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Genome organization and phylogenetic relationship of *Pineapple mealybug wilt associated virus-3* with family Closteroviridae members

Diane M. Sether · Michael J. Melzer · Wayne B. Borth · John S. Hu

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Abstract The nucleotide sequence of *Pineapple mealybug wilt associated virus-3* (PMWaV-3) (Closteroviridae: *Ampelovirus*), spanning seven open reading frames (ORFs) and the untranslatable region of the 3′ end was determined. Based on the amino acid identities with orthologous ORFs of PMWaV-1 (54%–73%) and PMWaV-2 (13%–35%), we propose PMWaV-3 is a new species in the PMWaV complex. PMWaV-3 lacks an intergenic region between ORF1b and ORF2, encodes a relatively small, 28.8 kDa, coat protein, and lacks a coat protein duplicate. Phylogenetic analyses were used to analyze seven different domains and ORFs from members of the family Closteroviridae. Two distinct clades within the recognized genus *Ampelovirus* were observed; one that includes PMWaV-3 and PMWaV-1 and several GLRaVs and another that includes PMWaV-2 and GLRaV-3, the type member of the genus *Ampelovirus*.

Keywords Virus genome · Pineapple · Mealybug wilt · *Ananas comosus* · Virus transmission

Introduction

A complex of pineapple mealybug wilt associated viruses (PMWaVs), have been identified in the *Ananas* (pineapple) and *Pseudoananus* genera throughout the pineapple growing regions of the world [1–8]. PMWaV-1, PMWaV-2, and PMWaV-3 are mealybug transmitted viruses [7, 9] in the genus *Ampelovirus* of the family Closteroviridae. The presence of PMWaV-2 in conjunction with the feeding of mealybugs has been shown to result in mealybug wilt of pineapple (MWP), a devastating disease of pineapple worldwide [10]. Pink and grey pineapple mealybugs, *Dysmicoccus brevipes* (Cockerell) and *D. neobrevipes* Beardsley, respectively, can transmit PMWaV-2 and induce MWP [10]. These mealybug species can also transmit PMWaV-1 and PMWaV-3, but these viruses in conjunction with mealybug feeding do not result in MWP [7, 10]. Also, mealybug feeding on PMWaV-free plants does not induce MWP [10, 11].

The family Closteroviridae is currently composed of three recognized genera, *Ampelovirus*, *Closterovirus*, and *Crinivirus*. Virus species within these genera are characteristically mealybug, aphid, or whitefly transmitted, respectively, although vector species have not been determined for some of the virus species in each group. The partial genome sequence of PMWaV-2 and, more recently, the complete genome sequence of PMWaV-1 have been determined [12, 13]. Sequence comparisons of the RNA-dependent RNA polymerase (RdRp), heat shock protein 70 homolog (Hsp70h), and coat protein (CP) regions suggested that PMWaV-1, *Plum bark necrotic stem-pitting-associated virus* (PBNSPaV), *Grapevine leafroll-associated virus-4* (GLRaV-4), GLRaV-5, GLRaV-6, and GLRaV-9 form a distinct branch from PMWaV-2, GLRaV-1, and GLRaV-3, the type member of the *Ampelovirus* genus. In this article, we characterize the genome organization and protein features of PMWaV-3 and compare these to orthologs from other PMWaVs and members of the family Closteroviridae. Phylogenetic analyses conducted on seven domains and open reading frames (ORFs) are used to further elucidate

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D. M. Sether · M. J. Melzer · W. B. Borth · J. S. Hu (✉)
Plant and Environmental Protection Sciences, University of Hawaii at Manoa, 3190 Maile Way, Honolulu, HI 96822, USA
e-mail: johnhu@hawaii.edu

two distinct clades that clearly separate PMWaV-2 from the other characterized PMWaVs in the genus *Ampelovirus*.

Materials and methods

Cloning and sequencing

PMWaV-3-infected pineapple plants were identified with reverse transcription (RT)-PCR and specific oligonucleotide primers 264 and 263 as previously described [7]. Double-stranded RNA (dsRNA) was purified from the leaf bases of these infected plants and used as a template for cDNA synthesis as previously described [3, 12]. Pineapple leaf bases were powdered in liquid nitrogen and RNA was purified from 100 mg of tissue with RNeasy™ Plant Mini Kits (Qiagen, Valencia, CA) following manufacturer's instructions and stored at −80°C until needed. From this initial clone, a step-by-step walking procedure [12, 13] was used to obtain additional sequence information from the 5′ and 3′ regions of the genome with overlapping clones (Fig. 1a). These RT-PCR amplicons were gel purified and ligated into the plasmid pGEM-T Easy (Promega, Madison, WI). Plasmids were sequenced in both directions by automated sequencing at the Center for Advanced Studies of Genomics, Proteomics, and Bioinformatics at University of Hawaii at Manoa. Nucleotide sequences were compared with the nonredundant amino acid sequence database at the National Center for Biotechnology Information (NCBI) website using the BLASTX algorithm [14, 15].

Sequence comparisons and phylogenetic analyses

The amino acid (aa) sequences from PMWaVs and other family Closteroviridae members were retrieved from the NCBI for the following domains and ORFs: ORF 1a, the methyltransferase (MTR) domain belonging to pfam01660 and the helicase (HEL) domain; ORF 1b, the RNA- dependent RNA polymerase (RdRp); ORF 2, a small hydrophobic protein (p5); ORF 3, the heat shock 70 protein homolog (Hsp70h) protein; ORF 4, a 61 kDa protein (p61); and ORF5, the coat protein (CP) and the conserved clostero_coat of pfam01785 present in the CP open reading frame. Ampelovirus sequences used for phylogenetic analyses and GAP alignment in SeqWeb were as follows: *Grapevine leafroll-associated virus-1* (GLRaV-1) AF195822, GLRaV-3 NP_813795, GLRaV-4 AM162279, GLRaV-5 AF233934, GLRaV-6 AM745345, GLRaV-9 AY297819/AY072797, GLRaV-10 AM182328,GLRaV-11 AM494935, *Little cherry virus-2* (LChV-2) AF416335, *Plum bark necrosis stem pitting-associated virus* (PBNSPaV) EF546442, PMWaV-1 NC_010178, PMWaV-2 AF283103, PMWaV-3 DQ399259, PMWaV-4 EU372003, PMWaV-5 EF467920, and *Tulip severe mosaic virus* (TSMV) EF203673. Closterovirus sequences used for phylogenetic analyses and alignment with the GAP function of SeqWeb (GCG Inc.) were as follows: *Beet yellow stunt virus* (BYSV) U51931, *Beet yellows virus* (BYV) X73476, *Citrus tristeza virus* (CTV) isolate T36 AY170468, *Fig leaf mottle-associated virus* (FLMaV) AM279677, FLMaV-2 AM286422, FLMaV-3 EF654103, GLRaV-2 EF012721, *Mint virus-1* (MV-1) AY792620, *Olive leaf yellowing-associated virus* (OLYaV) AJ440010, *Raspberry mottle virus* (RMoV) DQ357218, and *Strawberry chlorotic fleck-associated virus* (SCFaV) DQ860839. Crinivirus sequences used for phylogenetic analyses and GAP alignment were as follows: *Abutilon yellows virus* (AYV) AY422070, *Bean yellow disorder virus* (BYDV) NC_010560/NC_010561, *Beet Pseudo-yellows virus* (BPYV) AY330919/AY330919, *Blackberry yellow vein associated virus* (BYVaV) AY77335, *Cucurbit yellow stunting disorder virus* (CYSDV) AJ243000, *Lettuce infectious yellows virus* (LIYV) NP_733943, *Potato yellow vein virus* (PYVV) AJ557129, *Strawberry pallidosis associated virus* (SPaV) AY262159, *Sweet potato chlorotic stunt virus* (SPCSV) AJ428555, and *Tomato chlorosis virus* (TCV) AY903448. Sequences of unassigned members in the family Closteroviridae used for phylogenetic analyses and GAP alignment were as follows: LChV-1 NP_045006 and *Mint vein banding virus* (MVBV) AY548173.

Sequences were aligned with Clustal X v1.8 [16] using pairwise alignment parameters with a gap opening penalty of 35, a gap extension penalty of 0.75, and the Gonet protein weight matrix. Multiple alignment parameters had a gap opening penalty of 15 and gap extension penalty of 0.3. Divergent sequence delay was set at 25%. Unrooted phylogenetic trees based on 1000 replications were created with PAUP* v4.0b [17] using neighbor-joining analysis with bootstrapping and parsimony analysis with jackknifing.

Results

Genome organization of PMWaV-3

The sequence composed of 11891 nucleotides of PMWaV-3, was deposited in GenBank (accession DQ399259). The ORF nomenclature used here follows the convention used for GLRaV-3, the type member of the genus *Ampelovirus* [18]. The PMWaV-3 sequence spans seven ORFs including the untranslatable region at the 3′ end of the virus (Fig. 1b). The partial sequence of ORF1a contains a conserved viral methyltransferase (MTR) domain typical of pfam 01660 and viral helicase (HEL) domain typical of pfam 01443

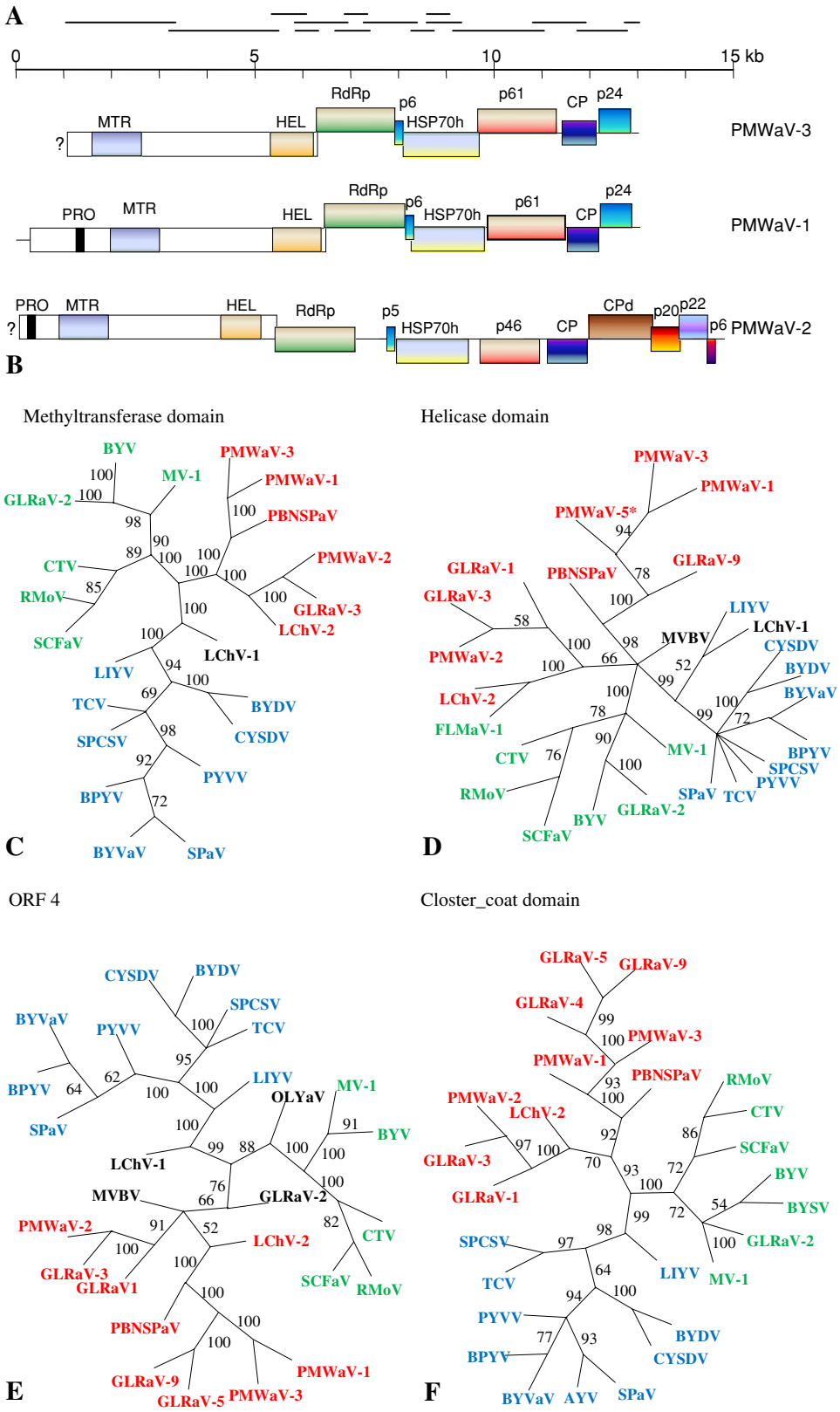

Fig. 1 Genome organization and phylogenetic analysis of *Pineapple mealybug wilt associated virus*-3. **a** Approximate size and location of clones generated to sequence *Pineapple mealybug wilt associated virus*-3 (PMWaV-3), **b** Genomic organization and approximate size of PMWaV-3, PMWaV-1, and PMWaV-2. Shared color indicates similar protein products. ORF1a: MTR, viral methyltransferase region; HEL, viral helicase region; ORF1b: RdRp, RNA dependent RNA polymerase; ORF2, small hydrophobic protein; ORF3 Hsp70h, heat shock protein 70 homolog; ORF4: p61 or p46 proteins; ORF5: CP, coat protein; ORF6, p24 or CPd, coat protein duplicate; ORF7, p20; ORF8, p22;ORF9, p6; Phylogenetic analyses of the **c** methyltransferase, **d** helicase, **e** ORF4, and **f** Closter_coat region of nonredundant sequences from members of the family Closteroviridae. * denotes partial sequence. Virus names and accession numbers are listed in the Materials and Methods section. Values along branches of neighbor joined trees are bootstrap values based on 1000 replications. Colors represent recognized ampeloviruses, closteroviruses, and criniviruses. Unassigned members in the family Closteroviridae are in black

[19]. The MTR region of PMWaV-3 shares ~58% identity with PMWaV-1 but less than 44% with other ampeloviruses, including PMWaV-2 (Table 1). The HEL domain shares ~96% with a partial sequence (130 nt) of an Australian isolate identified as PMWaV-3 (Table 1). The sequence GUUUAACG codes for the stop codon

Table 1 Percentage amino acid (aa) identity of *Pineapple mealybug wilt associated virus*-3 (PMWaV-3) and other representative PMWaV and genus *Ampelovirus* accessions for various domains or gene products

Coding region[a] (% aa identity/similarity)										
Virus	Origin	Accession	MTR	HEL	RdRp	ORF2	Hsp70h	ORF4	CP	ORF6
PMWaV-1	USA	AF414119	58.4	62.9	71.2	72.5	72.1	63.3	62.9	53.6
PMWaV-1	Australia	EF467923		70.2[y]	70.2[y]	74.5	80.3[b]			
PMWaV-1	Thailand	EF620774					77.0[b]			
PMWaV-2	USA	AF283103	29.3	32.9	32.7	12.8	35.0	21.2	24.7	
PMWaV-2	Thailand	EU016675					41.0[b]			
PMWaV-2	Australia	EF488757		34.7[b]	28.9[b]					
PMWaV-2	Cuba	DQ225114							24.7	
PMWaV-3	Australia	EF488750		95.5[b]						
PMWaV-3	Australia	EF467918			99.1[b]	100	98.b			
PMWaV-3	Australia	EF467919			93.8[b]	100	92.8[b]			
PMWaV-4	USA	EU372003					72.0	65.8[b]		
PMWaV-5	Australia	EF467922		68.6[b]	67.3[b]					
PMWaV-5	Australia	EF467920			65.3[b]	56.9	63.6[b]			
GLRaV-1		AF195822		29.3	33.3	15.7	35.0	21.4	20.5	
GLRaV-3		NP_813795	30.7	33.1	35.6	25.6	36.7	19.0	28.2	
GLRaV-4		AM162279					65.0[b]		65.3	
GLRaV-5		AF233934					58.1[b]	44.4	64.5	37.3
GLRaV-6		AM745345					64.4[b]			
GLRaV-9		AY297819/AY072797	43.9[b]	51.5	59.8[b]	26.2	59.3	47.3	63.7	37.4
GLRaV-10		AM182328					63.1[b]	46.6	62.6	36.2
GLRaV-11		AM494935					63.1[b]	48.0	67.2	43.4[b]
LChV-2		AF416335	29.7	31.5	35.8	16.3	34.3	23.0	22.8	
MVBV		AY548173	26.4	37.7	35.0	28.6	30.3	20.0	18.1	
PBNSPaV		EF546442	34.9	37.2	39.5	28.9	44.6	31.3	36.0	23.8
TSMV		EF203673					48.5[b]			

[a] *MTR*, viral methyltransferase (pfam 01660); *HEL*, viral helicase (pfam 01443); *RdRp*, RNA dependent RNA polymerase; *ORF2*, small hydrophobic protein; *Hsp70h*, heat shock protein 70 homolog; *ORF4*, includes in some viruses a motif identified as Viral_HSP90 pfam 03225) in GenBank; *CP*, coat protein; *ORF6*, open reading frame 6. Comparisons were made with GAP alignment tool in SeqWeb using a blosum62 matrix

[b] Based on partial sequence

(underlined) of ORF1a and is similar to the sequence presumed to be involved with the +1 ribosomal frameshift in PMWaV-1 [12] and BYV [20] but differing from that of GLRaV-3, the genus *Ampelovirus* type member, and PMWaV-2 [13]. The asparagine coded by AAC (italicized above) has been proposed to initiate translation of the overlapping ORF1b that codes for a 525 aa, 65.5-kDa RdRp that contains the RdRp_2 domain, Pfam00978 [19]. This domain shares 93.3%–99% aa identity with partial sequence accessions of PMWaV-3 from Australia. Amino acid identities with other ampeloviruses for the MTR, HEL, and RdRp regions were below 74% and lowest with PMWaV-2 (Table 1). PMWaV-3, like PMWaV-1, but unlike PMWaV-2, lacks an intergenic region between ORF1b and ORF2 (Fig. 1b). PBNSPaV is the only other ampelovirus characterized to date, that shares this feature with PMWaV-1 and PMWaV-3 [21].

ORF2 encodes a 5.7-kDa hydrophobic protein sharing 100% identity with two partial sequence accessions from Australia identified as PMWaV-3. Although PMWaV-2 has a similarly located 5.3-kDa hydrophobic protein, the identity is less than 13% (Table 1). The ORF2 of PMWaV-3 and PMWaV-1 both overlap ORF 1b by 17 nucleotides and are both comprised of 51 residues, whereas the closely related PMWaV-5 [2] is composed of 67 residues. ORF 2 of PMWaV-3 and PMWaV-1 lack the 16 residues at the 5′-end of ORF2 that are present in PMWaV-5.

ORF3 is composed of 533 aa and encodes a 57.6-kDa Hsp70h protein that contains the HSP70 pfam00012 motifs. ORF3 of PMWaV-3 overlaps ORF2 by 17 nucleotides whereas PMWaV-2 has an intergenic region between ORF2 and ORF3 (Fig. 1b). PMWaV-3 shares >64% aa identity with the Hsp70h of PMWaV-1, putative PMWaV-4, and PMWaV-5, whereas the aa identity with

PMWaV-2 is less than 41% (Table 1). PMWaV-3 shares nearly 99% homology in ORF3 with a 182 aa partial sequence (accession EF488755) of a PMWaV-3 isolate from Australia but only 92.8% identity with a 291 aa partial sequence also identified as PMWaV-3 (accession EF467919) from Australia. In contrast, the six GenBank accessions available for PMWaV-1 from the USA, Australia, and Thailand share greater than 95.2% identities for the Hsp70h within the PMWaV-1 species.

ORF4 overlaps ORF 3 by 20 nucleotides in PMWaV-3 and encodes a 61.0 kDa protein, p61 that is conserved in the family Closteroviridae [22, 23]. The p61 protein of PMWaV-3 shares >60% aa identity with its ortholog in PMWaV-1 and only 21% aa identity with PMWaV-2 (Table 1). The aa identity is >48% with other ampeloviruses (Table 1). The conserved domain searching tool available through GenBank identifies a motif presently designated as pfam03225, Viral_HSP90 [19, 24] in ORF4 of PMWaV-3 and PMWaV-1. Presently, this motif can be identified in at least 84 other family Closteroviridae accessions representing 30 distinct virus species in GenBank but is not detected in PMWaV-2 or GLRaV-1. Similarity of p61 orthologs to the cellular chaperone, HSP90, has been reported for other family Closteroviridae members including BYV, CTV, and GLRaV-2 [25–27] or through designation of the p61 orthologous proteins as HSP90 in GenBank accessions [28–30]. The significance of the similarity between p61 and established HSP90 cellular chaperones has been questioned by other researchers [21] and thus the designation HSP90 may be superfluous.

ORF5 is separated from ORF4 by 103 nucleotides. ORF5 encodes a 28.8-kDa putative coat protein containing the Clostero_coat protein motif in pfam01785 [19]. PMWaV-3 shares greater than 60% aa identity with PMWaV-1, GLRaV-4, GLRaV-5, and GLRaV-9 but less than 25% aa identity with PMWaV-2 and GLRaV-3, the type member of the ampelovirus genus (Table 1). The CP region contains the amino acid residues, S^{115}, R^{167}, and D^{208} that are conserved in all rod shaped and filamentous RNA plant viruses [31]. The molecular mass of the coat proteins of PMWaV-3, PMWaV-1, GLRaV-9, GLRaV-5, range from 28-30 kDa, the smallest of the ampeloviruses characterized to date.

ORF 6 encodes a 24.1-kDa protein (p24) of unknown function. This protein shares 54% aa identity with a similarly located protein in PMWaV-1 (Table 1). The protein coded by ORF6 of PMWaV-3 shares only 33% aa identity with the CP of PMWaV-3. PMWaV-3, like PMWaV-1, does not encode the Clostero_coat protein motif in ORF6. The orthologous protein to p24 in PBNSPaV has been referred to as a minor coat protein and as a coat protein duplicate in GLRaV-5 and GLRaV-9 although the Clostero_coat conserved regions are absent. In contrast, a similarly located protein in PMWaV-2 identified as a diverged coat protein (Cpd) [13], contains the the Clostero_coat protein motif. PMWaV-3 shares complete consensus of two sets of arginine and aspartate residues that we have previously reported to occur in PMWaV-1 [12]. Like PMWaV-1, PMWaV-3 lacks the conserved serine previously reported [21, 31] in other family Closteroviridae members (data not shown). ORF6 is followed by 132 nt untranslatable region, completing the 3′ terminus.

Phylogenetic analyses

Phylogenetic analyses with neighbor-joining (NJ) (Fig. 1c–f) and parsimony (data not shown) analyses were conducted using nonredundant sequences available for members of the family Closteroviridae in GenBank. Unrooted trees were developed for seven distinct domains or ORFs as follows: viral methyltransferase, helicase, RdRp, Hsp70h, ORF4, CP, and Clostero_coat region in the CP. Bootstrap values for NJ trees and jack-knife values for parsimony-derived trees based on 1000 replications showed the presence of two distinct clades within the *Ampelovirus* genus for each of the seven regions evaluated. Bootstrap and jack-knife values, which estimate the reliability of the groupings, strongly support the sequence divergence of PMWaV-3, PMWaV-1, and PBNSPaV from that of PMWaV-2, GLRaV-1, and GLRaV-3, the type member of the genus *Ampelovirus*. In several regions, additional ampelovirus sequences were available. NJ and parsimony trees clearly grouped GLRaV-4, GLRaV-5, GLRaV-6, GLRaV-9, GLRaV-10, GLRaV-11, PMWaV-1, and PMWaV-4, PMWaV-5 in the clade with PMWaV-3 (Fig. 1c–f). NJ analysis of complete coat proteins resulted in a polytomy composed of LChV-2, MVBV, and the two distinct branches that formed the PMWaV-3 clade and the GLRaV-3 clade within the ampeloviruses (data not shown). Bootstrap and jack-knife values for neighbor joined and parsimony trees strongly support the divergence of these two clades. Analyses with the clostero_coat region resolved the polytomies at the most basal level, resulting in two distinct branches within the ampeloviruses that were supported with statistical estimates >70% (Fig. 1f). MVBV and LChV-1 could not be included in the clostero_coat motif analyses because of the absence of the conserved pfam01785 region in their designated coat proteins. Interestingly, two clades in the genus *Closterovirus* were also supported with high bootstrap and jack-knife values, placing CTV, RMoV, and SCFaV together, but distinct from the other closteroviruses that include BYV, the type member of the genus (Fig. 1c–f).

Discussion

The aa sequence comparisons of PMWaV-3 with other characterized ampeloviruses show the virus is a distinct

species. One criterion for species demarcation criteria of ampeloviruses is amino acid sequences of relevant gene products (CP, CPm, Hsp70h) differ by more than 10% from that of other species [32]. The Hsp70h is generally the most conserved ORF among the ampeloviruses. Homologies of the complete Hsp70h genes of PMWaV-1, PMWaV-2, and PMWaV-4, and a partial sequence of PMWaV-5, with PMWaV-3 were all less than 72%, supporting the designation of PMWaV-3 as a new species.

The lack of intergenic regions between the RdRp ORF and the small hydrophobic protein encoded by ORF2, the presence of a conserved motif in ORF4, the relatively small size of the coat proteins <29 kDa, and the absence of a diverged coat protein ORF, clearly show that the genome of PMWaV-3 and PMWaV-1 are distinct from those of PMWaV-2 and the ampelovirus type member GLRaV-3. The p24 protein of PMWaV-3 and its ortholog in PMWaV-1 both lack a Clostero_coat region and neither have high homologies to their respective coat proteins. Other family Closteroviridae members, including PMWaV-2, encode proteins identified as CP duplicates or diverged CPs. These proteins retain specific S, R, and D residues in their CPs and in their minor, diverged, or duplicate CPs [21, 31]. However, PMWaV-1 and PMWaV-3 lack the conserved serine residues in the p24 protein. The lack of these residues in the p24 of PMWaV-1 and PMWaV-3 is a noticeable break from conservation and further suggests that p24 and the CP are not paralogs.

Phylogenetic analyses with a distance method (NJ) that uses an algorithm based on minimum-change, and parsimony analyses, a character-based tree searching method, consistently predicted two distinct clades in the genus *Ampelovirus.* These distinct groupings were detected across all seven conserved motifs and ORFs evaluated. Although these unrooted trees are not intended to predict directions of evolution, they do show phylogenetic relationships [33]. Both of these methods gave predictions similar to those of phylogenetic inference (a minimum evolution method), tree puzzle, and Baysian analyses, previously reported for the HEL, RdRp, Hsp70h, and CP regions of other available ampelovirus sequences [2, 12, 21]. In all of these analyses, two clades were generated in the genus *Ampelovirus*. This consistent identification of two clades within the current genus *Ampelovirus* may have broader evolutionary implications involving replication, movement, or vector relationships that have not yet been elucidated. This strong phylogenetic support for two clades combined with the significant differences in genome organization between GLRaV-3, the type member of the genus *Ampelovirus*, and several other ampeloviruses including PMWaV-1 and PMWaV-3 suggests the addition of another genus within the family Closteroviridae may be appropriate.

The division of the current genus *Ampelovirus* into two genera may provide a framework that leads to identifying further shared or diverged biological or physiological characteristics. This in turn may provide a better understanding of the relationships of these viruses, their host plants, vectors, and disease. MWP is not induced by PMWaV-3, PMWaV-1, or a combination of the two viruses when accompanied by pineapple mealybug feeding in Hawaii [7]. PMWaV-1 infection is strongly correlated with yield reduction that is manifested as a reduction in ratoon number [34, 35]. Similar yield studies have not been conducted on pineapple with PMWaV-3 infections alone. Greenhouse and field studies have shown that PMWaV-2 infection vectored by grey pineapple mealybugs does result in MWP in Hawaii [10, 11]. This difference in disease etiology between the PMWaV-2 and PMWaV-3 groups further suggests a strong biological distinction between the two clades of PMWaVs.

Additional PMWaV-3 variants that shared higher aa identity in the Hsp70h regions to the Australian accessions [2] were searched for in several different PMWaV-3-infected hybrids grown in Hawaii. Only one variant having a single amino acid substitution in position 174 was identified and no isolates identical to the partially sequenced PMWaV-3 from Australia were identified in Hawaii. Although only small numbers of PMWaV-3 infected plants from the hybrids in Hawaii were evaluated for variants, there is no evidence that the PMWaV-3 present in Australia is present on the islands of Oahu or Maui, in Hawaii.

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