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BRIEF REPORT

Genomic variation in pepper vein yellows viruses in Australia, including a new putative variant, PeVYV-10

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Abstract

Since the first identification and full sequence of the polerovirus pepper vein yellows virus in Australia in 2016, virus surveys of crops and weeds have sporadically identified PeVYV in different hosts and locations. Genomic comparisons of 14 PeVYV-like isolates using RT-PCR products spanning the 3' end of the RdRp region (ORF 2), the intergenic region, ORF 3a, ORF 4, and ORF 3 (1388 nt) showed that four of the PeVYV isolates might be a new variant or PeVYV-like virus. From six PeVYV-positive plants, eight PeVYV-like sequences were obtained by high-throughput sequencing, as two hosts, 5352 and 5634, contained two slightly different PeVYV-like isolates. Three of the PeVYV-like isolates were most closely related to PeVYV-6 and PeVYV-5, and two isolates were closely related to PeVYV-9 and PeVYV-2. The other three isolates shared only 69-74% nucleotide sequence identity across the whole genome with any of the other PeVYVs, despite sharing 73-98%, 87-91%, and 84-87% amino acid sequence identity in ORF 3a, ORF 3, and the RdRp (ORF 2), respectively, suggesting that this virus is a new PeVYV-like virus, which we have tentatively called PeVYV-10. This is also the first report of a PeVYV-like virus infecting garlic.

Pepper vein yellows virus (PeVYV), a polerovirus, was described and fully sequenced in Australia in 2016 [1] and subsequently called PeVYV-4 [2]. Poleroviruses (family *Solemoviridae*, formerly *Luteoviridae*) infect a wide range of plant hosts, are phloem-limited, and are transmitted predominantly by aphids (but a rare few are transmitted by whiteflies) in a persistent, circulative, non-propagative manner [3, 4]. The monopartite linear single-stranded RNA genome, encapsidated in an icosahedral shell [5, 6], is organised into seven open reading frames (ORFs). The 5' ORFs 0, 1, and 2 (RNA-dependent RNA polymerase; RdRp) are expressed via translation of genomic RNA and encode the proteins P0, a suppressor of gene silencing that influences host range and symptoms, and the P1 and P1-P2 fusion proteins, which are required for viral replication [7–9]. The intergenic region (IR) separates the 5' and 3' portions of the genome, with the

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latter region containing ORFs 3, 3a, 4, and 5, which encode P3 (coat protein), P3a, and P4 movement proteins and the P3-P5 readthrough domain (RTD) fusion protein, which is required for virus accumulation, circulation, and persistence within a vector [5, 10–13].

According to the International Committee on Taxonomy of Viruses (ICTV), the current species demarcation criteria for poleroviruses are 10% amino acid sequence divergence in any gene product and differences in host range and serological specificity. However, with the increased use of high-throughput sequencing and whole-genome analysis, greater genetic diversity within polerovirus species has been revealed [2, 14–17]. The regions of the polerovirus genome that are the most variable are ORF 0 and 5, and the most conserved regions are the RdRp (P2) and P3 coat protein [2, 14, 16]. This has been demonstrated for turnip yellows virus (TuYV), beet western yellows virus (BWYV) [14], and, to some extent, pepper vein yellows virus (PeVYV) [2]. Here, we present the diversity of PeVYV-like viruses in Australia as well as a new PeVYV-like variant.

During virus surveys of pulse and horticulture crops in Queensland, South Australia, and the Northern Territory in Australia between 2014 and 2020, random plant samples of tomato (3 samples), chickpea (1), garlic (1) [18], capsicum (3), and chilli (7) were collected (Table 1; Fig. 1). Total nucleic acid was isolated using a BioSprint 15 workstation

Table 1 Details of PeVYV strains, hosts, and collection locations used in this study

Isolate number	Host	Symptoms	Location	Collection year	PeVYV closest match; % nt ID	Accession no.
Ita7[Δ]	*Allium sativum*	Stem lesions, chlorotic leaves[Δ]	Gatton, QLD	2020	PeVYV-6 LT559483; 72%	OR225496
5396[♦]	*Capsicum annuum*	Chlorosis, vein banding	Darwin, NT	2017	PeVYV-CA MK931185; 96%	-
5628[♦]	*C. annuum var annum*	Leaf distortion, vein clearing*	Cairns, QLD	2020	PeVYV-CA MK931185; 96%	-
5629[♦]	*C. annuum var annum*	Leaf distortion*	Cairns, QLD	2020	PeVYV-CA MK931185; 96%	-
5630[♦]	*C. annuum var annum*	Leaf chlorosis	Erub, Torres Strait, QLD	2019	PeVYV-3 KP326573; 96%	-
5631	*C. annuum var annum*	Sever leaf chlorosis	Ugar, Torres Strait, QLD	2019	PeVYV-1 NC_015050; 89 %	OR225498
5632[♦]	*C. annuum var annum*	Yellowing, small leaves	Waiben, Torres Strait, QLD	2019	PeVYV-8 MK184554; 96%	-
5633[♦]	*C. annuum var annum*	Yellowing	Bungalow, QLD	2020	PeVYV-4 KU999109; 98%	-
35744[♦]	*C. chinense* × *C. frutescens*	N/R	Darwin, NT	2020	PeVYV-CA MK931185; 95%	-
5352-1[∇]	*C. annuum*	Mottling, curling leaves	Virginia, SA	2016	PeVYV-6 LT559483; 90%	OR225499
5352-2	*C. annuum*	Mottling, curling leaves	Virginia, SA	2016	PeVYV-2 HM439608; 89 %	OR225500
5357	*Cicer arietinum*	Reddening	Dalby, QLD	2016	PeVYV-6 LT559483; 72%	OR225495
J4702-2-1	*Solanum lycopersicum*	Yellowing of leaf margins	Bowen, QLD	2017	PeVYV-6 LT559483; 74%	OR225497
5634-1	*S. lycopersicum*	Stunting, yellowing leaf margins	Erub, Torres Strait, QLD	2020	PeVYV-6 LT559483; 89%	OR225501
5634-2	*S. lycopersicum*	Stunting, yellowing leaf margins	Erub, Torres Strait, QLD	2020	PeVYV-2 HM439608; 89%	OR225502
Ac-05[♦]	*S. lycopersicum*	Leaf yellowing, mottling	Townsville, QLD	2014	PeVYV-G MW161057; 90%	-

Queensland, QLD; South Australia, SA; Northern Territory, NT; not recorded, N/R. Isolates in bold were subjected to high-throughput sequencing

[Δ]This isolate is a pooled sample of 30 individual garlic plants with uniform symptoms as described and were co-infected with potyviruses, carlaviruses, and allexiviruses [18].

*Mixed infection. Isolate 5628 and 5629 also contained CMV.

[♦]Partial genomic fragment only containing partial ORF2, ORF3a, 3, and 4

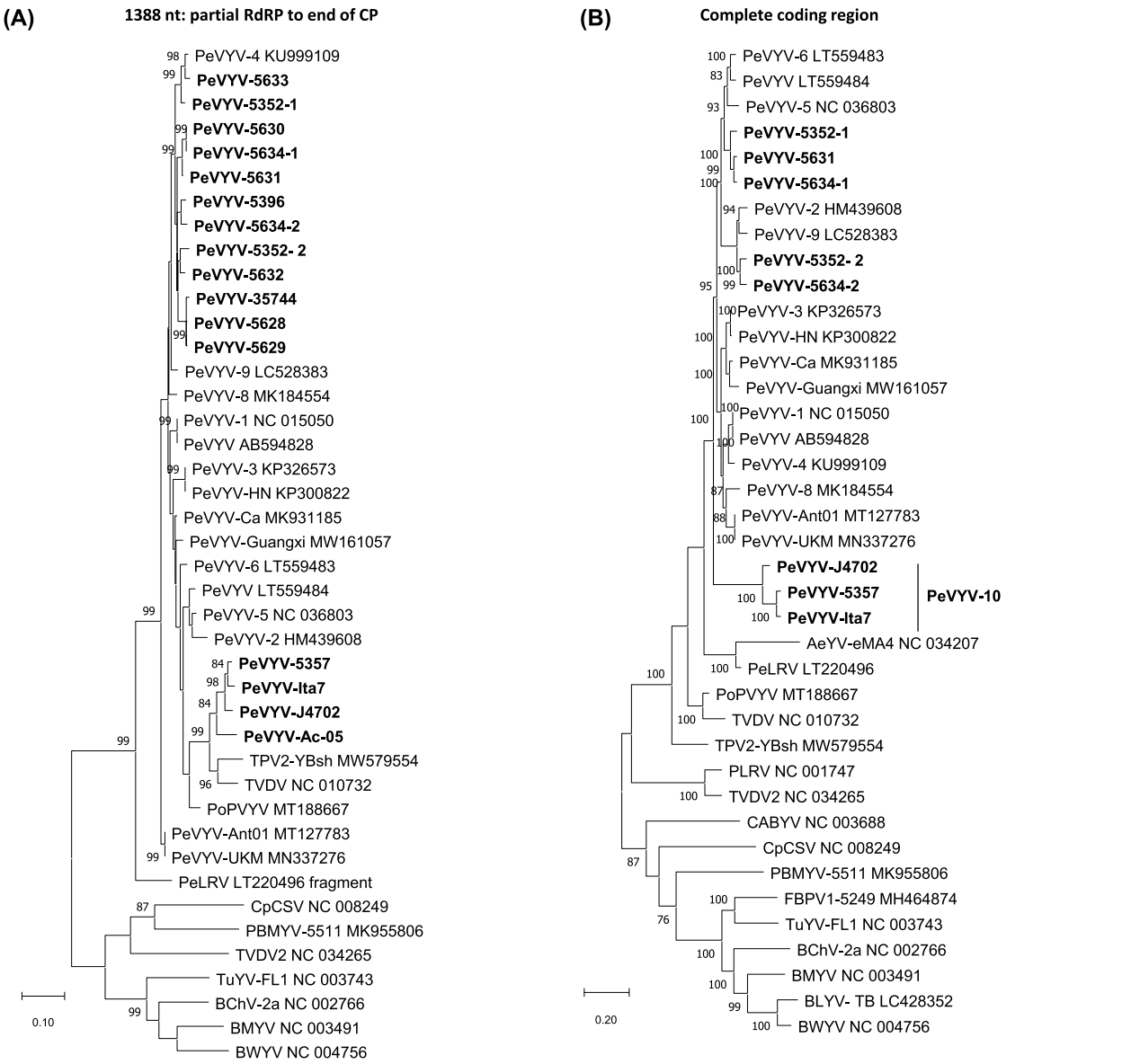
[∇]Near-complete coding sequence, missing 626 nt at the 5' end of ORF0

Fig. 1. Photographs of tomato (Ac-05, J4702, 5634), chilli, (5631, 5633), and capsicum (5352) infected with PeVYV. Isolates Ac-05 and J4702 were infected with PeVYV-10; 5634 and 5352 were infected with two different PeVYV isolates that were most closely related to PeVYV-6 and PeVYV-2 or -9; 5631 was infected with an isolate closest to PeVYV-1 and the partial genome fragment obtained from 5633 closely resembled PeVYV-4

with a BioSprint 15 Plant DNA Kit (QIAGEN, catalogue no. 941514) as per the manufacturer's instructions but without the use of RNase A, or a modified CTAB method as described by Sharman et al. [19]. Initial reverse transcription (RT) PCRs were done using polerovirus degenerate primers pol3870F [19] and AS3 [20], which amplify a conserved region of the CP, as described by Sharman et al. [19]. cDNA synthesis was done using primer AS3 with SuperScript IV reverse transcriptase (Thermo Fisher, USA) as per the manufacturer's instructions. PCR was carried out using Mango Taq DNA polymerase (Bioline, USA) using the following cycling parameters: 95 °C for 1 min, 35 cycles of 95 °C for 15 s, 62 °C for 20 s, 56 °C for 10 s, and 72 °C for 40 s, followed by 72 °C for 3 min. The approximately 350-bp PCR products were sequenced directly (Macrogen Inc. Seoul, Korea) and analysed using Geneious 10.02 (Biomatters). The CP fragment of all isolates shared 89-98% nucleotide (nt) sequence identity with other pepper vein yellows viruses. To confirm this result, a larger genome fragment containing approximately 568 nt of the RdRP (ORF2) 3' end, the intergenic region, P3a, ORF4, and ORF3 was obtained using the above primers together with PeVYV-2678F (CAR AAY CAG AAC AAG CGA GAA ATC GC), PeVYV-3813R (TTC KGT TCC TGC CTC CAN TTC GTC), Pol3167F [19], PolG-F, and PolG-R [21] as described above.

The phylogenetic relationships of the approximately 1388-bp RT-PCR fragment obtained for each of the isolates (Table 1), as well as the same region from other PeVYVs and poleroviruses, were determined by aligning nucleotide sequences (Geneious; ClustalW) and creating a maximum-likelihood (ML) phylogenetic tree in MEGA [22] using the Tamura-Nei model [23] with 500 bootstrap replicates. The phylogenetic tree for the 1388-nt fragment illustrated that 12 out of the 16 isolates were very similar to PeVYVs and shared 91-98% nucleotide sequence identity in this region (Fig. 2A). The other four isolates, Ac-05, J4702, 5357, and Ita7, formed a separate clade but still shared high similarity (88-91% nt sequence identity) with various PeVYVs, with the closest being PeVYV-9 (89-91%), PeVYV-6 (89-90%), and PeVYV-Ca (89-90%). The four isolates also shared high similarity in this region with TPV2-YBsh (89-90% identity) and TVDV (88-91% identity).

To characterise some of the isolates further, six of the PeVYV-like positive samples (Table 1) were selected for high-throughput sequencing (HTS) in separate runs. Total RNA was extracted using TRIzol Reagent (Invitrogen-Thermo Fisher Scientific, Waltham, USA) as described by



(C)

	PeVYV-5	PeVYV-6	5352-1	5634-1	5631	PeVYV-1	PeVYV-4	PeVYV-8	PeVYV-3	PeVYV-2	PeVYV-9	5352-_2	5634-2	5357	Ita7	J4702
PeVYV-5_NC_036803																
PeVYV-6_LT559483	91.31															
PeVYV-5352-1	89.95	90.02														
PeVYV-5634-1	89.32	89.50	94.59													
PeVYV-5631	88.82	88.97	93.12	95.74												
PeVYV-1_NC_015050	87.20	86.98	86.74	87.89	89.60											
PeVYV-4_KU999109	86.26	85.90	87.77	87.86	89.28	95.42										
PeVYV-8_MK184554	84.23	85.08	83.42	84.41	85.61	89.70	89.27									
PeVYV-3_KP326573	86.62	88.50	85.83	86.45	87.90	92.23	90.94	89.56								
PeVYV-2_HM439608	84.66	85.07	81.58	82.82	82.14	82.53	81.75	81.12	82.72							
PeVYV-9_LC528383	83.54	84.69	81.78	82.73	82.07	82.33	82.06	81.80	82.96	92.47						
PeVYV-5352-_2	83.63	83.36	84.77	85.97	87.14	85.68	86.03	83.25	84.70	89.39	89.49					
PeVYV-5634-2	83.58	83.70	84.90	85.07	85.03	83.77	84.27	80.67	82.35	91.47	91.47	92.21				
PeVYV-5357	71.09	71.52	71.89	70.58	69.74	69.25	69.29	69.43	69.58	71.22	71.33	69.73	71.64			
PeVYV-Ita7	71.07	71.57	71.95	70.59	69.71	69.37	69.53	69.17	69.47	71.39	71.64	69.79	71.90	96.10		
PeVYV-J4702	73.51	73.86	74.61	72.97	72.34	72.02	72.00	72.06	71.99	71.22	71.08	69.51	71.62	88.27	88.30	
PeLRV_LT220496	79.08	79.42	76.60	78.32	78.68	78.33	77.94	77.02	78.98	78.39	77.65	77.20	77.07	65.56	65.61	66.91

◂**Fig. 2** (**A** & **B**) Maximum-likelihood phylogenetic trees based on (**A**) a nucleotide sequence alignment of a 1388 nt fragment spanning part of the RdRP to the end of the CP and (**B**) nucleotide sequence alignments of the complete coding region, of PeVYV-like strains and other members of the genus *Polerovirus*. Only bootstrap values greater than 70 % are shown. (**C**) Distance matrix showing the percentage nucleotide identity of PeVYV-like genomes obtained through high throughput sequencing in this study and the main PeVYV isolates from GenBank. Sequences used for alignments: pepper vein yellows virus (PeVYV); pepper leaf roll virus (PeLRV; also known as PeVYV-7); pod pepper vein yellows virus (PoPVYV); beet chlorosis virus-2a (BChV-2a); chickpea chlorotic stunt virus (CpCSV); beet mild yellows virus (BMYV); beet western yellows virus (BWYV); cucurbit aphid-borne yellows virus (CABYV); phasey bean mild yellows virus (PBMYV); tobacco vein distorting virus (TVDV); tobacco vein distorting virus 2 (TVDV2); tobacco polerovirus 2 (TPV2); potato leaf roll virus (PLRV); beet leaf yellows virus (BLYV); faba bean polerovirus 1 (FBPV1); turnip yellows virus-FL1 (TuYV-FL1); African eggplant yellows virus (AeYV); beet mild yellows virus (BMYV)

Reinhart et al. [24] or using a TRIzol plus RNA Purification Kit as per the manufacturer's instructions. For plant samples containing isolates 5631, 5352, J4702-2-1, and 5634, library preparation and sequencing were done by Macrogen (Seoul, South Korea) using a TruSeq Stranded Total RNA with Ribo-Zero Plant Kit (Illumina) to remove excess ribosomal RNA, followed by 151-bp paired-end sequencing on an Illumina Platform System (NovaSeq). For the chickpea sample containing isolate 5357, sequencing was done at the Australian Genome Research Facility (AGRF) using a TruSeq Stranded RNA with Ribo-Zero Plant Kit (Illumina) for library preparation and 150-bp paired-end sequencing with an Illumina MiSeq sequencer as described by Filardo et al. [25]. Trimming for quality and adaptor and primer sequence removal was carried out using the Trim Galore tool [26] available on Galaxy Australia (https://usegalaxy.org.au/), and paired reads were assembled *de novo* using CLC Genomic Workbench 12.0 (CLCGW) (CLCbio) as described by Filardo et al. [25]. The garlic sample (isolate Ita7) was sequenced by AGRF as described [18], using a 100-bp paired-end NovaSeq SP 300-cycle reagent kit and an Illumina TruSeq Stranded Total RNA and Ribo-Zero Plant Kit for library preparation. Trimming for quality, adapter removal, and *de novo* assembly was done through Galaxy Australia following the pipeline of Bester et al. [27]. Contigs and BLAST (NCBI) results were further analysed in Geneious 10 (Biomatters). The total number of reads obtained before and after trimming, the total number of contigs produced, the final PeVYV contig length, the number of reads that were mapped to each isolate, and the average coverage are shown in Supplementary Table S1.

The 3′-terminal sequences of isolates 5357 and Ita7 were determined by addition of a poly(A) tail to the purified RNA using poly(A) polymerase (NEB, USA), followed by cDNA synthesis and PCR using the primers Potyvirid primer 1 [28] and PeVYV-5462F (GATCGCCGCTCAGACCCTTTAAG), which binds to both isolates, followed by a semi-nested PCR using Potyvirid primer 1 and PeVYV10-5682F (GGCATC ATTCGGCAAAACTCGGG). The 5′-terminal sequence was determined using a Roche 5'/3' RACE Kit (Sigma-Aldrich, USA) as per the manufacturer's instructions and using two nested reverse primers: PeVYV10-625R (GTGACCAGA TAAAAGATCCAACAACACATG) and PeVYV10_330-R (GCCACAGCGGCTCCACAGTAAAG). PCR conditions were as described above but with an annealing temperature of 58 °C for 30 s. Products were sequenced directly by Macrogen using the Sanger method.

Phylogenetic relationships of sequences obtained through HTS were determined as described above and included other PeVYVs and poleroviruses (Fig. 2B). Phylogenetic results showed that isolates 5352-1, 5631, and 5634-1 shared 93-95% nt sequence identity over the whole genome and were most closely related to PeVYV-6 and -5 (88-90% nt sequence identity; Fig. 2B and C). A second PeVYV isolate was found in both 5352 and 5634, due to mixed infections with two slightly different strains. These second isolates, 5352-2 and 5634-2 shared 92% nt sequence identity and were most closely related to PeVYV-9 and -2 (89-91% nt sequence identity). The isolates 5357, Ita-7, and J4702 shared 88-96% nt sequence identity over the whole genome. J4702 shared less nt sequence identity with 5357 and Ita7 due to a 764-nt region at the end of ORF 5, which shared only 43% nt sequence identity in the same genome region with 5357 and Ita7, and BLASTx (NCBI) results showed that the closest match for the 764- nt region was to a PeVYV isolate from Guangxi, China (MW161057), with 55% amino acid (aa) sequence identity. Despite this small difference in ORF 5 of J4702, it closely aligns with 5357 and Ita7. These three isolates are divergent from the other described PeVYV strains, as they share only 69-74% nt sequence identity over the whole genome to the other PeVYVs (PeVYV-1 to 9; Fig. 2B and C).

Comparing the open reading frames of each of the PeVYVs to 5357 – excluding ORFs 3, 4, and 5 of PeVYV-7, which are highly divergent from other PeVYVs and homologous to pepper whitefly-borne vein yellows virus [29] – ORF 5, ORF 0, and ORF 1 showed the highest variation, with 50-70%, 54-57%, and 60-62 % aa sequence identity, respectively. The most conserved regions were ORF 3a (73-98% aa sequence identity), ORF 3 (87-91% aa sequence identity), ORF 2 (RdRp; 84-87% aa sequence identity), and ORF 4 (80-83% aa sequence identity) (Supplementary Table S2). In the RdRp protein, most variation was observed at the N-terminal end within a 122-aa section (data not shown). The rest of the RdRP (478 aa) was more conserved, as shown by the results of the 1388-nt fragment.

Genetic diversity coupled with recombination events has made naming and distinguishing poleroviruses species

extremely complex. Recent analysis of genomes of the polerovirus turnip yellows virus (TuYV) in Australia demonstrated that TuYV is highly variable in ORF 0, P3a, and ORF 5, and Filardo et al. [14] suggested that these regions may not be informative for species demarcation. It was therefore suggested that whole-genome analysis coupled with conservation in the RdRP and ORF 3 regions (>89% aa sequence identity for both regions) could be used for polerovirus species demarcation.

In this study, we have identified a range of PeVYV-like isolates in Australia, with isolates 5357, Ita-7, and J4702 being a new potential PeVYV-like variant. If we use the criteria of Filardo et al. [14] for species demarcation, these isolates could possibly be described as PeVYV strains. The isolates share up to 87% and 98% aa sequence identity in the RdRP and ORF 3, respectively. It is noted that for TuYV isolates, the suggested cutoff for the RdRP was 89% aa sequence identity, and these isolates (5357, Ita7, and J4702) share slightly less aa sequence identity (84-87%) with other PeVYV RdRPs. In addition, for TuYV, it was suggested that members of the same species share at least 83% nucleotide sequence identity over the whole genome, yet these PeVYV-like isolates share only up to 74% nt sequence identity with other PeVYVs. This suggests that PeVYV-like viruses may be even more diverse than TuYV or that more genomic diversity is still to be discovered for TuYV.

Alternatively, Fiallo-Olivé et al. [2] and the ICTV have suggested, for PeVYV-like viruses only, that new PeVYV-like viruses (those showing less than 10% aa sequence identity in any ORF but with similar RdRP sequences) should be described as numbered species in order of discovery rather than giving them new species names. However, as the diversity of these isolates grows with the use of HTS, the number of PeVYV-like viruses will continue to grow, and the discussion of whether they are new viruses or just different strains will continue.

Despite the species demarcation difficulties, we have tentatively called this new PeVYV-like virus PeVYV-10, and further whole-genome sequencing of PeVYV-like isolates may one day resolve whether these are new viruses or strains of the same species.

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Author contributions FF wrote the manuscript and analysed the data. FF, SN, and PC performed high-throughput sequencing. FF and SN performed the 5' and 3' RACE. LJ contributed, identified, and photographed seven isolates. CG, SB, and MS contributed and identified isolates. PC provided supervision and grant funding. All authors read, revised, and approved the final manuscript.

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Declarations

Conflict of interest The authors declare there are no conflicts of interest.

Ethical approval This article does not contain any studies with human participants or animals performed by any of the authors.

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