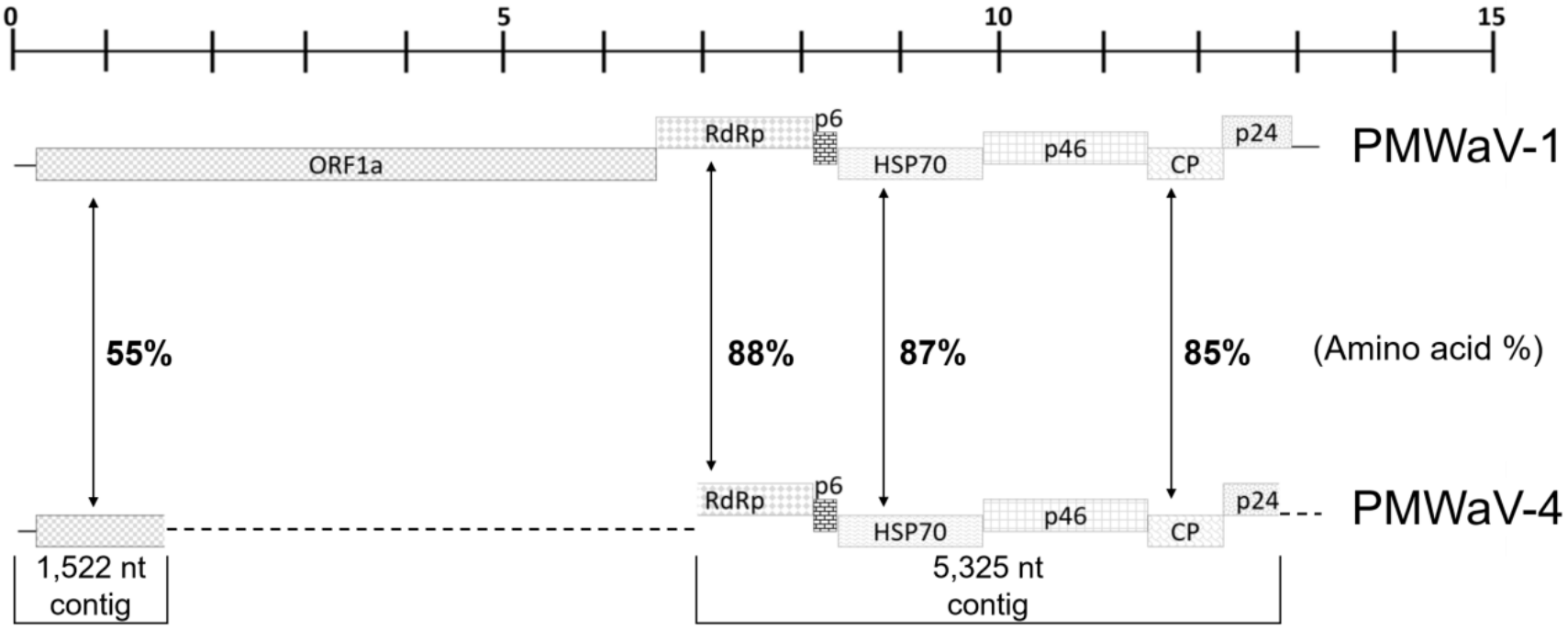

Figure 2.3 PMWaV-4 HTS assembly similarity to PMWaV-1
PMWaV-4 HTS dataset 1 PFS pipeline assembly contigs amino acid sequence similarity to PMWaV-1 (AF414119)

Table 2.2 PMWaV-4 contigs assembled from HTS dataset 1

Pineapple Accession	Most Similar BLAST hit	Contig Length (nt)	Dataset	Assembly Pipeline
Hana 160	PMWaV-1 (AF414119)	1522	1	PFS
Hana 160	PMWaV-4 (EU372003)	5325	1	PFS
Hana 160	PMWaV-1 (AF414119)	11244	1	Galaxy
Hana 160	PMWaV-1 (AF414119)	6592	1	Galaxy
Hana 160	PMWaV-1 (AF414119)	6116	1	Galaxy
Hana 160	PMWaV-1 (AF414119)	4332	1	Galaxy
Hana 160	PMWaV-1 (AF414119)	3112	1	Galaxy
Hana 160	PMWaV-1 (AF414119)	2242	1	Galaxy
Hana 160	PMWaV-1 (AF414119)	1027	1	Galaxy
Hana 160	PMWaV-1 (AF414119)	899	1	Galaxy
Hana 160	PMWaV-1 (AF414119)	660	1	Galaxy
Hana 160	PMWaV-1 (AF414119)	583	1	Galaxy
Hana 160	PMWaV-1 (AF414119)	556	1	Galaxy
Hana 160	PMWaV-1 (AF414119)	541	1	Galaxy
Hana 160	PMWaV-1 (AF414119)	524	1	Galaxy
Hana 160	PMWaV-1 (AF414119)	481	1	Galaxy
Hana 160	PMWaV-1 (AF414119)	274	1	Galaxy
Hana 160	PMWaV-1 (AF414119)	265	1	Galaxy
Hana 160	PMWaV-1 (AF414119)	253	1	Galaxy
Hana 160	PMWaV-1 (AF414119)	238	1	Galaxy

Table 2.3 PMWaV-4 HTS assembly similarity to PMWaV-1 (AF414119)

Gene	Amino Acid	Nucleotide
RdRp	89%	78%
p6	68%	71%
HSP70	87%	76%
p46	82%	74%
CP	85%	78%
p24	81%	78%

The BLAST tool[41] returned a 55% aa similarity (**Figure 2.3**) of a 1,522 nt contig (**Table 2.2**) sequence to ORF1a, the polyprotein encoding the MTR and HEL genes of PMWaV-1 (AF414119). The 5' end of PMWaV-1 is mostly comprised of one large polyprotein, ORF1a (Error! Reference source not found.). The 5' end, or ORF1a, of ampeloviruses, with exception of the MTR and HEL domains, is not highly conserved compared to the ORFs of the 3' end. To validate the 1,522 nt contig assembly we designed three pairs of PMWaV-4 specific primers using the 1,522 nt contig sequence from HTS dataset 1, PFS pipeline and the Primer-BLAST tool[49] to amplify different regions of ORF1a of PMWaV-4; we then cloned the PCR amplicons and sequenced with Sanger sequencing. Each of the 3 PMWaV-4 ORF1a specific primer pairs

amplified the expected product size (

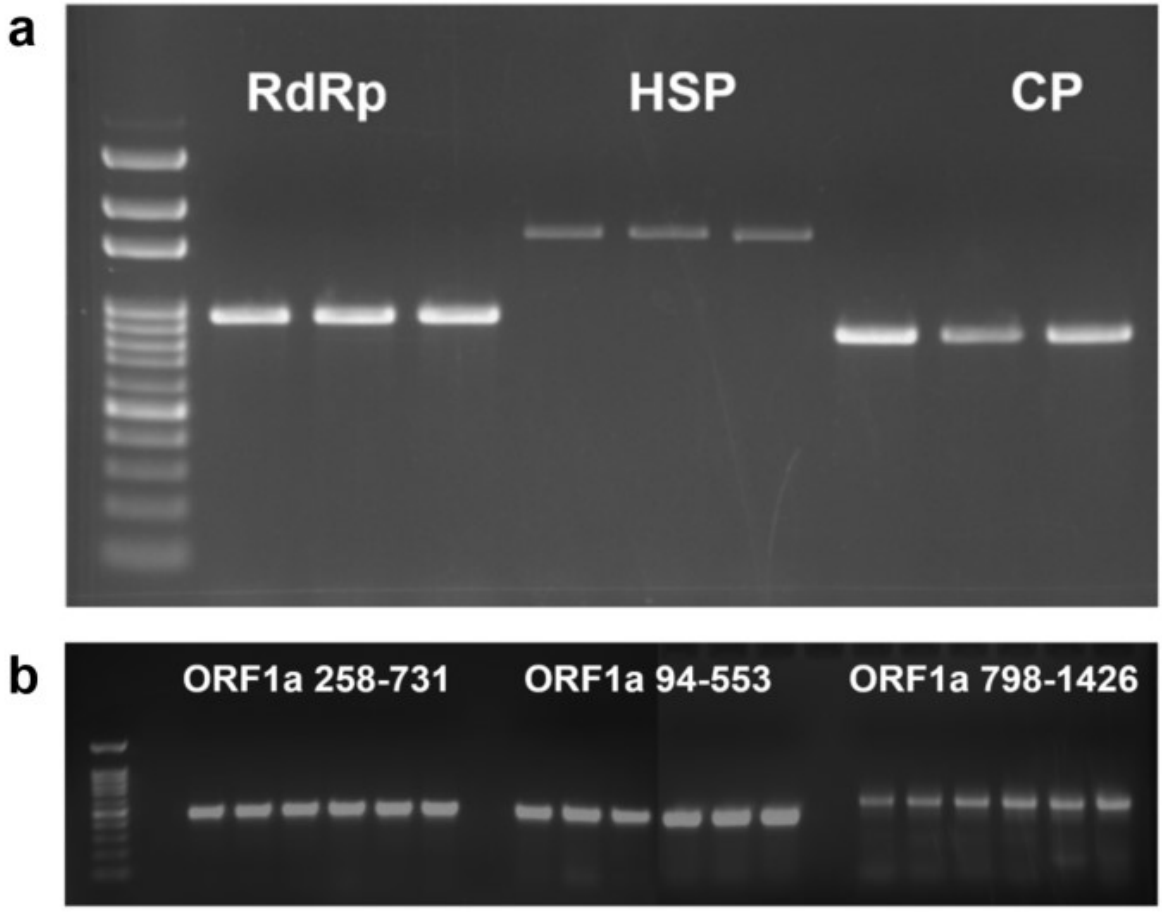

Figure 2.4) and Sanger sequencing results validated the HTS dataset 1, PFS pipeline assembly sequence of the 1,522 nt contig.

With Sanger sequencing results validating our HTS sequence we were confident that our HTS dataset was reliable, however, because our two contigs aligned to opposite termini of PMWaV-1 there was a substantial gap (**Figure 2.3**) between the two contigs. Due to the gap (**Figure 2.3**) we were unable to assume that both contigs represented sequences from the same virus. The pineapple accession Hana 160 from which the 1,522 and 5,325 nt contigs originated returned negative results when assayed with RT-PCR for PMWaV-1, PMWaV-2 and PMWaV-3 (**Figure 2.2**), but it was still possible that pineapple accession Hana 160 might harbor another yet uncharacterized virus in addition to PMWaV-4. To confirm the pineapple accession Hana 160 was only infected with one ampelovirus, PMWaV-4, we employed a degenerate primer set (**Table 2.1**) designed to amplify the HSP70 gene of ampeloviruses. The expected PCR product

was amplified, cloned and 29 clones were sequenced using Sanger sequencing. All 29 clones sequenced returned 99% nt and aa identity to the HSP70 gene of PMWaV-4 (EU372003) indicating that Hana 160 was probably only infected with one ampelovirus: PMWaV-4.

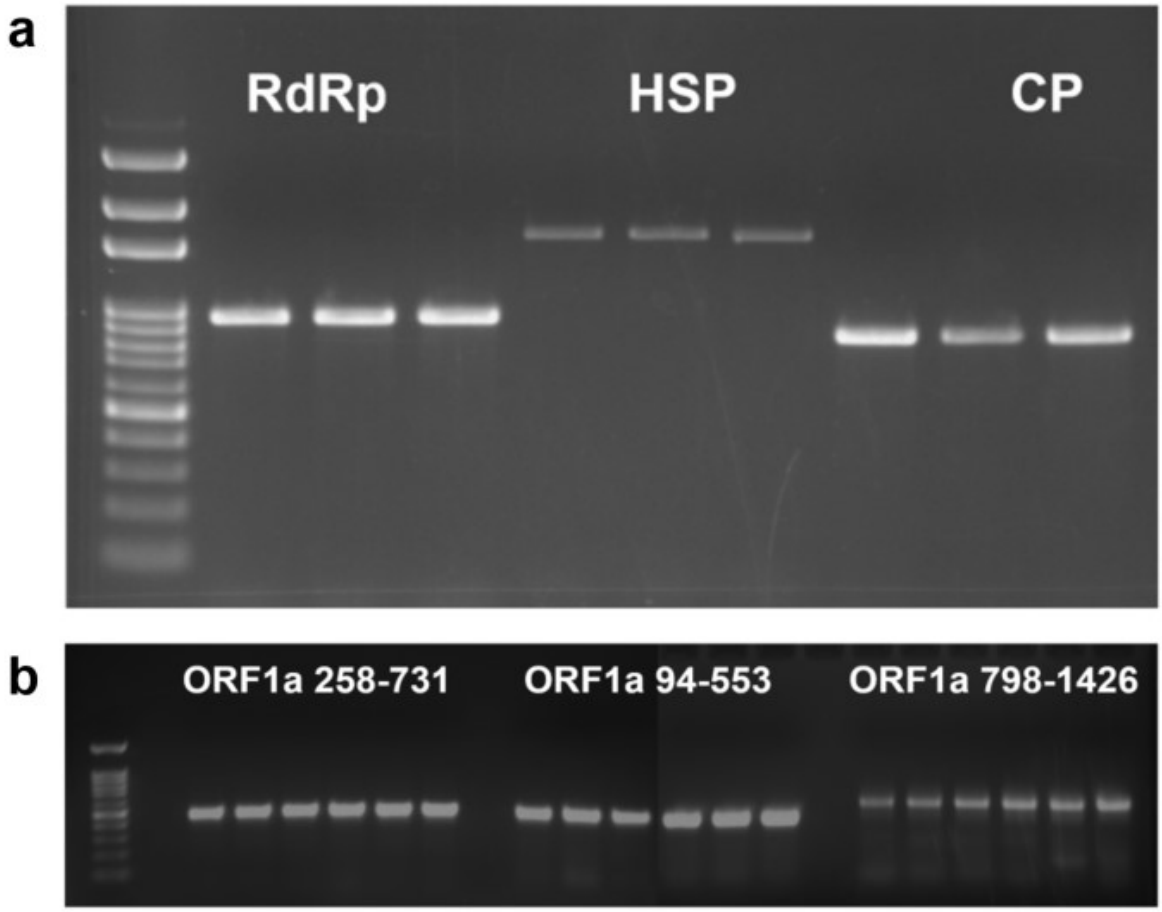

Figure 2.4 RT-PCR with PMWaV-4 HTS assembly specific primers
RT-PCR of PMWaV-4 using six pairs of HTS assembly specific primers from (**a**) a 5,325 nt contig for the RdRp, HSP70 and CP genes and (**b**) a 1,522 nt contig for three regions of ORF1a

Table 2.4 PMWaV-4 contigs assembled from HTS dataset 2

Pineapple Accession	Most similar BLAST hit	Contig Length (nt)	Dataset	Assembly Pipeline
Hana 160	PMWaV-4 (EU372003)	5325	2	PFS
Hana 160	PMWaV-1 (AF414119)	2268	2	PFS
Hana 160	PMWaV-1 (AF414119)	1988	2	PFS
Hana 160	PMWaV-1 (AF414119)	1395	2	PFS
Hana 160	PMWaV-1 (AF414119)	1239	2	PFS
Hana 160	PMWaV-1 (AF414119)	609	2	PFS
Hana 160	PMWaV-1 (AF414119)	372	2	PFS
Hana 160	PMWaV-1 (AF414119)	789	2	Galaxy
Hana 160	PMWaV-1 (AF414119)	817	2	Galaxy
Hana 160	PMWaV-1 (AF414119)	433	2	Galaxy
Hana 160	PMWaV-1 (AF414119)	2145	2	Galaxy

HTS Dataset 1 PFS + Galaxy Pipeline

We suspected that additional sequence information might be available in the Illumina TruSeq 500 raw reads data file of HTS dataset 1, so we requested the raw reads data file from UC-Davis PFS. Galaxy pipeline assembly is explained in section 2.3.6 De Novo Assembly and Mapping. Further bioinformatic analysis of the reads using the Galaxy pipeline resulted in an additional 18 contigs (**Table 2.2**). Mapping of the contigs from the PFS and Galaxy pipelines to the PMWaV-1 reference genome resulted in the assembly of a three large scaffolds, that if assumed to belong to the same virus, would represent approximately 11 kb or nearly the entire genome of PMWaV-4. When these scaffolds were mapped together to PMWaV-1 as a reference genome a gap of approximately 2,000 nt in the ORF1a and a gap of approximately 500 nt in the 5' end of the RdRp gene remained (**Figure 2.5**).

HTS Dataset 1 PFS + Galaxy and HTS Dataset 2 PFS Pipelines

We suspected further sequencing might fill the remaining gaps in our data set and additional TNA extractions from pineapple accession Hana 160 were sent to UC-Davis PFS, for sequencing. UC-Davis PFS returned the HTS dataset 2 that contained seven additional contigs (**Table 2.4**); the PFS pipeline assembly is explained earlier in section 2.3.6 De Novo Assembly and Mapping. The contigs from the HTS dataset 1 PFS and Galaxy pipelines as well as the contigs from the HTS dataset 2 PFS pipeline were all mapped to the PMWaV-1 reference genome. The resulting two scaffolds still contained an approximately 2,000 nt gap in ORF1a, but the approximately 500 nt gap in the 5' end of the RdRp gene was eliminated completing the CDS of the RdRp gene of PMWaV-4 (**Figure 2.5**).

HTS Dataset 1 PFS + Galaxy and HTS Dataset 2 PFS + Galaxy Pipelines

We again suspected that additional sequence information might be available in the Illumina TruSeq 500 raw reads data file of the HTS dataset from UC-Davis PFS, so we requested the HTS dataset 2 raw reads data file from UC-Davis PFS. Galaxy pipeline assembly is explained in section 2.3.6 De Novo Assembly and Mapping. Further bioinformatic analysis of the reads using the Galaxy pipeline resulted in an additional 4 contigs (**Table 2.4**). Mapping of

the contigs from the HTS datasets 1 and 2 - PFS and Galaxy pipelines to the PMWaV-1 reference genome resulted in the assembly of the entire genome of PMWaV-4 (**Figure 2.5**).

Amino acid Sequence Similarity and Multi-loci Analysis

The PMWaV-4 HTS genome assembly showed the expected high similarity to PMWaV-1 (AF414119, **Table 2.3, Table 2.5**). Multi-loci analysis of the aa sequence of concatenated RdRp, HSP70 and CP genes of Pineapple mealybug wilt-associated virus-4 (PMWaV-4) in relation to those of the recognized *Ampelovirus* genus member species. The multi-loci analysis resulted in a phylogenetic tree (**Figure 2.6**) constructed using the Maximum Likelihood method based on the Le Gascuel model[46]. PMWaV-4 clustered together with PMWaV-1 (AF414119), as expected, and other subgroup II ampeloviruses including: *Pea bark necrosis stem pitting-associated virus* (PBNSPaV, EF546442), *Grapevine leafroll-associated virus*-4 (GLRaV-4, ACS44657) and PMWaV-3 (DQ399259). PMWaV-4 showed expected low similarity to the subgroup II ampeloviruses including: *Blackberry vein banding-associated virus* (BVBaV, KC904540), (GLRaV-1, JQ023131), GLRaV-3 (AF037268), GLRaV-13 (LC052212), *Little cherry virus* 2 (LChV-2, AF531505) and PMWaV-2 (AF283103). The closterovirus type member *Beet yellow virus* (BYV, AAF14300) was used as the outgroup.

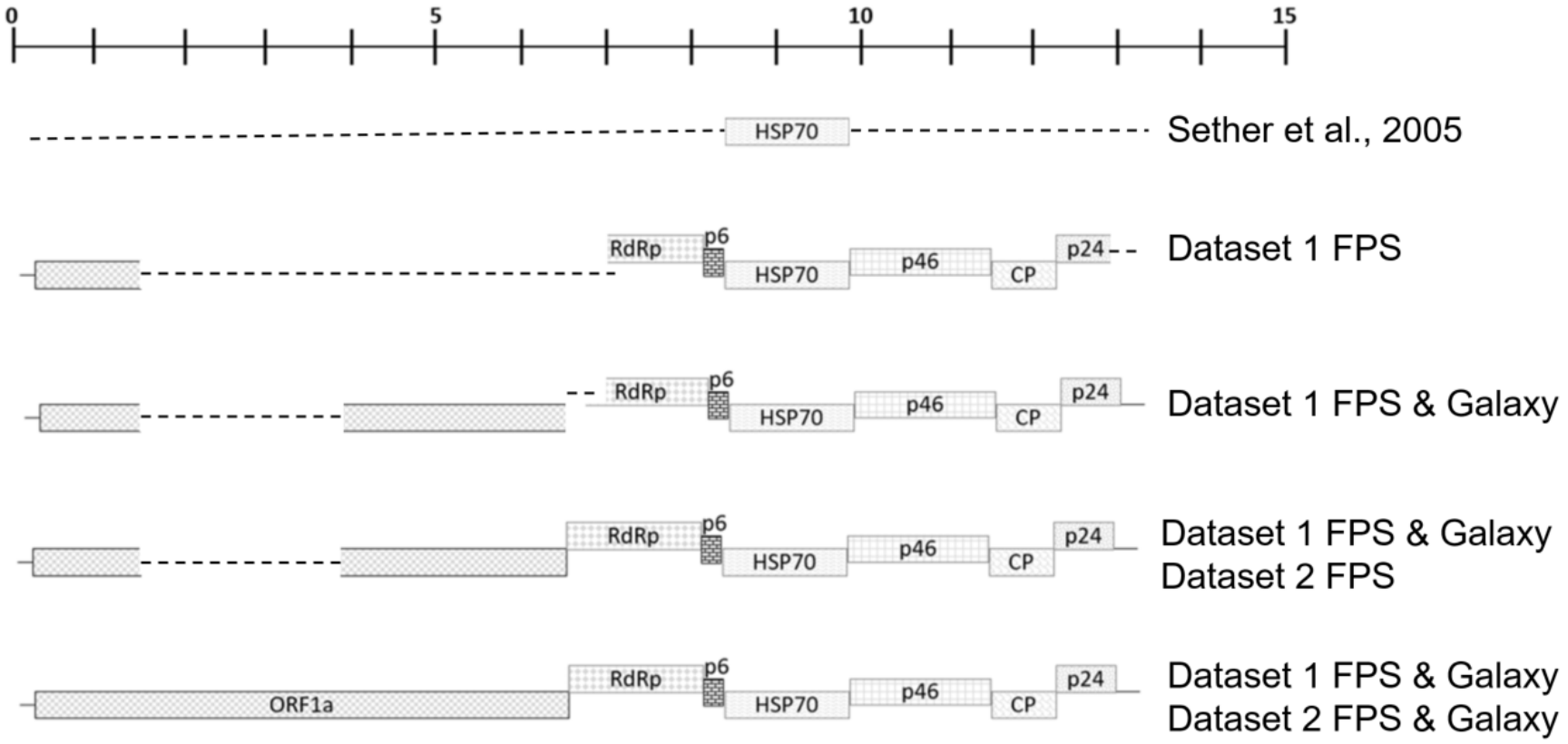

Figure 2.5 PMWaV-4 sequences throughout the stages of HTS genome assembly
PMWaV-4 sequences throughout the stages of genome characterization, including the initial characterization of the HSP70 gene (prior this this study) and the various stages of genome assembly as additional HTS datasets and assembly pipelines were incorporated. Dotted lines represent gaps

Table 2.5 Amino acid similarity of ampeloviruses to PMWaV-4

Virus	RdRp	HSP70	CP
Subgroup I			
PMWaV-2 (AF283103)	33%	33%	26%
GLRaV-3 (AF037268)	32%	37%	29%
GLRaV-1 (JQ023131)	33%	34%	26%
LChV-2 (AF531505)	33%	32%	26%
Subgroup II			
PBNSPaV (EF546442)	37%	44%	36%
GLRaV-4 (ACS44657)	59%	59%	60%
PMWaV-1 (AF414119)	88%	87%	85%
PMWaV-3 (DQ399259)	69%	73%	65%

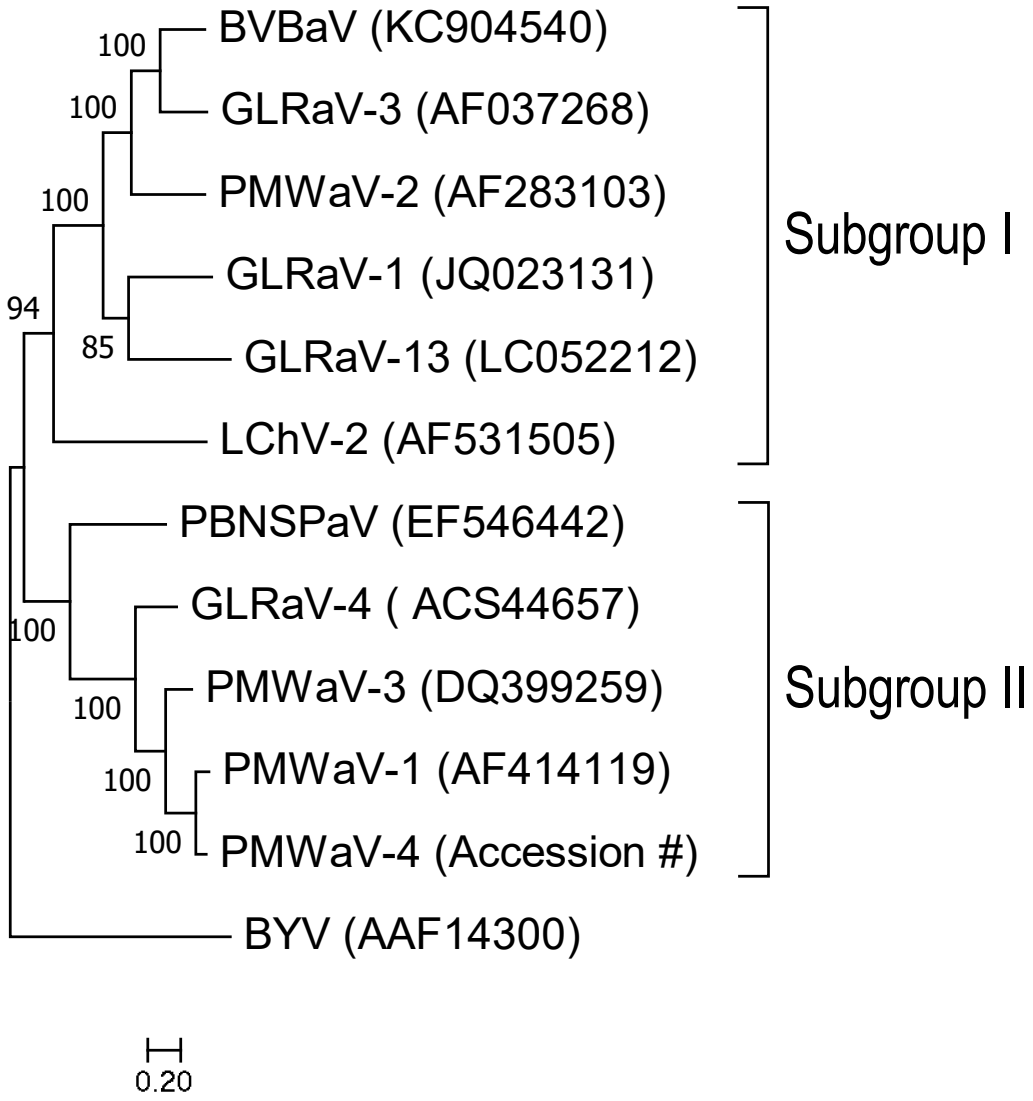

Figure 2.6 Multi-loci phylogenetic analysis of PMWaV-4 similarity to ampeloviruses
Multi-loci analysis using a concatenated amino acid sequence of RdRp, HSP70 and CP genes. The tree shows Pineapple mealybug wilt-associated virus-4 (PMWaV-4) in relation to those of the recognized *Ampelovirus* member species. The multi-loci analysis resulted in a phylogenetic tree constructed using the Maximum Likelihood method based on the Le Gascuel model. The tree is drawn to scale. Branch lengths were measured in the number of substitutions per site. There were a total of 1159 positions in the final dataset. The sequences of the following viruses were retrieved from GenBank and included in the analysis: *Blackberry vein banding-associated virus* (KC904540), *Grapevine leafroll-associated virus*-1 (GLRaV-1, JQ023131), GLRaV-3 (AF037268), GLRaV-4 (ACS44657), GLRaV-13 (LC052212), *Little cherry virus* 2 (LChV-2, AF531505), *Pea bark necrosis stem pitting-associated virus* (PBNSPaV, EF546442), PMWaV-1 (AF414119), (AF283103), PMWaV-3 (DQ399259) and the closterovirus type member *Beet yellow virus* (BYV, AAF14300) was used as the out group. PMWaV-4 clustered with its sister taxa PMWaV-1, and other ampelovirus subgroup II members as expected

2.4.3 Further Genome Characterization of PMWaV-1

PMWaV-1 is an *Ampelovirus* subgroup II member with a 13,071 nt genome first characterized by our lab, in HI, in 2008[20]. HTS data from our first round of sequencing, using the PFS assembly pipeline, included a 6,186 nt contig of the 5' terminus of PMWaV-1 from pineapple accession Hana 158 (**Table 2.6**). HTS data from our first round of sequencing, subjected to our Galaxy assembly pipeline resulted in the complete 13,071 nt genome. The PFS pipeline assembly of 6,186 nt and Galaxy pipeline assembly of 13,071 nt both showed an overall comparison of 99% nt similarity to the PMWaV-1 (AF414119) reference genome first characterized about 10 years ago (**Figure 2.7, Figure 2.8**)

2.4.4 Further Genome Characterization of PMWaV-2

PMWaV-2 is an *Ampelovirus* subgroup I member with a 14,861 bp of its genome first characterized by our lab, in HI, in 2001[19]. HTS data from our first round of sequencing, using the PFS assembly pipeline, included a 7,653 nt contig of the 3' end of PMWaV-2 from pineapple accession Hana 158 as well as a 14,684 nt contig (nearly the complete genome) lacking only several hundred bp of the 3' terminus from pineapple accession Hana 187 (**Table 2.7**). HTS data from our first round of sequencing, subjected to our Galaxy assembly pipeline resulted in a more complete 15,027 nt genome assembly that contained an additional 166 nt on the 5' end of the virus extending ORF1a. Although it has been nearly 20 years since the first genome characterization of PMWaV-2 was published the 15,027 nt Galaxy pipeline genome assembly and both contigs from the PFS pipeline show an overall comparison of 99% nt similarity to the PMWaV-2 (AF283103) reference genome (**Figure 2.7, Figure 2.8**).

2.4.5 Further Genome Characterization of PMWaV-3

PMWaV-3 is an *Ampelovirus* subgroup II member with 11,872 nt of its genome first characterized by our lab, in HI, in 2009[21]. HTS data from our first round of sequencing, using the PFS assembly pipeline, included a 6,036 nt contig of the 5' end of PMWaV-3 from pineapple accession Hana 187 (**Table 2.8**). HTS data from our first round of sequencing, subjected to our Galaxy assembly pipeline resulted in a more complete 12,502 nt genome assembly that contained

an additional 641 nt on the 5’ end of the virus extending ORF1a. HTS data from our first round of sequencing using both the PFS and Galaxy pipelines show an overall comparison of 97% nt similarity to the PMWaV-3 (DQ399252) reference genome first characterized nearly 10 years ago (**Figure 2.7, Figure 2.8**).

Table 2.6 PMWaV-1 contigs assembled from HTS dataset 1

Pineapple Accession	Most Similar BLAST Hit	Contig Length (nt)	Dataset	Assembly Pipeline
Hana 187	PMWaV-1 (AF414119)	6186	1	PFS
Hana 187	PMWaV-1 (AF414119)	13071	1	Galaxy

Table 2.7 PMWaV-2 contigs assembled from HTS dataset 1

Pineapple Accession	Most Similar BLAST Hit	Contig Length (nt)	Dataset	Assembly Pipeline
Hana 158	PMWaV-2 (AF283103)	7653	1	PFS
Hana 187	PMWaV-2 (AF283103)	14684	1	PFS
Hana 158+187	PMWaV-2 (AF283103)	15027	1	Galaxy

Table 2.8 PMWaV-3 contigs assembled from HTS dataset 1

Pineapple Accession	Most Similar BLAST Hit	Contig Length (nt)	Dataset	Assembly Pipeline
Hana 158	PMWaV-3 (DQ399259)	6036	1	PFS
Hana 158	PMWaV-3 (DQ399259)	12502	1	Galaxy

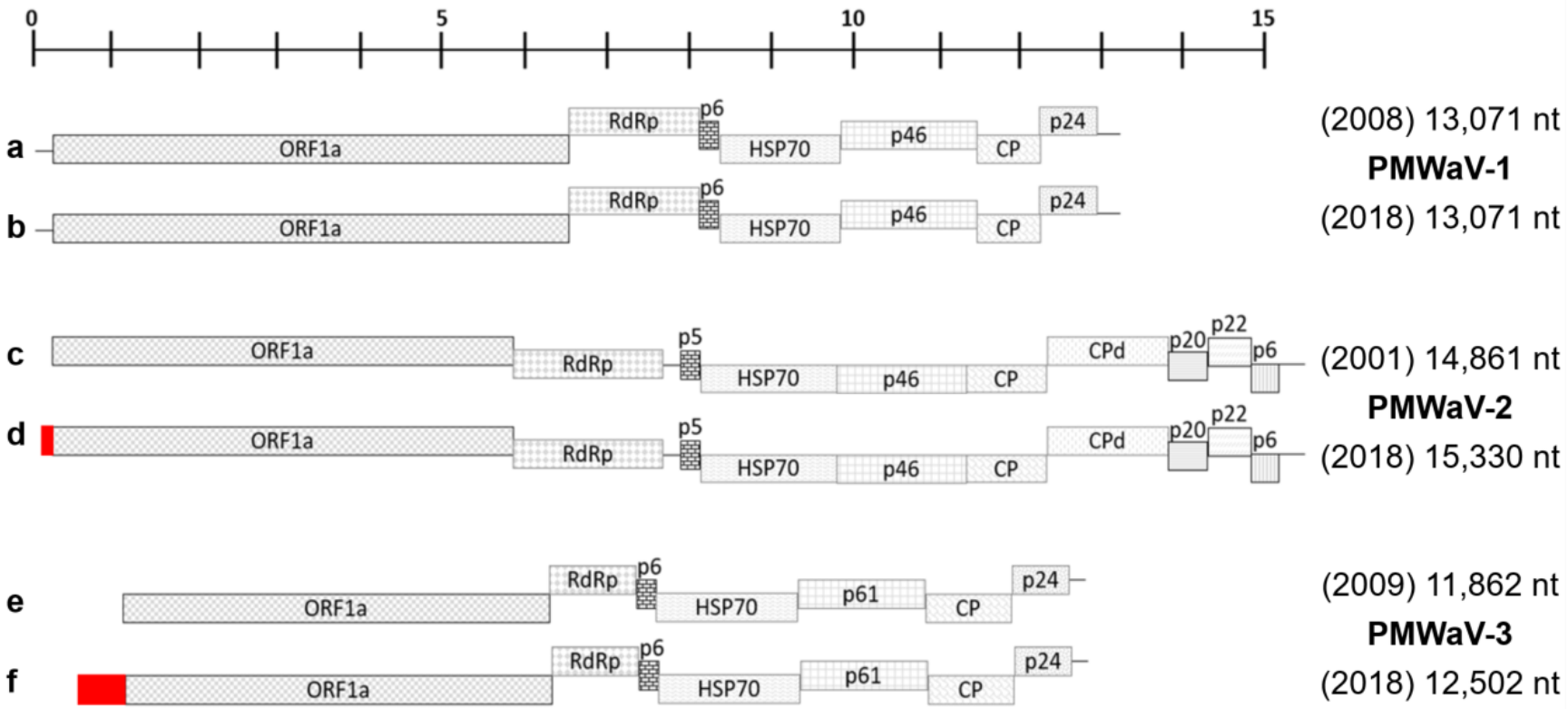

Figure 2.7 PMWaV-1, -2 & -3 HTS assembly comparisons
HTS assembly comparison of genome organization and nucleotide (nt) similarity to the previously published genomes of (**a**) *Pineapple mealybug wilt-associated virus*-1 (PMWaV-1, AF41411) reference genome from 2008 publication compared to (**b**) PMWaV-1 (2018) HTS assembly share 99% nt similarity; (**c**) PMWaV-2 (AF283103) reference genome from 2001 publication compared to (**d**) PMWaV-2 (2018) HTS assembly share 99% nt similarity; (**e**) PMWaV-3 (DQ399259) reference genome from 2009 publication compared to (**f**) PMWaV-3 (2018) HTS assembly share 97% nt similarity

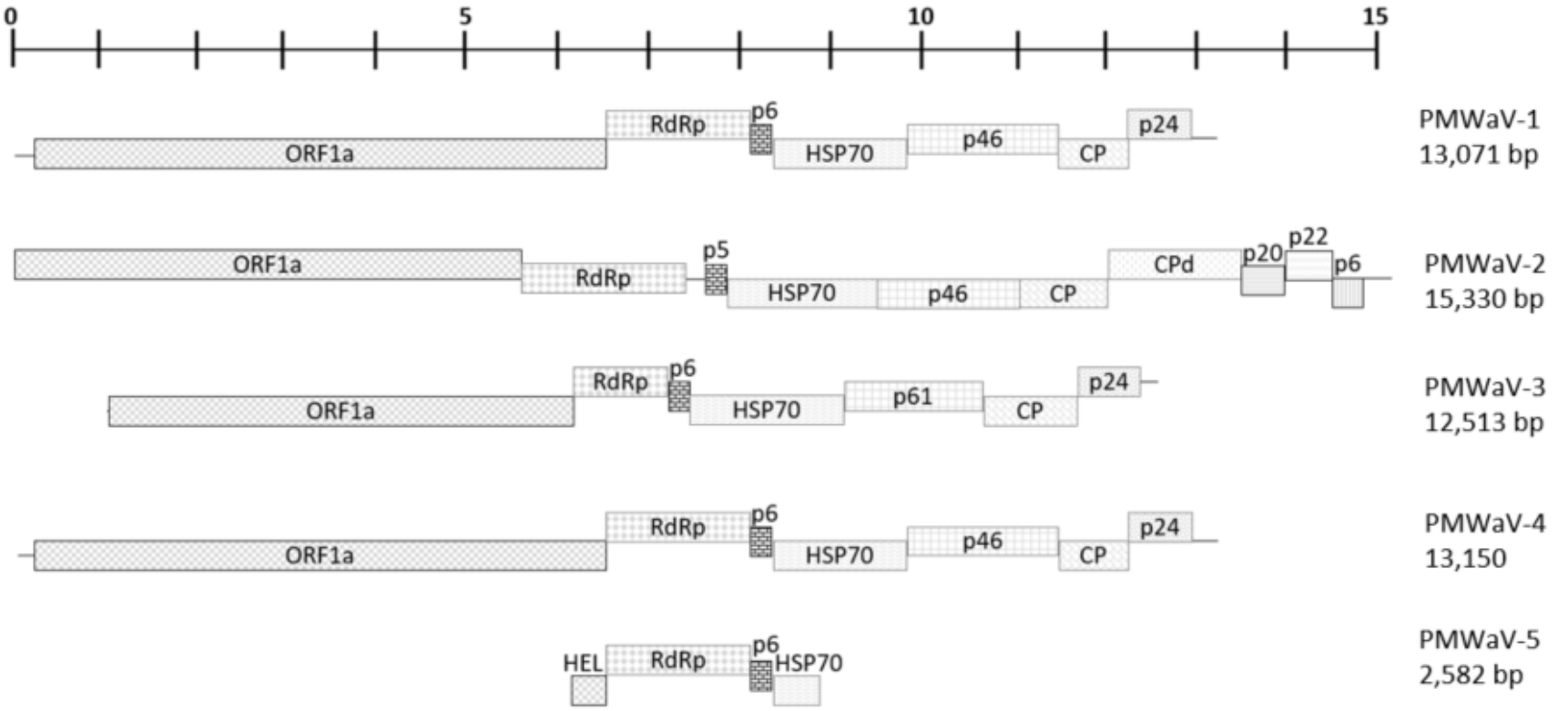

Figure 2.8 Genome organization of PMWaV HTS assemblies
HTS assembly genome organization of *Pineapple mealybug wilt-associated virus*-1 (PMWaV-1), PMWaV-2, PMWaV-3, PMWaV-4 and the available sequences of PMWaV-5 (EF488753, EF467920, EF467921, EF467922, EF488753)

2.4.6 Tissue Blot Immunoassay

Previously, antibodies were developed for a TBIA[17, 18] to detect PMWaV-1 and PMWaV-2. We used a TBIA with pineapple accessions Hana 158, 160 and 187 to determine if the antibody developed to detect PMWaV-1 can also detect the PMWaV-4 strain. We used leaf tissue from the same pineapple accessions previously assayed by RT-PCR for PMWaV presence. RT-PCR assay showed Hana 158 infected with PMWaV-2 and PMWaV-3, Hana 160 infected with PMWaV-4 and Hana 187 infected with PMWaV-1. The pineapple accession Hana 187 that was confirmed to be infected with RT-PCR returned positive results for PMWaV-1 with the TBIA. The pineapple accessions Hana 158 and 160 returned negative results for PMWaV-4 with the TBIA indicating the MAb developed to detect PMWaV-1 is not able to detect the PMWaV-4 strain of PMWaV-1.

2.5 Discussion

In the past, our lab employed a TBIA[17, 18, 23] using mAbs specific to PMWaV-1 or PMWaV-2 for pineapple virus surveys; if the pineapple TBIA result was negative for PMWaV-1 or PMWaV-2 then ampelovirus degenerate HSP70 primers (**Table 2.1**) were used to investigate the potential of novel PMWaV infections underlying the discovery of PMWaV-3[21], PMWaV-4[26] and PMWaV-5[33]. The HSP70 gene of putative PMWaV-4 (EU372003) has a 74% nt and 87% aa similarity to PMWaV-1 (AF414119)[26]. However, at the time, the criteria for *Ampelovirus* species demarcation was a 10% divergence of aa sequence of the RdRp, HSP70 and CP genes indicating the possibility of a new species of PMWaV. Further, it was reported in China that the PMWaV-1 isolate Hainan (PMWaV-1 HN) has an additional 72 bp in the 3' of the HSP70 gene compared to other PMWaV-1 isolates[36]. The additional sequence on PMWaV-1 HN encodes the aa residue: "ETGLLTLGRQQREIIYKRHGFESN" and interestingly has a 65% similarity to that of the 3' end the PMWaV-4 (EU372003) HSP70 gene which has the same increased length of coding region[36]. The PMWaV-4 HTS assembly aa sequence similarity to PMWaV-1 (AF414119) is 89, 87 and 85% for the RdRp, HSP70 and CP respectively. With the additional data generated from this study we now propose that putative PMWaV-4 is a strain of PMWaV-1 and not a separate species. In the future, we suggest using the same nomenclature adopted to clarify different strains

of *Grapevine leafroll-associated virus*-4 (GLRaV-4), where GLRaV-5, GLRaV-6 and GLRaV-9 were renamed GLRaV-4 strain 5, 6 or 9[39]; therefore, the designation of PMWaV-4 suggested is PMWaV-1 strain 4. In this way, PMWaV-1 strain 4 will be considered a strain of PMWaV-1 but is a distinct strain based on sequence comparisons and serology relationships with PMWaV-1.

In Australia, a putative PMWaV-5 was reported in 2008[33] prior to the revised *Ampelovirus* species demarcation criteria[35]. PMWaV-5 was detected in 42 separate plants from 4 separate locations in symptomless and symptomatic plants[33]. Four putative PMWaV-5 sequences were reported[33] encoding partial CDS of HEL, RdRp and HSP70 genes, complete CDS of the p6 (SHP) gene and share low aa similarity to PMWaV-1 and PMWaV-3 (**Table 2.9**). The low aa similarity of the PMWaV-5 RdRp and HSP70 genes to PMWaV-1 and PMWaV-3 indicates, PMWaV-5 could potentially be a distinct subgroup II ampelovirus, but due to the incomplete sequence of the RdRp, HSP70 and the lack of CP sequence and genome characterization of PMWaV-5 there is insufficient evidence to yet propose the putative PMWaV-5 is indeed a distinct ampelovirus species.

The complex etiology of PMWaV involving a complex of ants, mealybugs, virus particles and pineapple might be further explained in part by the distinction between the two ampelovirus subgroups. The lack of a clearly understood etiology of MWP disease might potentially be due to the differing genomic organization and length of the ampelovirus subgroups. The longer, more complex genome of PMWaV-2 which has been shown to encode RNA silencing suppressors[37] which might be considered as factors supporting PMWaV-2's ability, in concert with mealybugs, to develop symptoms of MWP disease in HI. Further complicating this PMWaV-2 was not clearly associated with development of disease symptoms in Australia[33], Ecuador[38] or Cuba[31]. Clarity in genomic organization and gene function of all type members of the PMWaV complex is necessary to unravel the poorly understood etiology of MWP disease. It may be that symptomatic plants testing positive for PMWaV-1 or 3 but testing negative for PMWaV-2 are infected with another virus from subgroup I that, while similar to PMWaV-2 is distinct enough to inhibit molecular detection by the existing RT-PCR primer pairs or serological detection by the existing MAb for PMWaV-2. This possibility indicates the importance of using the HSP70 degenerate primer set and sequencing of many clones to confirm

the identities of PMWaVs in mixed population infections and subsequent symptom development resulting from mixed populations.

In the future, it would be interesting to use HTS to study PMWaV populations in healthy asymptomatic pineapple plants and pineapple plants with typical MWP symptoms. The complex of factors involved including the virus particles from the varying PMWaVs, mealybugs should be studied further. After identifying the population (if any) of PMWaVs in both asymptomatic and symptomatic pineapple plants TNA could be used to construct cDNA libraries and Illumina HTS used to determine the differing factors between the many possible combinations of mixed populations and presence or absence of mealybugs. With the advent of new HTS technologies like those used in this study it would be prudent to pursue transcriptomic and/or proteomic studies in order to clarify the etiology of the poorly understood MWP disease. RNAseq transcriptome analysis to determine differing amounts of gene expression in symptomatic and asymptomatic pineapple plants exposed to and in the absence of PMWaVs and mealybugs individually and in each possible combination to determine the molecular factors necessary for symptom development in order to identify the causal agent of MWP disease as well as identify possible avenues towards developing resistant cultivars in breeding programs.

Table 2.9 PMWaV-5 amino acid similarity to PMWaV-1 and PMWaV-3

Virus	PMWaV-5 (EF467920)			PMWaV-5 (EF467921)	PMWaV-5 (EF467922)		PMWaV-5 (EF488753)
Gene	RdRp	p5	HSP70	HEL	HEL	RdRp	HSP70
PMWaV-1 (AF414119)	69%	57%	69%	55%	64%	72%	72%
PMWaV-3 (DQ399259)	65%	57%	63%	53%	71%	67%	67%

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Chapter 3

Detection of Viruses from Symptomatic Ginger in Hawaii

3.1 Abstract

Banana bract mosaic virus (BBrMV) and *Canna yellow mottle virus* (CaYMV) were previously identified infecting *Alpinia purpurata* (flowering ginger) in Hawaii (HI). Recently, farmers growing flowering ginger have reported severe disease symptoms from multiple farms on Oahu, HI. Symptomatic flowering ginger leaves and bracts were used as starting material for molecular and serological assays to detect the presence of the previously identified viruses in flowering ginger. Symptoms corresponding with those previously characterized as CaYMV were observed, such as streaking on the bracts, yet PCR detection failed to correlate the presence of symptoms with CaYMV infection. BBrMV symptoms were not observed, and BBrMV was not detected from any of the plants assayed. The lack of conclusive evidence linking virus presence with the symptomatic flowering ginger assayed during this study may indicate the presence of another pathogens involvement in the development of disease on flowering ginger. Further study is necessary to conclude the casual organism of the disease found on flowering ginger.

3.2 Introduction

Banana bract mosaic virus (BBrMV) is a member of the *Potyvirus* genus of the family *Potyviridae* and was first reported in *Musa* spp. (banana) from the Philippines in 1979[50]. BBrMV has since been detected in many parts of the world including India, Samoa, Sri Lanka, Thailand, Vietnam, Colombia, Ecuador and Hawaii (HI)[51, 52]. BBrMV can spread quickly and causes significant economic losses worldwide. BBrMV was first reported in HI in 2010 in a new host, *Alpinia purpurata* (flowering ginger), but has not yet been found infecting banana in HI[53]. Symptoms of BBrMV on flowering ginger include: mosaic, streaking, severe cupping of leaves, browning of flowers, reduction in size & shelf life (**Figure 3.1**). BBrMV, has flexuous, filamentous rods 720 to 850 nm long and 12 to 15 nm in diameter and ssRNA genome[54, 55]. Three characterized genomes of BBrMV have been reported from the Philippines[56], India[57] and HI[58]. Enzyme linked immunosorbent assay (ELISA), RT-PCR, immunocapture RT-PCR and Reverse transcription-loop-mediated isothermal amplification (RT-LAMP) assays are available for detection of BBrMV[55, 56, 58, 59].

Canna yellow mottle virus (CaYMV), a member of the *Badnavirus* genus of the *Caulimoviridae* family was first reported infecting *Canna* spp. in Japan[60]. Later, CaYMV was reported in the United States, Italy, the Netherlands, India and Kenya[60-64]. CaYMV was reported infecting a new host, flowering ginger, in HI[65]. Symptoms of flowering ginger infected with CaYMV include: yellow mottling & necrosis of the leaves, vein streaking & stunting (**Error! Reference source not found.**). The episomal form of CaYMV was found to be associated with severe symptoms on flowering ginger in HI[65]. CaYMV is a member of the badnavirus genus, badnaviruses are known to integrate their genome sequence into the host's genome known as endogenous pararetroviruses (EPRV)[66-69]. PCR, RT-PCR and rolling circle amplification (RCA) assays are available for the detection of CaYMV[65]. Diagnostic testing of symptomatic ginger is necessary to establish if the current disease threatening ginger farmers is related to BBrMV and/or CaYMV infection. Molecular assays for detecting CaYMV and BBrMV are available as previously reported[53, 58] and serological assays are also available using the potyvirus or BBrMV-specific ELISA kit's from Agdia.

3.3 Materials and Methods

3.3.1 DNA Isolation

Ginger leaf samples were obtained from three Kahalu'u farms, Iolani School and University of HI at Manoa on the island of Oahu, HI. Approximately 0.1 g of symptomatic leaf tissue per sample per nucleic acid extraction was used for total genomic DNA extraction. The DNeasy Plant Mini Kit (QIAGEN, Redwood City, CA, USA) was used following the manufacturer's instructions.

3.3.2 PCR of CaYMV

The PCR Reagents used were: 10 µl 2X GoTaq Green master mix (Promega, USA), 0.5 µl forward/reverse primers (10 µM, **Table 3.1**), 1.0 µl DNA and 8 µl nanopure H_2O. The cycle conditions used were: 95°C for 5min; 30 cycles at 95°C for 30s, 55°C for 30s, 72°C for 1min; and then 72°C for 10 min.

3.3.3 RNA Isolation

Approximately 0.1 g of symptomatic leaf tissue per sample per nucleic acid extraction was used for total RNA extraction. The RNeasy Plant Mini Kit (QIAGEN, Redwood City, CA, USA) was used following the manufacturers protocols to isolate total RNA for cDNA synthesis.

3.3.4 cDNA Synthesis

For cDNA synthesis: 2 µl of RNA and 1.0 µl of random primers (50 ng/µl, Promega, WI, USA), heated at 70°C for 5 min, and then quenched on ice immediately. Then 5 µl M-MLV 5X reaction buffer (Promega. USA), 5 µl dNTP's (2.5 µM), 0.5 µl recombinant RNasin ribonuclease inhibitor (40 U/µl, Promega. USA), 1.0 µl M-MLV reverse transcriptase (200 U/µl. Promega. USA) were mixed before bringing the reaction to a final volume of 25µl with nanopure H_2O. Finally, both reactions were added together and incubated for 60 min at 37°C.

3.3.5 RT-PCR Assay

For RT-PCR of BBrMV a set of degenerate potyvirus primers[70] amplifying a partial nuclear inclusion B (NIb) gene were used (**Table 3.1**). The PCR reagents used were:10 µl 2X GoTaq Green master mix (Promega, USA), 0.5 µl forward/reverse primers (10 µM), 1.0 µl cDNA and 8 µl nanopure H_2O. The cycle conditions used were: 95°C for 5 min; 35 cycles at 95°C for 30 s, 55°C for 30 s, 72°C for 1 min; and then 72°C for 10 min.

3.3.6 BBrMV ELISA

A BBrMV-specific ELISA kit (Agdia, Elkhart, IN, USA) was used according to the manufacturer's instructions.

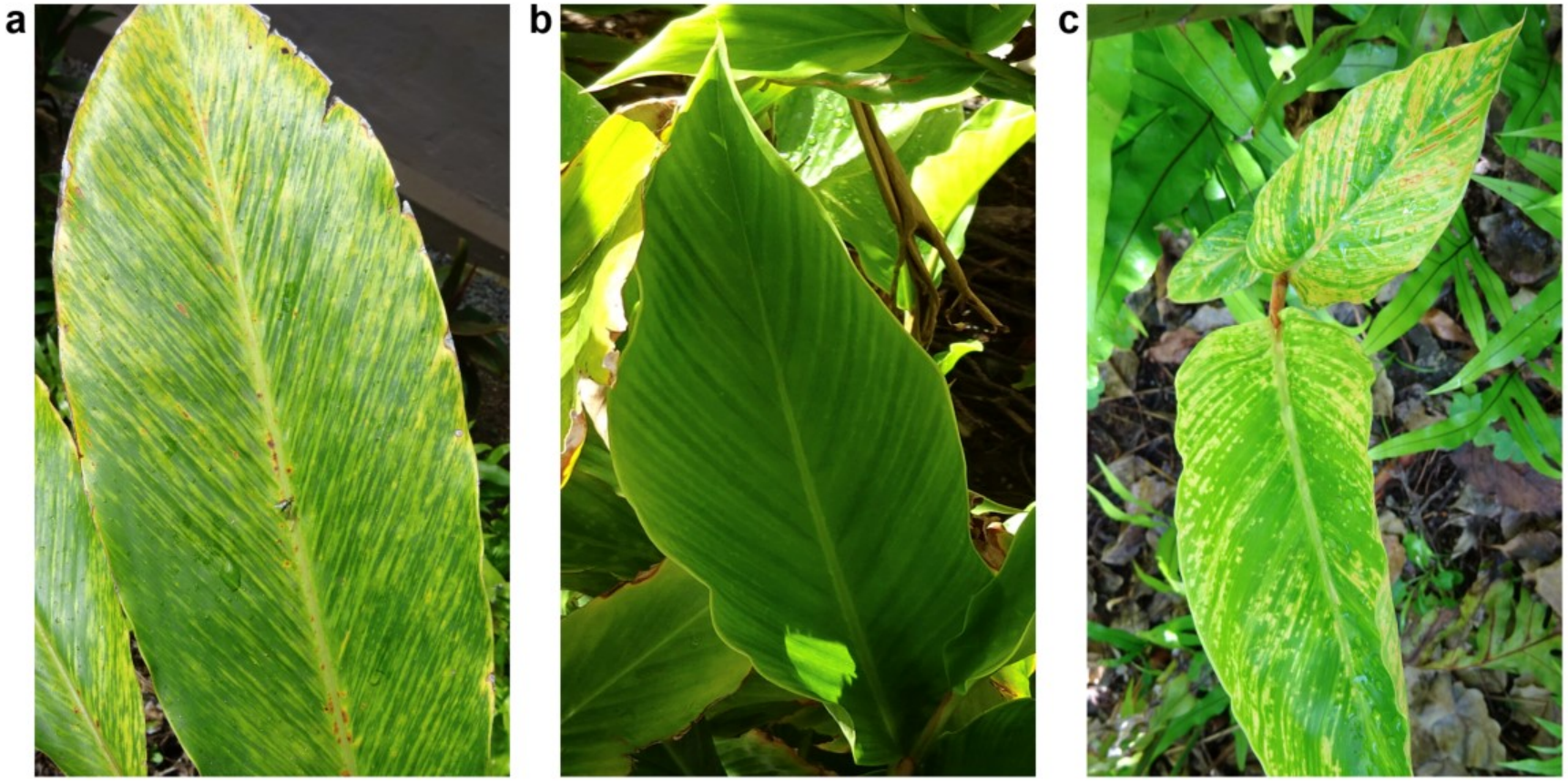

Figure 3.1 Symptoms of flowering ginger infected with BBrMV & CaYMV
Symptomatic flowering ginger: (**a**) BBrMV positive (**b**) CaYMV positive; (**c**) CaYMV and BBrMV positive

Table 3.1 Primers used to detect viruses in flowering ginger

Target Virus	Primer	Sequence (5' → 3')	Amplicon Size (nt)	Target Gene
CaYMV	1503	TGCTGGAACACTGGCTTTCA	667	ORF3
	1504	CCTCTTCATCCCCCACCA		
Degenerate Potyvirus	1130	GTITGYGTIGAYGAYTTYAAYAA	350	NIb
	1131	TCIACIACIGTIGAIGGYTGNCC		

3.4 Results

Symptomatic ginger leaves and bracts were assayed for the presence of viruses from several locations on Oahu including three farms in Kahalu'u and Iolani School in September of 2016 and April 2018.

3.4.1 BBrMV ELISA

Ginger leaf samples collected in September 2016 were assayed with the BBrMV-specific ELISA kit according to the manufacturer's instructions, no samples from any of the locations surveyed reported positive (**Table 3.2**).

3.4.2 PCR Assay of CaYMV

In September 2016 ginger leaves from 4 different locations in Oahu, HI were collected and assayed for the presence of CaYMV using a PCR assay. Samples from two of the three Kahalu'u farms and Iolani School tested positive for CaYMV; the K1 location has no positive samples for CaYMV (**Table 3.2**). In April 2018 the three Kahalu'u farms were revisited and all of the samples collected from two of the three Kahalu'u farms tested positive for CaYMV (**Table 3.2**). The results from September 2016 and April 2018 were consistent in that the same farm (K1) did not have any samples test positive for CaYMV (**Figure 3.2**).

3.4.3 RT-PCR Assay of Potyviruses

Ginger leaf samples collected in April 2018 were tested for potyviral infection with RT-PCR using degenerate potyvirus primers[70] (**Table 3.1**), no samples reported positive (**Table 3.2**).

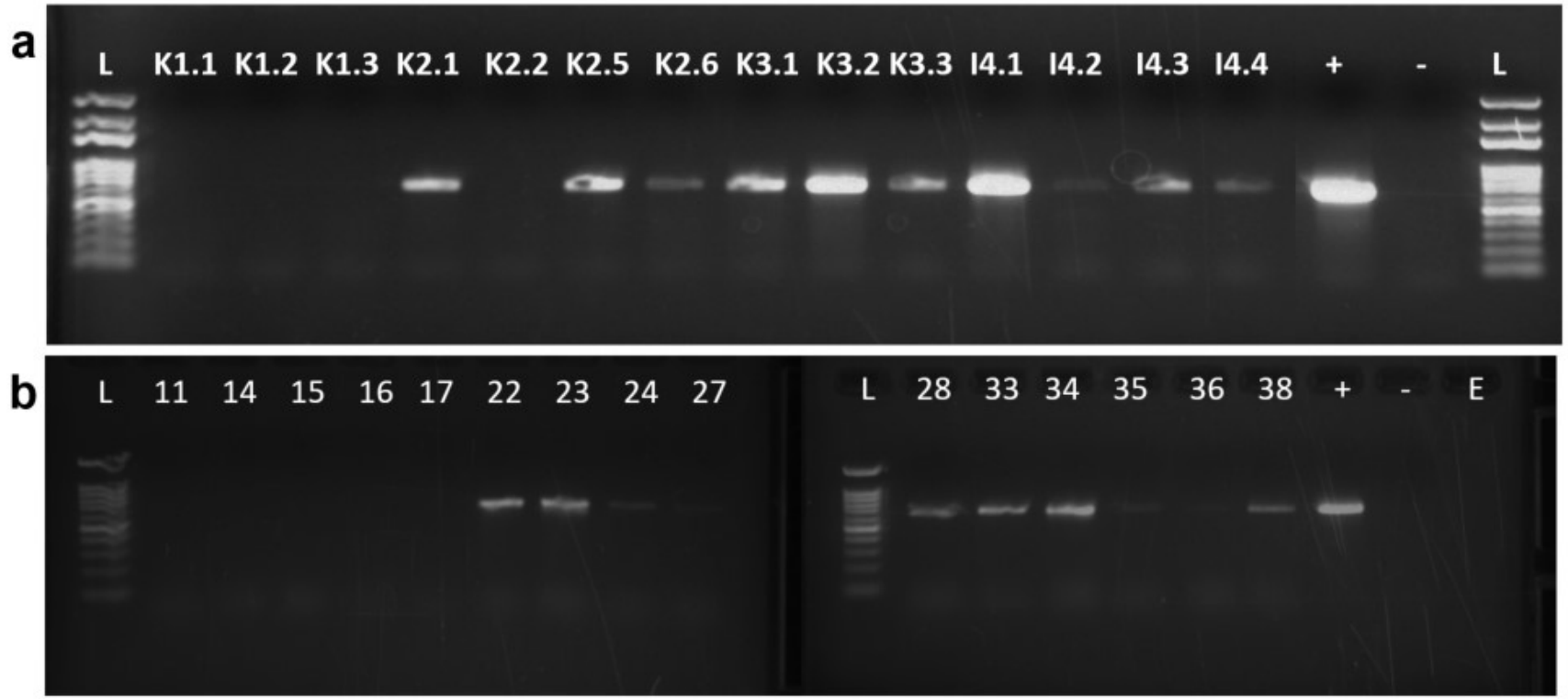

Figure 3.2 PCR of CaYMV
(**a**) PCR of CaYMV September 2016 survey samples with K1 location (K1.1-K1.3) only location with no CaYMV positive samples and (**b**) PCR of CaYMV April 2018 survey samples, again, K1 (11-17) only location with no CaYMV positive samples

Table 3.2 Surveys of BBrMV and CaYMV in flowering ginger

Sample # September 2016	BBrMV ELISA	CaYMV PCR	Sample # April 2018	Potyvirus RT-PCR	CaYMV PCR
K1.1	-	-	11	-	-
K1.2	-	-	14	-	-
K1.3	-	-	15	-	-
K2.1	-	+	16	-	-
K2.2	-	-	17	-	-
K2.5	-	+	22	-	+
K2.6	-	+	23	-	+
K3.1	-	+	24	-	+
K3.2	-	+	27	-	+
I4.1	-	+	28	-	+
I4.2	-	+	33	-	+
I4.3	-	+	34	-	+
I4.4	-	+	35	-	+

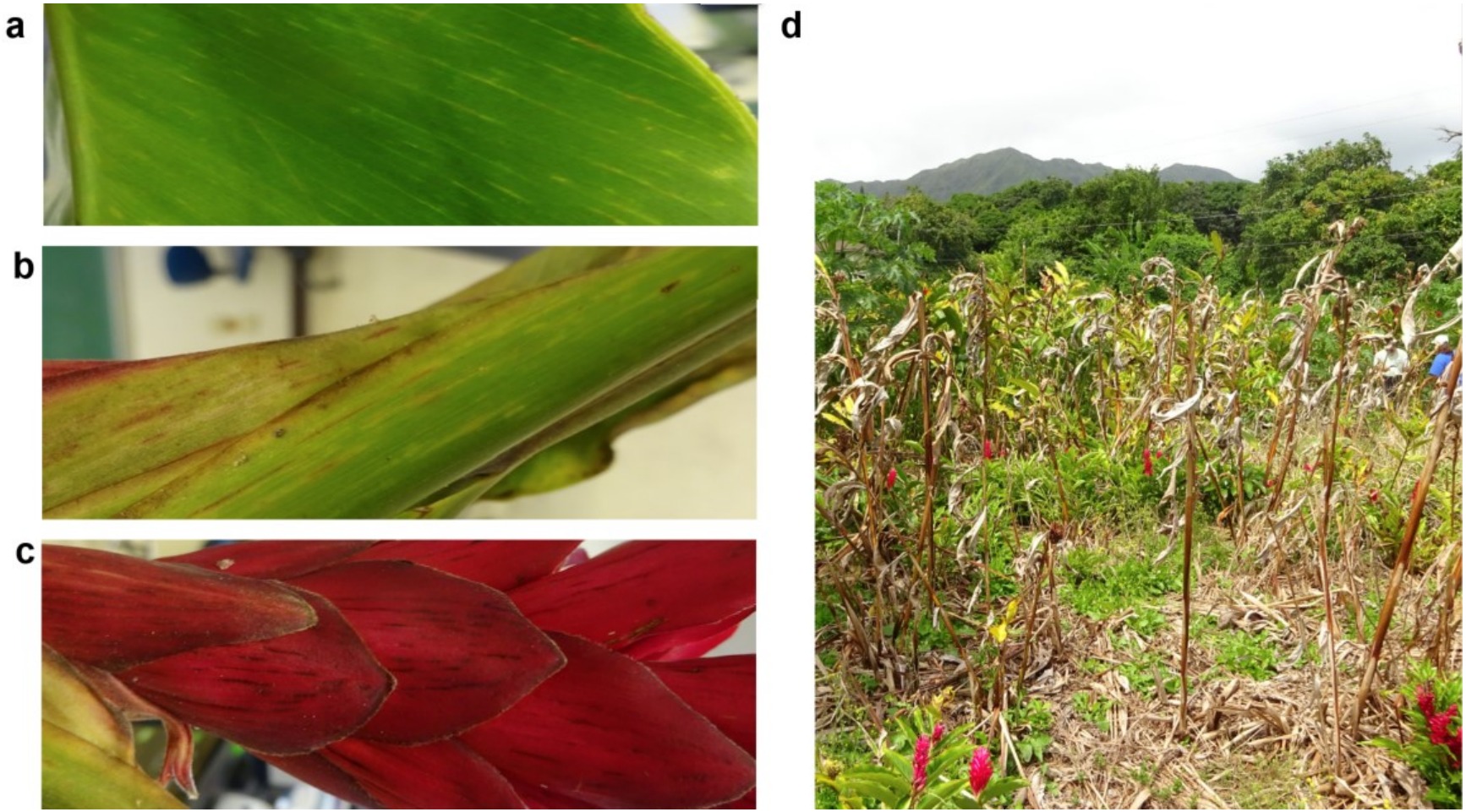

Figure 3.3 'Streaking' and 'dieback' symptoms on flowering ginger
Streaking symptoms present on (**a**) leaves, (**b**) stems and (**c**) bracts of flowering ginger from the K1 location that tested negative for CaYMV and BBrMV and (**d**) dieback symptoms from the K2 location

3.5 Discussion

Flowering ginger infected with CaYMV was found at different locations around Oahu, HI; symptoms were not consistent with previous reports (**Figure 3.1**). None of the samples assayed tested positive for BBrMV by either BBrMV-specific ELISA or potyvirus-specific RT-PCR. The new 'streaking' and 'dieback' symptoms (**Figure 3.3**) seen in the samples positive for CaYMV were also observed in samples that tested negative for CaYMV and BBrMV. Severe 'dieback' symptoms were present at the K2 location where CaYMV was prevalent (**Figure 3.3**). The 'streaking' symptoms in flowering ginger (**Figure 3.3**) have been associated with virus infection in previous studies, but bracts exhibiting the streak symptoms assayed for virus presence in this study returned negative results. These results might suggest the presence of another or multiple virus co-infections. It is possible these symptoms which are seemingly unrelated to CaYMV may be caused by another badnavirus; use of degenerate badnavirus primers to detect other badnaviruses infecting flowering ginger is warranted. Another member of the lab pursued this avenue and identified the *Banana streak virus* (BSV) a member of the *Badnavirus* genus of the *Caulimoviridae* family from the K1 location, where streaking symptoms were observed (**Figure 3.3**) and no CaYMV was detected. As the investigation of BSV and its role in disease of flowering ginger continues by other, future members of the lab it may be warranted to utilize HTS tools as a diagnostic for characterization of the BSV strain found in HI and any other potential viruses that might play a role in this disease of flowering ginger that have yet to be identified. In the field, this yet uncharacterized disease causes devastating loss of production and is has the potential to become extremely harmful to local growers of flowering ginger and the cut flower industry.

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Chapter 4

Identification of New Plant Hosts/Viruses in Hawaii

4.1 Abstract

Oahu, Hawaii (HI) is home to many community gardens which function to provide many urban dwellers the privilege of access to land for growing of edible and horticultural plants. Unfortunately, these areas are often 'hotbeds' of disease and pathogen dispersal. Recently, we identified several different plants infected with viruses from diverse crop and horticultural species including *Bean common mosaic virus* (BCMV) infecting *Phaseolus lunatus* (lima bean) in one community garden in Honolulu, HI. Symptomatic lima bean leaves were assayed for the presence of member species of the *Potyvirus* genus of the *Potyviridae* family using a potyvirus-specific ELISA, positives were confirmed with a degenerate potyvirus RT-PCR assay, PCR amplicons were cloned and sequenced using Sanger sequencing. The sequence homology of the HI BCMV isolate showed a high degree (92% nucleotide and 100% amino acid of similarity to a BCMV isolate from China. A BCMV-specific ELISA assay was used to reconfirm the sequencing results. The unusually diverse amount of plants grown in a community garden as well as the high volume of individuals with access to the area provide the ideal circumstances for dissemination of plant pathogens.

4.2 Introduction

The *Potyvirus* genus of the *Potyviridae* family is one of the largest virus genera that includes over a hundred recognized member species, several dozen tentative species noted by the ICTV and several new species reported annually[71]. In response to the great abundance of potyviruses several sets of degenerate primers have previously been developed for reliable and efficient identification of potyvirus infection in plants. Among these, notably, the potyvirid 2 degenerate primer pair[72] targeting the nuclear inclusion protein b (NIb) gene, the CN48 degenerate primers[73] targeting the coat protein gene and the NIb2F and NIb3R degenerate primers[70] targeting the NIb gene have been widely used. Our lab has used the NIb2F and NIb3R primer set primarily to detect a wide number of potyviruses previously and use this potyvirus specific RT-PCR assay regularly to screen plants exhibiting virus-like symptoms. Many virus-like symptoms were observed in community gardens in Honolulu, HI (**Figure 4.**). We collected samples from many ornamental and crop plants to test with molecular and serological potyvirus detection assays.

4.3 Materials and Methods

4.3.1 Potyvirus ELISA

Symptomatic leaf tissue collected from the community garden in Honolulu, HI was used for the potyvirus-specific ELISA (Agdia, Elkhart, IN, USA) following the manufacturer's instructions.

4.3.2 RNA Isolation

Approximately 0.1 g of symptomatic leaf tissue per sample per nucleic acid extraction was used for total RNA extraction. The RNeasy Plant Mini Kit (QIAGEN, Redwood City, CA, USA) was used following the manufacturers protocols to isolate total RNA for cDNA synthesis.

4.3.3 cDNA Synthesis

For cDNA synthesis: 2 µl of RNA and 1.0 µl of random primers (50 ng/µl, Promega, WI, USA), heated at 70°C for 5 min, and then quenched on ice immediately. Then 5 µl M-MLV 5X

reaction buffer (Promega. USA), 5 µl dNTP's (2.5 µM), 0.5 µl recombinant RNasin ribonuclease inhibitor (40 U/µl, Promega. USA), 1.0 µl M-MLV reverse transcriptase (200 U/µl. Promega. USA) were mixed before bringing the reaction to a final volume of 25µl with nanopure H2O. Finally, both reactions were added together and incubated for 60 min at 37°C.

4.3.4 RT-PCR Assay

For RT-PCR a set of degenerate potyvirus primers[70] amplifying the partial NIb gene were used (**Table 4.1**). The PCR reagents used were:10 µl 2X GoTaq Green master mix (Promega, USA), 0.5 µl forward/reverse primers (10 µM), 1.0 µl cDNA and 8 µl nanopure H2O. The cycle conditions used were: 95°C for 5 min; 35 cycles at 95°C for 30 s, 55°C for 30 s, 72°C for 1 min; and then 72°C for 10 min.

4.3.5 Sanger Sequencing

PCR products amplified from infected leaf tissue were cloned into pGEM-T Easy vector (Promega, Madison, WI, USA), heat shock transformed in *E. coli* DH5α, red/white screened using MacConkey agar and sequenced using Sanger sequencing.

4.3.6 BCMV ELISA

A BCMV-specific ELISA (Agdia, Elkhart, IN, USA) was used following the manufacturer's instructions.

Table 4.1 Primers used to detect potyviruses

Target Virus	Primer	Sequence (5'→3')	Amplicon Size (nt)	Target Gene
Degenerate potyvirus	NIb2F	GTITGYGTIGAYGAYTTYAAYAA	350	NIb
	NIb3R	TCIACIACIGTIGAIGGYTGNCC		

4.4 Results

Ornamental and crop plants exhibiting virus like symptoms were collected from the Ala Wai community garden in Honolulu, HI (**Figure 4.**). Among the plants collected were: *Phaseolus lunatus* (lima bean), *Araceae* spp., *Colocasia esculenta*, *Cordyline fruticosa*, *Crinium* spp., *Heliconia* spp., *Passiflora* spp. (passionfruit), *Polyscias* spp. and *Syngonium podophyllum.*

4.4.1 Potyvirus-specific ELISA

Leaf tissue from the collected plants were assayed for the presence of potyviruses using a potyvirus-specific ELISA according to the manufacturer's instructions with *Carica papaya* (papaya) infected with *Papaya ringspot virus* (PRSV) a member of the *Potyvirus* genus of the *Potyviridae* family was used as a positive control. ELISA detected potyvirus antigen in the lima bean, passionfruit and papaya samples (**Table 4.2**).

4.4.2 RT-PCR and Sanger Sequencing

To confirm the potyvirus-specific ELISA results with molecular detection a RT-PCR potyvirus detection assay was used with a degenerate potyvirus primer pair[70] (**Table 4.1**) and both samples returned the expected 350-bp amplicon. The sanger results from the *Passiflora* spp. returned the expected high similarity to *Watermelon mosaic virus* (WMV), a member of the *Potyvirus* genus of the *Closteroviridae* family was previously reported infecting *Passiflora* spp. in HI[74]. Lima bean sequencing results (KY473075) revealed a 92% nt sequence identity and 100% aa sequence identity to the NIb gene of a BCMV isolate from China.

4.4.3 BCMV ELISA

To further confirm the potyvirus-specific ELISA and RT-PCR results seven symptomatic lima bean samples collected from the same community garden in Honolulu, HI as well as seven lima bean samples collected from another location in Honolulu, HI for a BCMV-specific ELISA assay. All seven samples from the community garden and one of seven from the second location tested positive for BCMV.

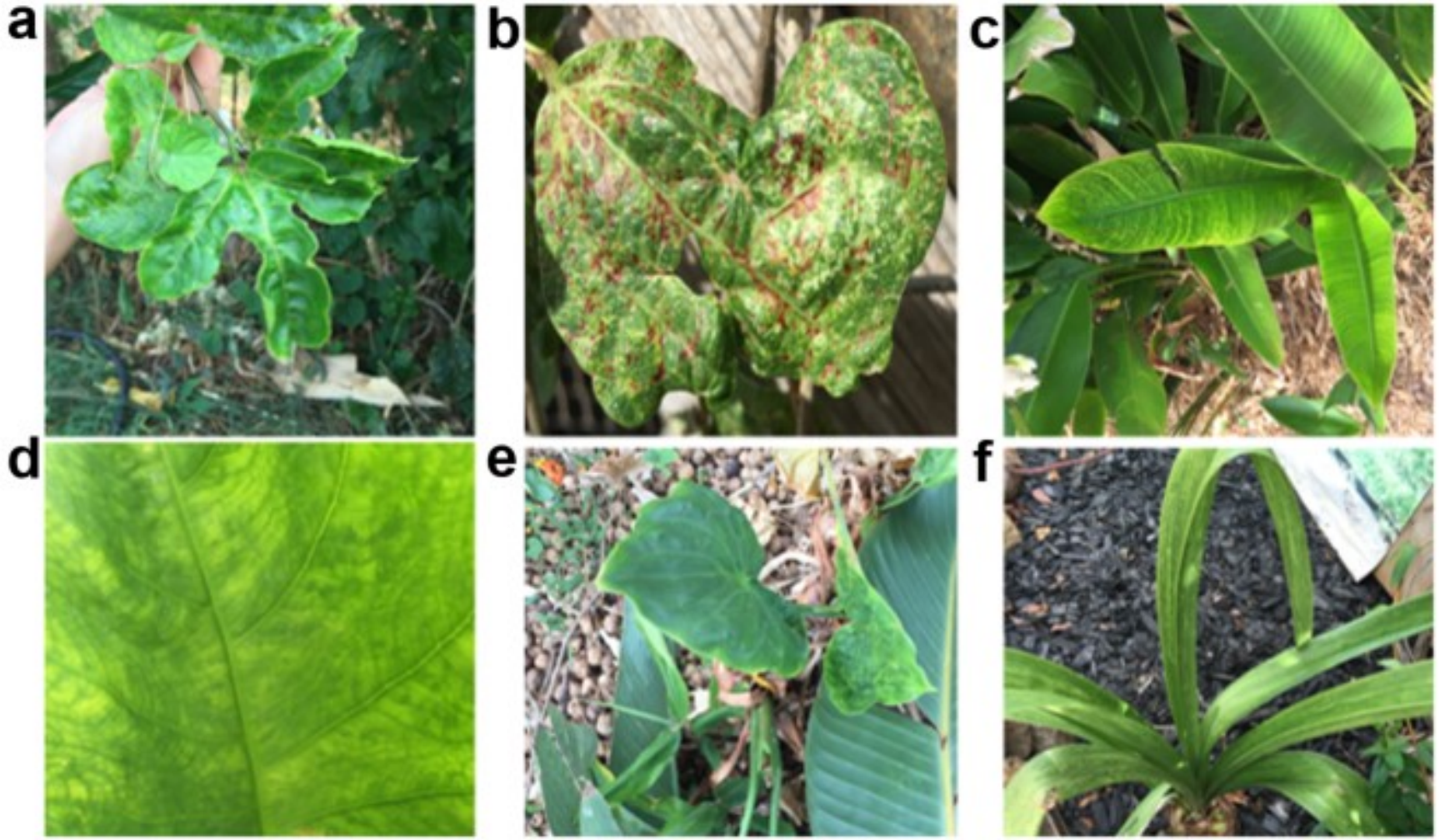

Figure 4.1 Symptomatic plants from a community garden in Honolulu, HI
(**a**) *Passiflora* spp. (**b**) *Phaseolus lunatus* (**c**) *Heliconia* spp. (**d**) *Syngonium podophyllum* (**e**), *Araceae* spp. and (**f**) *Crinium asiaticum*

Table 4.2 Virus infection in symptomatic plants

Plants	Potyvirus RT-PCR	Potyvirus ELISA	BCMV ELISA
Syngonium podophyllum	-	-	-
Polyscias spp.	-	-	-
Araceae spp.	-	-	-
Phaseolus lunatus	+BCMV	+BCMV	+BCMV
Colocasia esculenta	-	-	-
Passiflora spp.	+WMV	+WMV	-
Heliconia spp.	-	-	-
Cordyline fruticosa	-	-	-
Crinum asiaticum	-	-	-
Carica papaya (+C)	+PRSV	+PRSV	-

4.5 Discussion

Of the plants collected only two returned positive results from molecular and serological potyvirus assays; lima bean infected with BCMV and passion fruit infected with WMV previously reported[74]. Interestingly, the symptomatic heliconia were found to be infected with *Banana bunchy top virus* (BBTV), a member of the *Babuvirus* genus of the *Nanoviridae* family, by another member of the lab group[75]. While heliconia is in the same order, *Zingiberales*, as bananas (*Musa* spp.) this is the first report of BBTV infecting a host other than bananas and may have serious implications for management of BBTV by farmers and backyard growers. The remaining plants assayed exhibited severe virus symptoms but did not return positive results from either molecular or serological potyvirus assays. It is possible that these symptomatic plants were infected with other viruses.

The use of more powerful tools like HTS might be prudent to identify any possible virus infections in symptomatic plants. It is possible to pool nucleic acid content for sequencing runs to minimize the overhead of using HTS as a diagnostic tool. After HTS and data analysis, nucleic acid isolated from the individual plants prior to pooling can be assayed to determine the source of any potential viruses identified through sequencing. It is important to note that all the viruses discussed in this chapter were identified from a single location, a community garden in Honolulu, HI. These community gardens, while providing a niche service for amateur agriculturists and horticulturalists in urban areas represent ‘hot beds’ of infection for many plant pathogens and should be closely monitored by resident plant pathologists to curb the influx and subsequent escape of any potentially devastating plant diseases.

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Chapter 5

Discussion and Conclusion

Currently and historically viruses have had a severe impact upon agricultural stakeholders in HI and many severe virus diseases represent serious challenges and threaten the livelihood of agricultural stakeholders throughout the islands. PRSV is of serious economic concern to papaya growers, fortunately the successful application of transgenics has allowed the once threatened papaya industry to recover after resistant varieties were developed[76]. Impact of agricultural viruses is further evidenced by the recent closure of HI's largest banana producer Hamakua Springs, which shut down its operations, citing losses due to BBTV as the primary reason for ceasing production.

CaYMV and BBrMV are viruses found infecting flowering ginger in HI. The lack of a clear association between symptoms observed and virus positive plants indicates the disease ginger farmers are facing may be caused by another pathogen, perhaps another virus like BSV recently identified in flowering ginger. The use of HTS technologies represents a powerful tool capable of identifying any virus infections present in plants. It is possible to pool nucleic acid content for sequencing runs to minimize the overhead of using HTS as a diagnostic tool and prudent to do so to detect any other potential viruses infecting ginger. After HTS and data analysis, nucleic acid isolated from the individual plants prior to pooling can be assayed to determine the source of any potential viruses identified through sequencing

MWP is a limiting factor in pineapple production resulting in reduction of yields representing a threat to pineapple growers. The unusual and mysterious etiology involving PMWaV-2 and mealybugs causing disease symptoms in HI, but elsewhere in the world, Australia, Cuba and Ecuador, a similar correlation is not found. With the advent of new HTS technologies like those used in this study it is possible and would be prudent to pursue transcriptomic and/or proteomic studies in order to elucidate the etiology of the rather unique nature of mealybug wilt of pineapple disease. The complex of factors involved including the virus particles from the varying PMWaVs, mealybugs and should be studied further. RNAseq

transcriptome analysis to determine differing amounts of gene expression in symptomatic and asymptomatic pineapple plants exposed to and in the absence of virus particles and mealybugs individually and in each possible combination to determine the molecular factors necessary for symptom development as well as identify possible avenues towards developing resistant cultivars in breeding programs.

Community gardens and in general urban areas are ‘hot beds’ of infection for many plant pathogens and should be closely monitored by resident plant pathologists to curb the influx and subsequent escape of any potentially devastating plant diseases. It is important to note that all the viruses discussed in Chapter 4 were identified from a single location, a community garden in Honolulu, HI. These community gardens, while providing a niche service for amateur agriculturists and horticulturalists in urban areas represent transmission and dispersal ‘highways’ for plant pathogens. The use of powerful tools like HTS as a diagnostic tool might be prudent to identify any possible virus infections in symptomatic plants.

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References

1. Larsen, L.D. Diseases of pineapple. Hawaii Sugar Planters Association Pathol. Physiol. Ser. *Experimental Station Bulletin* **10**, 1-72 (1910).
2. Carter, W. Studies of populations of Pseudococcus brevipes (Ckl.) occurring on pineapple plants. *Ecology* **13**, 296-304 (1932).
3. Carter, W. The pineapple mealy bug, Pseudococcus brevipes, and wilt of pineapples. *Phytopathology* **23**, 207-242 (1933).
4. Carter, W. Studies on the biological control of Pseudococcus brevipes and wilt of pineapple. *Phytopathology* **28**, 1037-1041 (1935).
5. Carter, W. Some etiological aspects of mealybug wilt. *Phytopathology* **35**, 305-315 (1944).
6. Illingworth, J.F. Preliminary report on evidence that mealy bugs are an important factor in pineapple wilt. *J. of Eco. Ento.* **24**, 877-889 (1931).
7. Carter, W. The feeding sequence of pseudococcus brevipes-(ckl) in relation to mealybug wilt of pineapples in hawaii. *Phytopathol.* **41**, 769-780 (1951).
8. Carter, W. Injuries to plants caused by insect toxins. II. *The Bot. Rev.* **18**, 680-721 (1952).
9. Ito, K. Additional immunological evidence supporting the virus nature of mealybug wilt. *Pineapple Res. Inst. News* **10**, 158-162 (1962).
10. Carter, W. Mealybug wilt of pineapple; a reappraisal*. *Ann. of the New York Acad. of Sci.* **105**, 741-764 (1963).
11. Gunasinghe, U. & German, T. in Phytopathol., Vol. 76 1073-1073 (amer phytopathological soc 3340 pilot knob road, st paul, mn 55121, 1986).
12. Gunasinghe, U. & German, T. in Phytopathol., Vol. 77 1776-1776 (amer phytopathological soc 3340 pilot knob road, st paul, mn 55121, 1987).
13. Gunasinghe, U. & German, T. Purification and partial characterization of a virus from pineapple. *Phytopathol.* **79**, 1337-1341 (1989).
14. Borroto, E.G., Cintra, M., González, J., Borroto, C. & Oramas, P. First report of a closterovirus-like particle associated with pineapple plants (Ananas comosus cv. Smooth Cayenne) affected with pineapple mealybug wilt in Cuba. *Plant Dis.* **82**, 263-263 (1998).
15. Maramorosch, K., Guan, T. & Gosh, B.K. in Proceedings of the 6th International Congress of Virology (1984).
16. Hu, J., Gonsalves, A., Sether, D. & Ullman, D. in I Int. Pineapple Symp. 334 411-416 (1992).
17. Hu, J., Sether, D. & Ullman, D. Detection of pineapple closterovirus in pineapple plants and mealybugs using monoclonal antibodies. *Plant Pathol.* **45**, 829-836 (1996).
18. Hu, J. et al. Use of a tissue blotting immunoassay to examine the distribution of pineapple closterovirus in Hawaii. *Plant Dis.* **81**, 1150-1154 (1997).
19. Melzer, M., Karasev, A., Sether, D. & Hu, J. Nucleotide sequence, genome organization and phylogenetic analysis of pineapple mealybug wilt-associated virus-2. *J. of Gen. Virol.* **82**, 1-7 (2001).

20. Melzer, M., Sether, D., Karasev, A., Borth, W. & Hu, J. Complete nucleotide sequence and genome organization of pineapple mealybug wilt-associated virus-1. *Arch. of virol.* **153**, 707-714 (2008).
21. Sether, D.M., Melzer, M.J., Borth, W.B. & Hu, J.S. Genome organization and phylogenetic relationship of Pineapple mealybug wilt associated virus-3 with family Closteroviridae members. *Virus genes* **38**, 414-420 (2009).
22. Sether, D., Ullman, D. & Hu, J. Transmission of pineapple mealybug wilt-associated virus by two species of mealybug (Dysmicoccus spp.). *Phytopathol.* **88**, 1224-1230 (1998).
23. Sether, D. et al. Differentiation, distribution, and elimination of two different pineapple mealybug wilt-associated viruses found in pineapple. *Plant Dis.* **85**, 856-864 (2001).
24. Sether, D. & Hu, J. Closterovirus infection and mealybug exposure are necessary for the development of mealybug wilt of pineapple disease. *Phytopathol.* **92**, 928-935 (2002).
25. Sether, D.M., Melzer, M.J., Busto, J., Zee, F. & Hu, J.S. Diversity and Mealybug Transmissibility of Ampeloviruses in Pineapple. *Plant Dis.* **89**, 450-456 (2005).
26. Sether, D., Melzer, M., Subere, C. & Hu, J. in XIII Int. Congr. of Virol., Vol. 89 121-122 (American Society for Microbiology, Washington, DC; 2005).
27. Gambley, C., Steele, V., Geering, A. & Thomas, J. The genetic diversity of ampeloviruses in Australian pineapples and their association with mealybug wilt disease. *Aust. Plant Pathol.* **37**, 95-105 (2008).
28. Gambley, C.F., Geering, A.D. & Thomas, J.E. Development of an immunomagnetic capture-reverse transcriptase-PCR assay for three pineapple ampeloviruses. *J Virol Methods* **155**, 187-192 (2009).
29. Subere, C.V.Q. (University of Hawaii at Manoa, 2009).
30. Dey, K.K., Lin, H., Borth, W.B., Melzer, M.J. & Hu, J.S. A highly sensitive single-tube nested PCR assay for the detection of Pineapple mealybug wilt associated virus-2 (PMWaV-2). *J Virol Methods* **183**, 215-218 (2012).
31. Hernandez-Rodriguez, L. et al. Geographic distribution of mealybug wilt disease of pineapple and genetic diversity of viruses infecting pineapple in Cuba. *Crop Protection* **65**, 43-50 (2014).
32. Gambley, C.F., Geering, A.D., Steele, V. & Thomas, J.E. Identification of viral and non-viral reverse transcribing elements in pineapple (Ananas comosus), including members of two new badnavirus species. *Arch Virol* **153**, 1599-1604 (2008).
33. Gambley, C.F., Steele, V., Geering, A.D.W. & Thomas, J.E. The genetic diversity of ampeloviruses in Australian pineapples and their association with mealybug wilt disease. *Aust. Plant Pathol.* **37**, 95 (2008).
34. Shen, B. et al. Occurrence and Molecular Characterization of Three Pineapple Mealybug Wilt-Associated Viruses in Pineapple in Taiwan. *Plant Disease* **93**, 196 (2009).
35. Martelli G.P. et al. (eds.) Family Closteroviridae, Vol. Ninth Rep. of the Int. Comm. on Taxon. of Viruses. (Elsevier-Academic Press, Amsterdam, The Netherlands; 2011).
36. Yu, N. et al. Complete genomic sequence of a Pineapple mealybug wilt-associated virus-1 from Hainan Island, China. *Eur. J. Plant Pathol.* **141**, 611-615 (2015).
37. Dey, K.K., Borth, W.B., Melzer, M.J., Wang, M.-L. & Hu, J.S. Analysis of Pineapple Mealybug Wilt Associated Virus -1 and -2 for Potential RNA Silencing Suppressors and Pathogenicity Factors. *Viruses* **7**, 969-995 (2015).

38. Alvarez RA, Martin RR & DF, Q.-A. First report of Pineapple mealybug wilt associated virus-1 in Ecuador. *New Disease Reports* **31** (2015).
39. Martelli, G.P. et al. Taxonomic revision of the family closteroviridae with special reference to the grapevine leafroll-associated members of the genus ampelovirus and the putative species unassigned to the family. *J. of Plant Pathol.* **94** 7-19 (2012).
40. , Vol. 2018, Edn. version 8.5.1 (QIAGEN, clcbio.com.
41. Altschul, S.F., Gish, W., Miller, W., Myers, E.W. & Lipman, D.J. Basic local alignment search tool. *Journal of molecular biology* **215**, 403-410 (1990).
42. Afgan, E. et al. The Galaxy platform for accessible, reproducible and collaborative biomedical analyses: 2016 update. *Nucleic Acids Res* **44**, W3-w10 (2016).
43. S., A. (http://www.bioinformatics.babraham.ac.uk/projects/fastqc; 2010).
44. Bolger, A.M., Lohse, M. & Usadel, B. Trimmomatic: a flexible trimmer for Illumina sequence data. *Bioinformatics* **30**, 2114-2120 (2014).
45. Kearse, M. et al. Geneious Basic: An integrated and extendable desktop software platform for the organization and analysis of sequence data. *Bioinform.* **28**, 1647-1649 (2012).
46. Le, S.Q. & Gascuel, O. An improved general amino acid replacement matrix. *Molecular biology and evolution* **25**, 1307-1320 (2008).
47. Kumar, S., Stecher, G. & Tamura, K. MEGA7: Molecular Evolutionary Genetics Analysis Version 7.0 for Bigger Datasets. *Molecular biology and evolution* **33**, 1870-1874 (2016).
48. Coordinators, N.R. Database resources of the National Center for Biotechnology Information. *Nucleic Acids Research* **44**, D7-D19 (2016).
49. Ye, J. et al. Primer-BLAST: a tool to design target-specific primers for polymerase chain reaction. *BMC Bioinformatics* **13**, 134 (2012).
50. Magnaye, L.V. & Valmayor, R.V. (ed. B.o.P. Industry) (Los Baños, Laguna, Philippines.
51. Kumar, P.L., Selvarajan, R., Iskra-Caruana, M.L., Chabannes, M. & Hanna, R. Biology, etiology, and control of virus diseases of banana and plantain. *Adv Virus Res* **91**, 229-269 (2015).
52. Quito-Avila, D.F. et al. First Report of Banana bract mosaic virus in 'Cavendish' Banana in Ecuador. *Plant Disease* **97**, 1003-1003 (2013).
53. Wang, I.C., Sether, D.M., Melzer, M.J., Borth, W.B. & Hu, J.S. First Report of Banana bract mosaic virus in Flowering Ginger in Hawaii. *Plant Disease* **94**, 921-921 (2010).
54. Rodoni, B.C., Ahlawat, Y.S., Varma, A., Dale, J.L. & Harding, R.M. Identification and Characterization of Banana Bract Mosaic Virus in India. *Plant Disease* **81**, 669-672 (1997).
55. Thomas, J.E., Geering, A.D., Gambley, C.F., Kessling, A.F. & White, M. Purification, properties, and diagnosis of banana bract mosaic potyvirus and its distinction from abaca mosaic potyvirus. *Phytopathology* **87**, 698-705 (1997).
56. Ha, C. et al. Design and application of two novel degenerate primer pairs for the detection and complete genomic characterization of potyviruses. *Arch Virol* **153**, 25-36 (2008).
57. Balasubramanian, V. & Selvarajan, R. Complete genome sequence of a banana bract mosaic virus isolate infecting the French plantain cv. Nendran in India. *Arch Virol* **157**, 397-400 (2012).

58. Zhang, J. et al. Deep sequencing of banana bract mosaic virus from flowering ginger (Alpinia purpurata) and development of an immunocapture RT-LAMP detection assay. *Arch Virol* **161**, 1783-1795 (2016).
59. Iskra-Caruana, M.L., Galzi, S. & Laboureau, N. A reliable IC One-step RT-PCR method for the detection of BBrMV to ensure safe exchange of Musa germplasm. *J Virol Methods* **153**, 223-231 (2008).
60. al., Y.e. *Ann Phytopahol. Soc. Jpn* **45**, 484 (1979).
61. Lockhart, B. & Autrey, L. Occurrence in sugarcane of a bacilliform virus related serologically to banana streak virus. *Plant Disease* **72**, 230-233 (1988).
62. Marino, M.T., Ragozzino, E., Lockhart, B.E.L., Miglino, R. & Alioto, D. First report of Canna yellow mottle virus (CaYMV) in Italy and in the Netherlands. *Plant Pathology* **57**, 394 (2008).
63. Kumari, A., S., K. & Raj, S.K. First report of Canna yellow mottle virus on Canna from India. *New Disease Reports* **29** (2014).
64. Agneroh, T.A., Bratsch, S.A. & Lockhart, B.E. First report of Canna yellow mottle virus in Kenya. *Plant Health Progress*, PHP-BR-14-0037 (2015).
65. Zhang, J. et al. Characterization of Canna yellow mottle virus in a New Host, Alpinia purpurata, in Hawaii. *Phytopathology* **107**, 791-799 (2017).
66. Geering, A.D., Olszewski, N.E., Dahal, G., Thomas, J.E. & Lockhart, B.E. Analysis of the distribution and structure of integrated Banana streak virus DNA in a range of Musa cultivars. *Mol Plant Pathol* **2**, 207-213 (2001).
67. Hany, U., Adams, I.P., Glover, R., Bhat, A.I. & Boonham, N. The complete genome sequence of Piper yellow mottle virus (PYMoV). *Arch Virol* **159**, 385-388 (2014).
68. Seal, S. et al. The prevalence of badnaviruses in West African yams (Dioscorea cayenensis-rotundata) and evidence of endogenous pararetrovirus sequences in their genomes. *Virus Res* **186**, 144-154 (2014).
69. Wang, Y., Cheng, X., Wu, X., Wang, A. & Wu, X. Characterization of complete genome and small RNA profile of pagoda yellow mosaic associated virus, a novel badnavirus in China. *Virus Res* **188**, 103-108 (2014).
70. Zheng, L., Rodoni, B.C., Gibbs, M.J. & Gibbs, A.J. A novel pair of universal primers for the detection of potyviruses. *Plant Pathology* **59**, 211-220 (2010).
71. Fauquet, C.M., Mayo, M., Maniloff, J., Desselberger, U. & Ball, L.A. Virus taxonomy: VIIIth report of the International Committee on Taxonomy of Viruses. (Academic Press, 2005).
72. Gibbs, A. & Mackenzie, A. A primer pair for amplifying part of the genome of all potyvirids by RT-PCR. *J Virol Methods* **63**, 9-16 (1997).
73. Pappu, S.S. et al. A polymerase chain reaction method adapted for selective amplification and cloning of 3' sequences of potyviral genomes: application to dasheen mosaic virus. *Journal of Virological Methods* **41**, 9-20 (1993).
74. Watanabe, S. et al. A distinct lineage ofWatermelon mosaic virusnaturally infects honohono orchid (Dendrobium anosmum) and passionfruit (Passiflora edulis) in Hawaii. *New Disease Reports* **34**, 13 (2016).
75. Hamim, I. et al. First Report of Banana bunchy top virus in Heliconia spp. on Hawaii. *Plant Disease* **101**, 2153 (2017).
76. Gonsalves, D. Control of papaya ringspotvirus in papaya: A case study. *Annu. Rev. Phytopathol.* **36**, 415-437 (1998).

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