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e-Xtra*

Phylogenetics and Evolution of Potato Virus V: Another Potyvirus that Originated in the Andes

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Abstract

Potato virus V (PVV) causes a disease of potato (*Solanum tubersosum*) in South and Central America, Europe, and the Middle East. We report here the complete genomic sequences of 42 new PVV isolates from the potato's Andean domestication center in Peru and of eight historical or recent isolates from Europe. When the principal open reading frames of these genomic sequences together with those of nine previously published genomic sequences were analyzed, only two from Peru and one from Iran were found to be recombinant. The phylogeny of the 56 nonrecombinant open reading frame sequences showed that the PVV population had two major phylogroups, one of which formed three minor phylogroups (A1 to A3) of isolates, all of which are found only in the Andean region of South America (Peru and Colombia), and the other formed two minor phylogroups, a basal one of Andean isolates (A4) that is paraphyletic to a crown cluster containing all the isolates found outside South America (World). This suggests that PVV originated in the Andean region, with only one minor phylogroup spreading elsewhere in the world. In minor phylogroups A1 and A3, there were two subclades on long branches containing isolates from *S. phureja* evolving more rapidly than the others, and these interfered with dating calculations. Although no temporal signal was directly detected among the dated nonrecombinant sequences, PVV and potato virus Y (PVY) are from the same potyvirus lineage and are ecologically similar, so "subtree dating" was done via a single maximum likelihood phylogeny of PVV and PVY sequences, and PVY's well-supported 157 CE "time to most common recent ancestor" was extrapolated to date that of PVV as 29 BCE. Thus the independent historical coincidences supporting the datings of the PVV and PVY phylogenies are the same; PVV arose ≥2,000 years ago in the Andes and was taken to Europe during the Columbian Exchange, where it diversified around 1853 CE, soon after the European potato late blight pandemic. PVV is likely to be more widespread than currently realized and is of biosecurity relevance for world regions that have not yet recorded its presence.

Keywords: potato, virus disease, potato virus V, South America, Andean crop domestication center, Europe, high-throughput sequencing, phylogenetics, population genetics, Andean lineages, dating, interpretation, evolution, prehistory, biosecurity significance

In 1984, a new potyvirus named potato virus V (PVV; genus *Potyvirus*, family Potyviridae) was described infecting potato (*Solanum tuberosum*) plants in Peru (Fribourg and Nakashima 1984; Jones and Fribourg 1986). It had been found earlier in Western Europe but was confused with potato virus Y (PVY; genus *Potyvirus*, family *Potyviridae*) biological strain group PVY^C. This confusion arose because, although it was only distantly related serologically to PVY, it caused a severe necrotic phenotype when inoculated to potato cultivars with PVY hypersensitivity gene *Nc* (Calvert et al. 1980; Cockerham 1943, 1970; De Bokx et al. 1975; Rozendaal et al. 1971). However, such cultivars were later found to carry an additional hypersensitivity gene, *Nv*, with which the virus was actually interacting (Jones 1987, 1990; Jones and Fribourg 1986; Jones and Fuller 1984; Oruetxebarria and Valkonen 2001). Early synonyms for PVV include PVY^C strain GL (GL = from the Dutch cultivar 'Gladblaadje') from the Netherlands, PVY^C strain AB (AB = from the Scottish cultivar 'Arran Banner') from Northern Ireland, and PVY strain B from France (Boudazin et al. 1984; Calvert et al. 1980; De Bokx et al. 1975; Rozendaal et al. 1971). Although most potato cultivars develop severe necrotic symptoms when inoculated with PVV, these hypersensitive phenotypes rarely occur in the field. The small proportion of cultivars often found infected are asymptomatic, or they develop leaf pallor and distortion or mosaic (Calvert et al. 1980; Fribourg and Nakashima 1984; Jones 1987; Jones and Fribourg 1986; Jones and Fuller 1984). So far, PVV has been reported infecting potato crops in eight European countries (Ireland, Finland, France, Germany, Norway, Sweden, the Netherlands, and the United Kingdom) (CABI 2020; Jones 1987, 1990; Jones and Fribourg 1986; Jones and Fuller 1984; Mortensen et al. 2010; Oruetxebarria and Valkonen 2001; Oruetxebarria et al. 2000; Spetz et al. 2003), the Andean region of South America (Peru, Colombia, Argentina) (Álvarez et al. 2016; Clausen et al. 2005; Fribourg and Nakashima 1984; Gutiérrez et al. 2016), Central America (Costa Rica) (Vásquez et al. 2006), and the Middle East (Iran) (Shamsadden-Saeed et al. 2014). It has also been found in another solanaceous crop in the Andean region, cape gooseberry (*Physalis peruviana*) in Colombia (Álvarez et al. 2018). Although PVV was reported infecting tamarillo (*S. betaceum*) in Ecuador (Insuasti et al. 2016), the virus found was later shown to be closer to Ecuadorian rocoto virus (Álvarez et al. 2018). In North America, PVV was detected infecting plants derived from Andean potato germplasm growing in quarantine (Shiel et al. 2004).

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PVV is currently considered of limited economic importance to potato production outside the Andean region. This is because most *S. tuberosum* subsp. *tuberosum* potato cultivars carry PVV hypersensitivity gene *Nv* and a few carry extreme resistance gene *Ry*, which is elicited by PVV, PVY, and potato virus A (PVA), so field infections with PVV occur in only a small number of cultivars that lack either gene (Barker 1997; Jones 1987, 1990; Jones and Fribourg 1986; Jones and Fuller 1984; Jones and Vincent 2018; Oruetxebarria and Valkonen 2001; Solomon-Blackburn and Barker 2001); and its foliage symptoms are easily missed during seed potato crop inspections because they are often subtle or absent in PVV-susceptible cultivars (Jones 1987, 1990; Oruetxebarria and Valkonen 2001). However, it can cause tuber cracking symptoms when present in mixed infection with PVA (Carnegie and McCreath 2010). Its detection during healthy seed potato production requires routine sample testing, but apart from in a few European countries, healthy seed potato schemes rarely test for its presence (Stevenson et al. 2001; UNECFE 2004), and the losses in potato tuber yield it causes have not yet been quantified. However, it is likely to be more widespread than currently realized and seems of biosecurity relevance for world regions that have not yet recorded its presence in the field, such as Australasia, Africa, the Indian subcontinent, East and Southeast Asia, and North America.

The properties of PVV virions are those typical of a potyvirus. Its virions consist of flexuous filaments about 760 nm long. Its genome (isolate DV 42; NC_004010.1) is a plus-sense single stranded RNA with 9,851 nucleotides encoding a 3,067-amino acid polyprotein in a large open reading frame (ORF) with nine putative cleavage sites that yield 10 proteins and a minor overlapping ORF, P3N-*pipo*, in the -1 frame (Gibbs et al. 2020; Oruetxebarria et al. 2000). PVV is within the PVY lineage of the genus *Potyvirus*, where it is closest to wild potato mosaic virus (WPMV), so far found only in Peru, and Ecuadorian rocoto virus (Fribourg et al. 2019; Jones and Fribourg 1979). PVV-infected plants develop cylindrical and pinwheel inclusions within their cell cytoplasm. The aphid vector species *Myzus persicae*, *Brachycaudus helichrysi*, *Macrosiphum euphorbiae*, and *Rhopalosphoninus latysiphon* transmit it nonpersistently (Bell 1983, 1988; Calvert et al. 1980). Apart from a few chenopodiaceous species that developed local lesions in their inoculated leaves after sap inoculation, it infected several other species of Solanaceae but not plants of ≥10 other families (Calvert et al. 1980; Fribourg and Nakashima 1984; Jones and Fribourg 1986; Jones and Fuller 1984). Two distinct biological strain groups (= pathotypes) of PVV were distinguished by the different phenotypes that they elicit in differential potato hosts cultivar 'Pentland Ivory' and clone A6 (*S. demissum* × S. *tuberosum* cultivar Aquila) (Oruetxebarria et al. 2000; Spetz et al. 2003). In inoculated leaves of cultivar Pentland Ivory, PVV-1 elicits necrotic local lesions, whereas PVV-2 causes asymptomatic infection, neither of them infecting it systemically. In clone A6, PVV-1 causes systemic mosaic or yellowing symptoms, whereas PVV-2 elicits systemic veinal necrosis. European PVV isolates belonged to PVV-1 but Peruvian isolates to PVV-2 (Oruetxebarria et al. 2000; Spetz et al. 2003). Cultivar Pentland Ivory carries resistance gene *Ny* (Jones 1990), so the two Peruvian isolates used by Spetz et al. (2003) overcame this gene. When the original Peruvian PVV isolate (UF) of Fribourg and Nakashima (1984) was inoculated to 18 cultivars of *S. tuberosum* subsp. *tuberosum* and one of *S. tuberosum* subsp. andigena, systemic necrosis developed in two of the former ('Maris Peer' and 'Pentland Dell') and the latter ('Tomasa Condemayta') but not in Pentland Ivory. This finding suggests that another PVV hypersensitive resistance gene (putative *Nv2*) may be present in Maris Peer and Pentland Dell, elicited by isolate UF, which might belong to a possible third strain group (PVV-3).

When Mortensen et al. (2010) compared the coat protein (CP) genes of 13 European, two Peruvian, and one Andean quarantine-detected isolate of PVV, phylogenetic analysis found that all of the European isolates grouped together and two Peruvian isolates were nearby, but the Andean quarantine isolate was more distant. These findings resembled those obtained in earlier phylogenetic analyses with fewer PVV isolates (Oruetxebarria et al. 2000; Shiel et al. 2004; Spetz et al. 2003). Later, when the CP genes of five Iranian PVV isolates were compared with all available PVV isolates, they were close to the European isolates, and there was 91% nucleotide identity between the complete genomic sequences of an Iranian and a Scottish isolate (Shamsadden-Saeed et al. 2014). Subsequently, CP analysis including sequences from six Colombian PVV isolates from *S. phureja* placed these and the quarantine isolate in a separate phylogroup from the European isolates and a Peruvian isolate (second Peruvian isolate absent) (Álvarez et al. 2016); a phylogroup is a phylogenetically distinguished group of isolates. In addition, three complete Colombian PVV genomes from *S. phureja* grouped separately from those of the Iranian and Scottish isolates (Álvarez et al. 2016; Gutiérrez et al. 2016). When CP (three) and complete genomes (one) of Colombian PVV isolates from *Physalis peruviana* (cape gooseberry) were included in a further analysis, they grouped with the Colombian *S. phureja* isolates (Álvarez et al. 2018).

This article is the sixth in a series of recent articles that compare the genomic sequences of viruses from the potato crop's Andean domestication center with those from other world regions. Three of these articles involve other potyviruses (PVA, PVY, and WPMV) (Fribourg et al. 2019; Fuentes et al. 2019, 2021a), and two of them involve viruses from other genera: potato virus S (PVS, genus *Carlavirus*, family *Betaflexiviridae*) and potato virus X (PVX, genus *Potexvirus*, family *Alphaflexiviridae*) (Fuentes et al. 2021b; Santillan et al. 2018). This article examines the phylogenetics and evolution of PVV by comparing 42 new genomes from Peru in the Andean region and eight new ones from historical (two) or more recent (six) European isolates with nine previously sequenced genomes from GenBank. It provides new information on the diversity of PVV and its evolution before and after the movement of this crop to the rest of the world after the Spanish conquest of Peru in 1532.

Materials and Methods

Virus isolates and plants. For each PVV isolate sequenced, where available, the potato cultivars they originally came from, years of first isolation, geographic origins, and first isolate references are listed in Table 1. They included historical European isolates from The Netherlands (GL) and Northern Ireland (AB) from a collection of freeze-dried virus isolates kept at Fera Plant Science Ltd, York, United Kingdom, four more recent UK isolates (ES, PR, 20910972, 20911536), and one isolate each from Switzerland (613) and Belgium (Seviplant-44_L205-01-04). Isolates from Peru (42) were obtained from leaves of potato plants collected between 2016 and 2018 in the northern, central, and southern Andean highlands of Peru (Fig. 1). The potato plants sampled showed foliage symptoms indicative of virus infection and came from five Peruvian departments (north: Cajamarca, $n = 14$; center: Huanuco, $n = 6$, and Huancavelica, $n = 6$; south: Apurimac, $n = 15$, and Cusco, $n = 1$). Each sample was placed in a separate labeled paper filter bag, nine of which were placed together in a zippered plastic bag with 100 g of dehydrated silica gel for rapid desiccation. The silica gel was changed after 24 to 48 h and the combined samples taken to International Potato Center in Lima for processing. Table 1 shows the Department of Peru from which each sample came and the year it was isolated; a simplified searchable spreadsheet version of this and other PVV data is in Supplementary Table S2.

High-throughput sequencing. In Peru, total RNA was extracted from each potato leaf sample with TRIzol as instructed by the manufacturer (Invitrogen). The large RNA fraction was precipitated by adding an equal volume of 4 M LiCl at about 4°C (on ice) overnight, followed by centrifugation. The remaining small RNA fraction was subsequently precipitated by adding 1 volume of isopropanol followed by centrifugation. Small RNAs were separated on 3.5% agarose gels, and bands corresponding to about 20 to 25 nt excised and purified via Quantum Prep Freeze 'N Squeeze Columns (BioRad, Hercules, CA). Small RNA libraries were prepared according to the protocol of Chen et al. (2012) and sent for sequencing on a HiSeq4000 by a commercial provider (Fasteris Life Sciences SA, Switzerland). Small RNA sequences were analyzed in VirusDetect version 1.6 (Zheng et al. 2017) to identify all viruses infecting the plants, and samples in which PVV was identified were selected for further analysis. A total of 192

Table 1. Origins and accession numbers of the potato virus V isolates used

Isolate	Source potato cultivar	Species	Where collected	Isolation year	Accession no.	Isolate reference
GL	Gladblaadje	*Solanum tuberosum* subsp. *tuberosum*	The Netherlands	c. 1970 (Received ex J.A. De Bokx)	MZ286362	De Bokx et al. 1975; Jones 1990; Rozendaal et al. 1971
AB	Arran Banner	*S. tuberosum* subsp. *tuberosum*	Northern Ireland	1973 (Received ex E. L. Calvert)	MZ286361	Calvert et al. 1980; Jones 1990
ES	Estima	*S. tuberosum* subsp. *tuberosum*	United Kingdom	2009	MZ286357	This article
PR	Premiere	*S. tuberosum* subsp. *tuberosum*	United Kingdom	2009	MZ286358	This article
613 (PVA isolate contaminant)	Advira	*S. tuberosum* subsp. *tuberosum*	Switzerland	Received 1985 ex Paul Gugerli	MZ286356 (100 nt gap in middle)	This article
20910972	Unknown	*S. tuberosum* subsp. *tuberosum*	United Kingdom	2010	MZ286360	This article
20911536	Unknown	*S. tuberosum* subsp. *tuberosum*	United Kingdom	2009	MZ286359	This article
Seviplant-44_L205-01-04	Rosa	*S. tuberosum* subsp. *tuberosum*	Luxembourg Province, Belgium	2020	MZ570609	This article
Apu041	Huayro	*Solanum* × *chaucha*	Apurimac, Peru	2018	MZ503925	This article
Apu042	Huayro	*S.* × *chaucha*	Apurimac, Peru	2018	MZ503926	This article
Apu044	Huayro	*S.* × *chaucha*	Apurimac, Peru	2018	MZ503927	This article
Apu066	Huayro	*S.* × *chaucha*	Apurimac, Peru	2018	MZ503928	This article
Apu069	Huayro	*S.* × *chaucha*	Apurimac, Peru	2018	MZ503929	This article
Apu070A	Huayro	*S.* × *chaucha*	Apurimac, Peru	2018	MZ503930	This article
Apu079	Peruanita	*Solanum stenotomum* subsp. *goniocalyx*	Apurimac, Peru	2018	MZ503931	This article
Apu080A	Peruanita	*S. stenotomum* subsp. *goniocalyx*	Apurimac, Peru	2018	MZ503932	This article
Apu082	Huayro	*S.* × *chaucha*	Apurimac, Peru	2018	MZ503933	This article
Apu083	Huayro	*S.* × *chaucha*	Apurimac, Peru	2018	MZ503934	This article
Apu084	Huayro	*S.* × *chaucha*	Apurimac, Peru	2018	MZ503935	This article
Apu085	Huayro	*S.* × *chaucha*	Apurimac, Peru	2018	MZ503936	This article
Apu087	Huayro	*S.* × *chaucha*	Apurimac, Peru	2018	MZ503937	This article
Apu089	Huayro	*S.* × *chaucha*	Apurimac, Peru	2018	MZ503938	This article
Apu090A	Huayro	*S.* × *chaucha*	Apurimac, Peru	2018	MZ503939	This article
Cca012	Chaucha	*Solanum phureja*	Cajamarca, Peru	2016	MZ503940	This article
Cca018	Chaucha	*S. phureja*	Cajamarca, Peru	2016	MZ503941	This article
Cca020	Chaucha	*S. phureja*	Cajamarca, Peru	2016	MZ503942	This article
Cca097	Chaucha	*S. phureja*	Cajamarca, Peru	2016	MZ503943	This article
Cca100B	Chaucha	*S. phureja*	Cajamarca, Peru	2016	MZ503944	This article
Cca101	Chaucha	*S. phureja*	Cajamarca, Peru	2016	MZ503945	This article
Cca102	Chaucha	*S. phureja*	Cajamarca, Peru	2016	MZ503946	This article
Cca103	Chaucha	*S. phureja*	Cajamarca, Peru	2016	MZ503947	This article
Cca104	Chaucha	*S. phureja*	Cajamarca, Peru	2016	MZ503948	This article
Cca105	Chaucha	*S. phureja*	Cajamarca, Peru	2016	MZ503949	This article
Cca106	Chaucha	*S. phureja*	Cajamarca, Peru	2016	MZ503950	This article
Cca107	Chaucha	*S. phureja*	Cajamarca, Peru	2016	MZ503951	This article
Cca120B	Chaucha	*S. phureja*	Cajamarca, Peru	2016	MZ503952	This article
Cca120C	Chaucha	*S. phureja*	Cajamarca, Peru	2016	MZ503953	This article
Cus098	Canchan	*S. tuberosum* subsp. *tuberosum* × *S. tuberosum* subsp. *andigena*	Cusco, Peru	2016	MZ503954	This article
Hco002B	Tumbay	*S. stenotomum* subsp. *goniocalyx*	Huanuco, Peru	2016	MZ503955	This article
Hco004B	Tumbay	*S. stenotomum* subsp. *goniocalyx*	Huanuco, Peru	2016	MZ503956	This article
Hco005B	Tumbay	*S. stenotomum* subsp. *goniocalyx*	Huanuco, Peru	2016	MZ503957	This article
Hco008	Yungay	*S. tuberosum* subsp. *andigena* × *tuberosum*	Huanuco, Peru	2016	MZ503958	This article
Hco036	Yungay	*S. tuberosum* subsp. *andigena* × *tuberosum*	Huanuco, Peru	2016	MZ503959	This article
Hco037	Yungay	*S. tuberosum* subsp. *andigena* × *tuberosum*	Huanuco, Peru	2016	MZ503960	This article
Hua041	Unknown	*Solanum* sp.	Huancavelica, Peru	2018	MZ503961	This article
Hua042	Unknown	*Solanum* sp.	Huancavelica, Peru	2018	MZ503962	This article
Hua043	Unknown	*Solanum* sp.	Huancavelica, Peru	2018	MZ503963	This article
Hua044	Unknown	*Solanum* sp.	Huancavelica, Peru	2018	MZ503964	This article
Hua048	Unknown	*Solanum* sp.	Huancavelica, Peru	2018	MZ503965	This article
Hua049	Unknown	*Solanum* sp.	Huancavelica, Peru	2018	MZ503966	This article

of 994 of samples (19%) from the Peruvian potato virome (Kreuze et al. 2020) contained PVV sequences. From them, the ones showing the highest coverage (with respect to the reference genome), from VirusDetect results, were selected for assembling the viral genomes. The 42 new PVV complete genomic sequences assembled were from samples with mixed infection. In addition to PVV, the following viruses were sometimes present in the same samples: PVA, PVY, PVX, PVS, Andean potato mottle virus (genus *Comovirus*, family *Secoviridae*), potato virus B (genus *Nepovirus*, family *Secoviridae*), Andean potato latent virus (genus *Tymovirus*, family *Tymoviridae*), and Andean potato mild mosaic virus (genus *Tymovirus*, family *Tymoviridae*). In the Geneious R11.1.3 software package, the PVV contigs produced by VirusDetect were extracted for each positive sample and a consensus generated. The small RNAs were then mapped back to the consensus to confirm the quality of the assemblies and make any corrections necessary. The 42 new genomic sequences with complete ORFs were 9,844 to 9,859 nts long.

In the United Kingdom, samples of freeze-dried or fresh PVV-infected leaf material containing one each of eight isolates were subjected to high-throughput sequencing in 2016 to 2019 (Table 1). Total RNA was extracted from each sample with the Total RNA kit (Qiagen, UK), including the optional DNAase treatment. An indexed sequencing library was produced from the total RNA with a ScriptSeq complete plant leaf kit (Illumina) and sequenced on a MiSeq instrument (Illumina), with a 600-cycle V3 kit. The methods followed were described in more detail by Fox et al. (2019). Seven of the isolates provided a complete PVV ORF. No other virus sequences were associated with these complete ORFs, except with isolate 613 (PVA sequence present and used by Fuentes et al. (2021a)). The seven new genomic sequences with complete ORFs were 9827 to 9877 nt long, but 613 had a 100-nt gap in its middle, which was revealed by comparison with other genomes.

In Belgium, 50 leaf samples of potato cultivar 'Rosa' were collected from a field in Luxembourg province. They were pooled together and lyophilized, and their virus nucleic acids were enriched with virion-associated nucleic acids via a method adapted as described previously (Hammond et al. 2021; Palanga et al. 2016), and adjusted for dehydrated material with a ratio of 33 ml of Hank's buffered salt solution to 1 g of dry tissue. A library was prepared with a NEBNext Ultra II DNA Library Prep Kit for Illumina at GIGA Genomics (University of Liege, Belgium), where the samples were sequenced by Illumina NovaSeq. The resulting 1,042,294 sequence reads were trimmed, paired, and merged in the Geneious R11 software platform (https://www.geneious.com; Biomatters, Inc.) and de novo assembled in SPAdes (Bankevich et al. 2012). From the contigs, BLASTx identified three potyviruses, PVA, PVY, and PVV. The largest PVV contig obtained was 10,061 nt in length. To retrieve the PVV-infected samples, we designed primers PVV-5141F (GTGCCATATAGAGCTTC GAGTGA) and PVV-5798R (TCATCTCTCCTGTGAACCAAGTG) from PVV isolate GL sequence MZ286362. Fifteen potato tubers from the same source were germinated, and RNA extracted from them with an RNeasy Plant Mini Kit (Qiagen) was tested by reverse transcription PCR with the enzyme Tetro (Bioline) with random hexamers. PCR was performed with EmeraldAmp GT PCR master mix (Takara) at 35 cycles of 98°C for 10 s, 60°C for 30 s, and 72°C for 20 s. The anticipated amplicon size of 657 bp was recovered from all 15 samples. Sanger sequencing of one positive amplicon was done by Macrogen Europe (Amsterdam, The Netherlands). The sequence

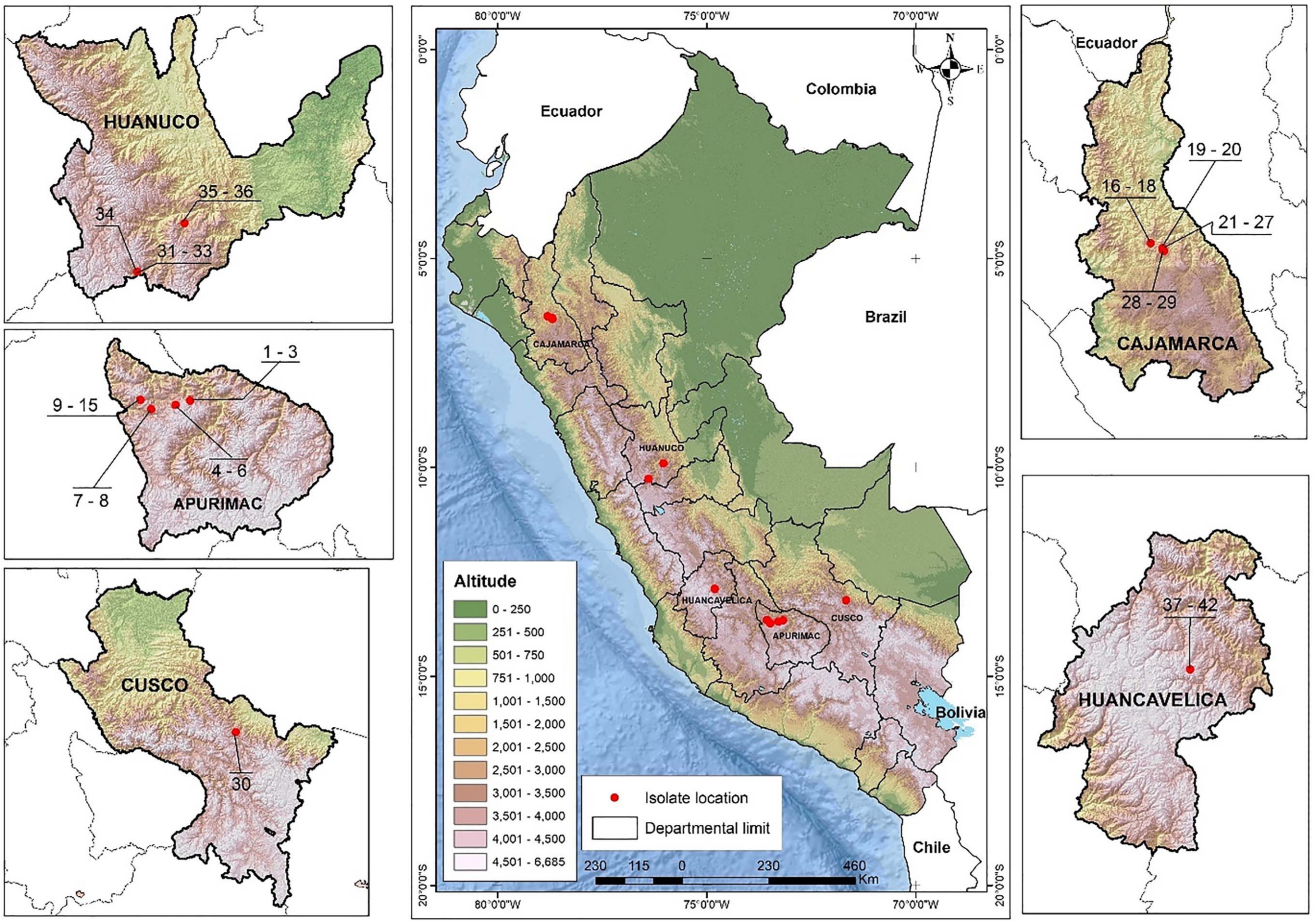

Fig. 1. Map of sample collection sites in the Andean Highlands showing where potato leaf samples were obtained. Peru's Andean highlands are shown in brown, the country's coastal desert and Amazonian jungle regions in green, and surrounding countries in white. The red dots on the main map represent the locations sampled, and the names on it are those of the countries' regional departments sampled (black lines are departmental boundaries). The red dots on the individual department maps show each collection site. Individual collection sites are numbered. The names of the departments also provide the first three letters of the Peruvian isolate names (Supplementary Table S2, column B).

obtained showed 99.7% nucleotide homology to the high-throughput sequence.

Final nucleotide sequences of these 50 new isolates were submitted to GenBank (accession nos. MZ286356 to MZ286609) (Table 1).

Sequence analysis. New (n = 50) and downloaded (n = 9) PVV genomic sequences were aligned and edited in MAFFT (Katoh and Standley 2013) and BioEdit (Hall 1999) to extract their ORFs. These were aligned, with the encoded amino acids used as a guide and the TranslatorX online server (Abascal et al. 2010; http://translatorx.co.uk) with its MAFFT option (Katoh and Standley 2013). Because the genomic sequences of historical isolates AB and GL were represented by duplicated accession numbers in GenBank, MZ28361 (AB) and MZ28362 (GL) were retained (Table 1), whereas MK756119 (AB) and MK756120 (GL) were removed from the alignment. To search for non-PVV sequences with which to compare and root phylogenies, we used the BLASTn online facilities of GenBank (Altschul et al. 1990) with the sequences representing the basal divergences of the phylogeny (i.e., the most distantly related PVVs). Sequences were tested for the presence of phylogenetic anomalies via the full suite of options in RDP version 5.5 with default parameters (Boni et al. 2007; Gibbs et al. 2000; Holmes et al. 1999; Lemey et al. 2009; Martin and Rybicki 2000; Martin et al. 2005, 2015; Maynard-Smith 1992; McGuire and Wright 2000; Padidam et al. 1999; Posada and Crandall 2001). The best-fit substitution models for PVV nucleotide sequences were assessed in MEGA-X (Tamura et al. 2021) and found to be GTR + γ_4 + I (Tavare 1986).

Phylogenetic trees were calculated via the neighbor joining option in ClustalX (Jeanmougin et al. 1998) and by PhyML version 3.0 (Guindon and Gascuel 2003). In PhyML, the statistical support for their topologies was assessed via the SH method (Shimodaira and Hasegawa 1999). The temporal signal in alignments of dated nonrecombinant ORFs was assessed in TempEst version 1.5 (Rambaut et al. 2016) to examine dated ML trees and also estimated via the RelTime with Dated Tips method (Miura et al. 2018) in MEGA-X (Tamura et al. 2021).

The statistical significance of correlation coefficients was calculated in the Social Science Statistics online facility (https://www.socscistatistics.com/pvalues/pearsondistribution.aspx). Trees were drawn in Figtree version 1.3 (http://tree.bio.ed.ac.uk/software/figtree/; 12 May 2018) and a commercial graphics package. The positions of basal nodes of subtrees in maximum likelihood (ML) phylogenies (Hajizadeh et al. 2019; Mohammadi et al. 2018) were compared in PATRISTIC (Fourment and Gibbs 2006). This converts tree files to matrices of their pairwise patristic distances that can then be interrogated in MS Excel; the mean pairwise patristic distance between all tips connected through each selected node is calculated. PATRISTIC was also used to check for mutational saturation by comparing the patristic distances of the nucleotide phylogenies with those of the amino acids they encoded. The program DnaSP version 6.10.01 (Rozas 2009) was used to analyze genetic differences between parts of the PVV population.

The 56 nonrecombinant PVV sequences were separated into the five minor phylogroup populations (Fig. 2), and the complete polyprotein ORFs and 11 individual genes of those populations were analyzed separately in DnaSP version 6.10.01 (Rozas 2009). The average pairwise nucleotide diversity (π), number of synonymous sites (SS), number of nonsynonymous sites (NS), mean synonymous substitutions per synonymous site (dS), mean nonsynonymous substitutions per nonsynonymous site (dN), and ratio of nonsynonymous nucleotide diversity to synonymous nucleotide diversity (dN/dS) were estimated. It was concluded that the virus genes were under positive, neutral, or negative selection when their dN/dS ratios were >1, 1 and <1, respectively. In addition, three population genetic tests of neutral evolution (Tajima's D^*, Fu and Li's D^*, and Fu and Li's F^* tests) were used to determine whether the observed variation of the populations was more or less than expected if the populations had been evolving randomly (Fu and Li 1993; Gao et al. 2017; Tajima 1989).

DnaSP version 6.10.01 was also used to assess whether the PVV populations were more or less genetically linked, as measured as the amount of gene flow between them. This was done via F_{ST} (the interpopulational component of genetic variation or the standardized variance in allele frequencies across populations) (Hudson et al. 1992) and the gene flow parameter Nm (the product of the effective population number and rate of migration between populations) (Wright 1943). Because the statistical significance of F_{ST} values cannot be evaluated in DnaSP, Arlequin version 3.5.2.2 (Excoffier and Lischer 2010) was used to do the permutations tests needed to obtain them.

Results

Recombination analysis. The 59 PVV genomic sequences (50 new and nine from GenBank, the RefSeq sequence being a replicate) were edited and converted as described previously to an alignment of ORFs 9,198 nt long. The sequences were checked for phylogenetic anomalies in RDP version 5.5, and three were found to have significant recombinant (rec) regions. The clearest recombination signal was in the sequence of the isolate from Iran, KC433411. The majority of its sequence is closest to that of an isolate from Belgium (MZ570609), but it has a phylogenetically anomalous region from nt 160 to 2,246. The 5′-terminal part of that region (nt 160 to 1,432) is closest to that of MZ503929, which is the Peruvian isolate that forms the long basal branch of the A2 population; it was identified by seven RDP methods, and the statistical likelihood of it being a random anomaly ranged from 3.3×10^{-16} to 2.4×10^{-97}. The 3′ region (nt 1,433 to 2,246) of the minor parent was "unknown" (likelihoods ranging from 4.2×10^{-14} to 1.7×10^{-25}). However, when the whole "minor parent" region was excised and compared with the GenBank database via BLASTn, it was found to be closest, although with only about 83% nucleotide identity, to those of the A1 isolates from Colombia.

Two Peruvian isolates also had phylogenetically anomalous regions. MZ503961 had a region between nt 892 and 1,336, found by seven methods to be closest to MZ503966 (detected by seven methods with likelihoods of a random cause ranging from 2.7×10^{-4} to 2.8×10^{-12}), and a second region between nt 5,672 and 7,277, also closest to MZ503966 (six methods with likelihoods ranging from 1.1×10^{-4} to 9.0×10^{-11}). The other Peruvian recombinant is MZ503962, which had a single recombinant region closely similar to the second region of MZ503961 from nt 5,505 to 7,194 (six methods with likelihoods ranging from 1.1×10^{-4} to 8.9×10^{-11}). There were also two other Peruvian isolates with sequences (MZ503963 and MZ503964) that have a small recombinant region between nt 6,822 and 7,083 that are most closely related to the homologous region of MZ503928, but this may be a chance similarity because it was detected by fewer than five methods with a chance probability of $>10^{-5}$. For our phylogenetic analyses the three sequences with statistically significant anomalies were removed, leaving an alignment of 56 nonrecombinant sequences.

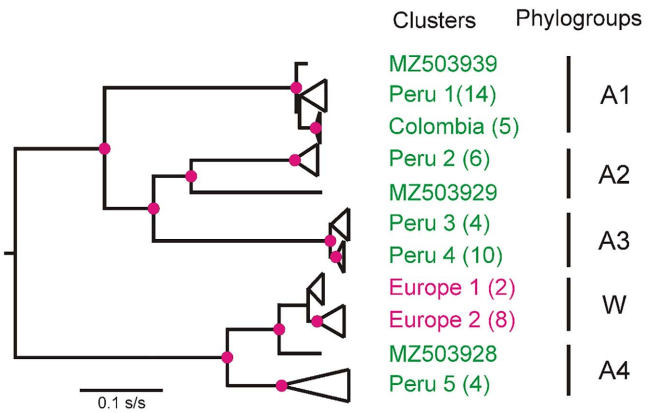

Fig. 2. A maximum likelihood phylogeny of 56 potato virus V nonrecombinant ORF sequences showing the collapsed clusters and three singletons and their assignment to five minor phylogroups; red disks mark the nodes with >0.95 statistical support, and the number of sequences in each cluster is shown in parentheses. Sequences from South American isolates shown in green, and from European isolates in red; details are in Supplementary Table S2. Scale bar: s/s means substitutions/site.

Phylogeny. An ML phylogeny (Fig. 2) of the 56 nonrecombinant ORFs showed that the known world PVV population had two major phylogroups, and these subdivided into five minor phylogroups that had complete statistical support. One major phylogroup had isolates only from the Andes and formed three distinct minor phylogroups, A1, A2, and A3. The other had a basal minor phylogroup of Andean isolates (A4) that was paraphyletic to a crown cluster containing all 11 isolates found outside South America (World [W]). This topology indicates that PVV probably originated in the Andean region in or near Peru, and only one of its major phylogroups spread from there to the remainder of the world (but see discussion of the Iranian recombinant isolate below).

Population genetic structure and test of neutrality. The five minor phylogroups of nonrecombinant ORFs and the 11 individual genes within them were analyzed in DnaSP version 6.10.01. The results (Table 2) show that phylogroups A2 and A4 have much larger average number of differences between sequences (399.7 and 471.1), than the other phylogroups. This is the result of including distinct basal singletons in those populations (MZ503929 and MZ503928, respectively). The π estimates confirmed this difference as populations A2 and A4 are more genetically diverse (0.043 and 0.051, respectively) than the other three PVV minor phylogroups (mean 0.012), as were all their individual genes (Supplementary Table S1). Notably, the P1 and *pipo* genes were the most (0.181) and least (0.076) variable genes, respectively.

As found in most virus genomes, the dN/dS ratios of the polyprotein ORF (Table 2) and the separate genes, except *pipo* in population A2, are <1 (Supplementary Table S1), indicating that they have been under purifying (negative) selection; the smallest dN/dS ratio (mean 0.022) was that of the 6K1 gene, and the largest mean dN/dS ratio (0.591) was that of the *pipo* gene. These values are similar to those found for other potyviruses (Gibbs et al. 2020; Hajizadeh et al. 2019), including the fact that, in most potyviruses, the *pipo* gene is either under positive selection or the smallest negative selection of all potyvirus genes (Gibbs et al. 2020).

We applied Tajima's D^* and Fu and Li's D^* and F^* tests to the polyprotein (Table 2) and individual genes of the PVV population (Supplementary Table S1) to test whether their observed variation was more or less than expected if they had been evolving randomly, and we obtained values for all but one of these metrics, which are not significantly different from those expected by chance. The exception is for the HC-Pro gene in minor phylogroup W, which gave significantly negative values with Tajima's D^* and Fu and Li's D^* and F* metrics, indicating that there had been an increase of the population size after a bottleneck or a selective sweep. Furthermore, although the Tajima's D^* value was significantly negative for the NIa-Pro genes of phylogroup A1, this result was not confirmed by Fu and Li's D^* and F^* tests.

DnaSP version 6 was also used to assess the genetic linkage or gene flow between PVV minor phylogroup populations via the F_{ST} and *Nm* metrics (Hudson et al. 1992; Wright 1943); maximal linkage is indicated by the smallest positive value of F_{ST} and by the largest positive value of Nm. It can be seen (Table 3) that the maximum linkage detected by both metrics was between the A4 and W phylogroup populations; F_{ST} metric 0.583, and the *Nm* metric 0.18, with all other comparisons yielding greater or smaller values, respectively. In Arlequin, the F_{ST} metric values obtained were slightly different from those derived from DnaSP version 6. All were highly significant statistically ($P < 0.000$).

Dating. The collection dates of the isolates supplying 56 of the nonrecombinant PVV ORFs are known (Supplementary Table S2). It was therefore possible to test their ML phylogeny for a temporal signal by the TempEst method. The correlation between the collection date and evolutionary distance was negative with an *x*-intercept (i.e., Time to Most Recent Common Ancestor [TMRCA]) of 25,966 BCE, but this date was not supported statistically (correlation coefficient, 0.142). The residuals graph of this relationship showed a large cluster of anomalous values specifically indicating that the A1 and A3 populations had evolved more quickly than the A2, A4, and W populations, as can also be seen by their branch lengths in Figure 2. These anomalous populations were mostly isolates obtained from the diploid potato species *S. phureja* rather than the tetraploid species *S. tuberosum* (Supplementary Table S2), and, when removed, the remaining 38 gave a TMRCA of 641.7 BCE, although this was still not supported statistically. Analyzing the same populations via the RelTime With Dated Tips method in MEGA-X gave a TMRCA of 1097.2 CE (95% CI, 719.6 to 1362.5 CE) for the node linking the A1 population to the A2 and A3 populations and, by extrapolation, a TMRCA of 731.3 CE for

Table 2. Genetic diversity of the potato virus V open reading frames[a]

Phylogroup	*N*	Hd	S	K	π	SS	NS	dS	dN	dN/dS	Tajima's D^*	Fu and Li's D^*	Fu and Li's F^*
A1	20	1.000	723	143.95	0.016	2,057.51	7,137.49	0.060	0.003	0.046	−1.316 ns	−1.938 ns	−2.043 ns
A2	7	1.000	1221	399.667	0.043	2,056.31	7,138.69	0.168	0.008	0.045	−1.266 ns	−1.285 ns	−1.422 ns
A3	14	1.000	379	94.396	0.010	2,066.73	7,128.27	0.039	0.002	0.045	−0.959 ns	−1.028 ns	−1.160 ns
W	10	1.000	387	95.933	0.011	2,012.00	6,979.00	0.034	0.004	0.116	−1.522 ns	−1.574 ns	−1.765 ns
A4	5	1.000	968	471.10	0.051	2,056.87	7,138.13	0.194	0.010	0.052	−0.074 ns	−0.008 ns	−0.023 ns
All	56	1.000	3127	1069.52	0.119	2,015.49	6,975.51	0.431	0.029	0.067	1.165 ns	0.598 ns	0.985 ns

[a] *N*, no. of sequences; Hd, haplotype diversity; S, no. of segregating sites; K, average no. of nucleotide differences between sequences; π, nucleotide diversity; SS, no. of synonymous sites; NS, no. of non-synonymous sites; dS, synonymous nucleotide diversity; dN, non-synonymous nucleotide diversity; dN/dS: the ratio of non-synonymous nucleotide diversity to synonymous nucleotide diversity; Tajima's D^*, comparison of estimates of the no. of segregating sites and the mean pairwise difference between sequences; Fu and Li's D^*, comparison of the no. of derived singleton mutations and the total no. of derived nucleotide variants (asterisk indicates "without outgroup"); Fu and Li's F^*, comparison of the no. of derived singleton mutations and the mean pairwise difference between sequences (asterisk indicates "without outgroup"); ns = not statistically significant.

Table 3. Genetic differentiation measurement for potato virus V populations[a]

Phylogroup	F_{ST} (DnaSP)	F_{ST} (Arlequin)	*P* (Arlequin)	*Nm*
A1 (n = 20) vs. A2 (n = 7)	0.790	0.834	0.000***	0.07
A1 (n = 20) vs. A3 (n = 14)	0.908	0.905	0.000***	0.03
A1 (n = 20) vs. W (n = 10)	0.924	0.910	0.000***	0.02
A1 (n = 20) vs. A4 (n = 5)	0.799	0.861	0.000***	0.06
A2 (n = 7) vs. A3 (n = 14)	0.780	0.823	0.000***	0.07
A2 (n = 7) vs. W (n = 10)	0.837	0.841	0.000***	0.05
A2 (n = 7) vs. A4 (n = 5)	0.711	0.715	0.000***	0.10
A3 (n = 14) vs. W (n = 10)	0.938	0.929	0.000***	0.02
A3 (n = 14) vs. A4 (n = 5)	0.817	0.876	0.000***	0.06
W (n = 10) vs. A4 (n = 5)	0.583	0.633	0.000***	0.18

[a] *** means significant at $P < 0.001$.

the whole PVV population and of 1945 and 1966 CE for the terminal clusters of the A1 and W phylogroups. Thus, attempts to date the PVV phylogeny directly were inconclusive.

PVV is closely related to PVY (Gibbs et al. 2020), for which well-supported TMRCAs of 156 and 158 CE have been published (Gao et al. 2020; Gibbs et al. 2017). These dates were used to estimate a TMRCA of 166 CE for potato virus A (Fuentes et al. 2021a) via the subtree comparison method described by Hajizadeh et al. (2019) and Mohammadi et al. (2018). This method requires a single ML phylogeny including sequences that represent the basal divergences of their individual phylogenies to be compared. In this way, the phylogenetic diversities of the PVV, PVA, and PVY phylogenies were analyzed. First, an alignment of the representative nucleotide sequences and the amino acid sequences they encoded was used to calculate ML phylogenies. The patristic distances of these phylogenies were compared in PATRISTIC (Supplementary Fig. S1) and showed that, although the slope of the between-species relationships was half that of the within-species relationships, there was no translational saturation (Supplementary Fig. S1). Finally, the patristic distances within the ML nucleotide phylogeny (Fig. 3) were used to calculate the mean pairwise patristic distance of tips connected through the basal nodes of the populations of the four potyviruses of South American plants of the Solanaceae, PVA, PVV, PVY, and WPMV. The extrapolation indicated that the three main viruses of potato had similar TMRCAs; PVY 157 CE, PVV 29 BCE, and PVA 166 CE, a range of ± about 6%. Furthermore, the basal divergence of the W phylogroup of PVV was estimated to be about 1863 CE.

Discussion

As previously found with our research on other viruses of potatoes, phylogenetic analysis of genomic PVV sequences from the potatoes' center of domestication in the Andean region of South America again revealed far greater diversity there than found elsewhere. With PVV, this diversity included a major phylogroup consisting solely of isolates from the Andean region and another not only of isolates from the Andean region but also of more recently diversified ones from elsewhere (in this case Europe). Therefore, like PVY (Fuentes et al. 2019) and PVA (Fuentes et al. 2021a), PVV first infected potato crops when potatoes were domesticated in the Andean region of South America. All three of these potyviruses are transmitted nonpersistently by aphids during short feeds and vegetatively in infected potato tubers. Also, they were subjected to similar selective forces during the crop domestication process in which potato landraces were selected from wild potato species. The TMRCA of the PVV population was closely similar to those found earlier with the other two potato potyviruses, PVY and PVA. We therefore conclude that PVV's prehistory is similar to that of PVA and PVY and that it has been influenced by the same historical events.

In our analysis, all five PVV isolates from Colombia formed a distinct cluster on a long branch of the A1 population, and all but one of them came from *S. phureja*. In addition, 14 other isolates from S. *phureja* form the A3 population and are also on a long branch. These long branch lengths indicate that *S. phureja* isolates are evolving more quickly than other PVV isolates. As with the nonpotato PVY isolates in major phylogroup PVY^{C} (Fuentes et al. 2019; Gibbs et al. 2017), this makes dating more difficult. The subtree comparison method (Hajizadeh et al. 2019; Mohammadi et al. 2018) previously used to date PVA (Fuentes et al. 2021a) was therefore used again with PVV. Despite fewer sequences being available, the estimated dating we obtained (29 BCE) is close to those for PVY (157 CE) and PVA (166 CE). It is important to realize that the TMRCA of a virus population is not necessarily the date when the virus first established itself within a host population but is an estimate of the age of the single original parent of the two oldest lineages that are present among the samples. Thus, the TMRCA may also represent the date when a host population became large enough to maintain two lineages until the present day. The three viruses may have entered the potato crops independently during the preceding 6,000 years, possibly on more than one occasion.

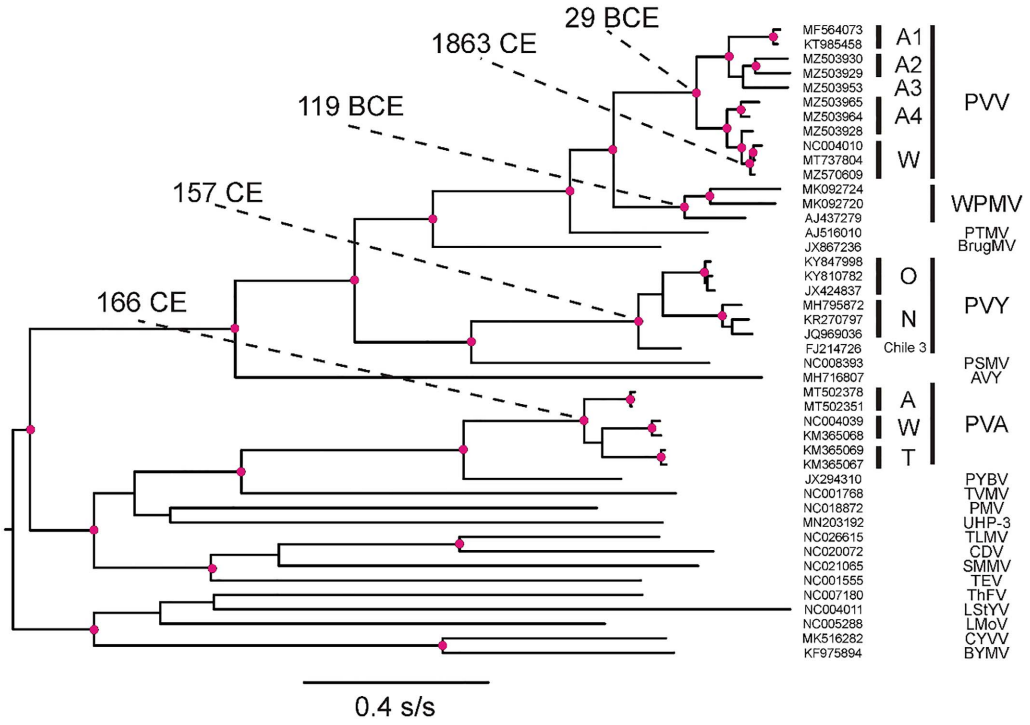

Fig. 3. A maximum likelihood phylogeny of the nonrecombinant principal open reading frame (ORF) sequences of representative isolates of potato virus V (PVV), wild potato mosaic virus (WPMV), potato virus Y (PVY) and potato virus A (PVA), and of appropriate outgroup viruses namely arracacha virus Y (AVY), Brugmansia mosaic virus (BrugMV), bean yellow mosaic virus (BYMV), Colombian datura virus (CDV), clover yellow vein virus (CYVV), lily mottle virus (LMoV), leek yellow stripe virus (LYStV), pokeweed mosaic virus (PMV), pepper severe mosaic virus (PSMV). Peru tomato mosaic virus (PTMV), potato yellow blotch virus (PYBV), sunflower mosaic virus (SMMV), tobacco etch virus (TEV), Thunberg fritillary virus (ThFV), tamarillo leaf malformation virus (TLMV), tobacco vein mottle virus (TVMV), and Potyvirus UHP-3 (UPH-3). Nodes with a red dot are fully statistically supported (SH = 1). The average patristic distances of all tips linked through basal nodes of the PVV, WPMV, and PVA populations were used to calculate possible dates by extrapolation from the well-supported date of 157 CE for the basal node of PVY.

Therefore, the fact that their TMRCAs are so similar suggests that they record a change that occurred almost two millennia ago in the potato crops they shared. The estimated TMRCAs for the three viruses coincide with the Tiahuanaco Empire's early formative period between 110 and 300 CE in the Lake Titicaca regions (Browman 1978; Fuentes et al. 2019, 2021a). Potato growing expanded in Bolivia and southern Peru during the Tiahuanaco Empire, which continued until approximately 1000 CE (Browman 1978; Hawkes 1978, 1990). In 1526 CE, the Spaniards invaded Peru, and the Columbian Exchange (Nunn and Qian 2010) commenced, which included introduction of the potato from the Andean region to Europe and later to other continents (Hawkes 1978, 1990). Potato viruses were introduced elsewhere with the transported potato tubers (Glendinning 1983). The phylogenies of all three potato potyviruses (PVA, PVV, PVY) revealed a recent major diversification in their populations (Fuentes et al. 2019, 2021a and this study) that occurred shortly after the European potato late blight (*Phytophthora infestans*) pandemic in 1845 to 1849 CE (Donnelly 2002; Zadoks 2008). This pandemic decimated European crops because the potato cultivars grown then were all inbred and highly blight susceptible (Glendinning 1983). The subsequent PVY and PVA diversification coincided with release of new potato cultivars bred from diverse potato germplasm introduced to Europe from the Andean region and the Isle of Chiloe to breed for blight resistance (Fuentes et al. 2019, 2021a; Glendinning 1983; Gutaker et al. 2019). With PVV, this same diversification is illustrated graphically by the sudden appearance of minor phylogroup W. A similar diversification of European PVS and PVX populations also occurred shortly after the potato late blight pandemic (Duan et al. 2018; Fuentes et al. 2021b; Santillan et al. 2018). Interestingly, when F_{ST} and *Nm* metrics were used to measure the genetic linkage between the different PVV minor phylogroup populations, the greatest linkage was between A4 and W, reflecting W's divergence from A4 in Europe. Moreover, the significantly negative values obtained with the Tajima's D^* and Fu and Li's D^* and F^* metrics in the HC-Pro gene of W isolates suggested an increase of the population size after a bottleneck.

Only three of the 59 genomic sequences of PVV had recombinant genomes (5%), two Peruvian and one Iranian. By contrast, nine of 63 PVA genomes were recombinants (14%) (Fuentes et al. 2021b), and 270 of 460 PVY genomes were recombinants (59%) (Fuentes et al. 2019). This large difference in recombination rates may reflect sampling bias rather than world incidence, because the recombinants of PVY are predominantly of non-Andean origin (223 of 460 [48%]) and were from the R1 and R2 phylogroups, which arose in Europe and attracted the attention of potato pathologists as they caused potato tuber necrotic ringspot disease, whereas only 47 (10%) were intraphylogroup or other interphylogroup recombinants and hence similar in frequency to those found for PVV and PVA.

Although minor phylogroup W was the only one of PVV's five nonrecombinant isolate minor phylogroups found, for certain, infecting potatoes outside the Andean region, the Iranian recombinant (KC433411) has a minor "parent" that is distantly related (about 84% identity) to isolates of the A1 minor phylogroup. This indicates that PVV isolates from the A1 minor phylogroup may already be present outside South America. This finding is significant because our analyses have shown that the most distantly related PVV isolates (i.e., those of the A1, A2, and A3 minor phylogroups vs. those of the A4 and W minor phylogroups) are as evolutionarily distant (about 4,000 years of separate evolution) as the parents of the damaging necrogenic R1 and R1 recombinant phylogroups of PVY (Fuentes et al. 2019; Gibbs et al. 2017). This evolutionary distance is about 4,000 years long because each of the lineages that goes back to the single ancestor at the TMRCA is about 2,000 years in duration, so there are 4,000 years of evolution separating the current isolates of the two basal lineages. Therefore, the possibility exists that, both in South America and, separately, in the other major potato growing countries of the world, recombinants of PVV may be generated that are potentially as damaging as the necrogenic PVY isolates causing the potato tuber necrotic ringspot disease.

The only major biological study of an Andean PVV isolate, which was with Peruvian isolate UF, was by Fribourg and Nakashima (1984). This included establishing the phenotypes that developed in wild potato species and in a range of potato cultivars from different countries. Unfortunately, no preserved specimen of this isolate remains, so its genes have never been sequenced. Limited biological research was done more recently with two other Peruvian isolates by Spetz et al. (2003), who suggested that introduction of a strain group PVV-2 isolate may pose a biosecurity risk to European potato crops. The same might also be so for putative strain group PVV-3. Moreover, our findings demonstrate far greater genetic diversity within Andean PVV isolates in minor phylogroups A1 to A4 than in the isolates from elsewhere in minor phylogroup W. This finding suggests that biological studies are needed with isolates from minor PVV phylogroups A1 to A4 to determine whether they pose a potential biosecurity risk to potato production should they become established elsewhere in the world. Moreover, because 19% of samples from the Peruvian potato virome contained PVV, this implies it may occur commonly, so such studies with A1 to A4 in potato cultivars and landraces are also needed in Andean countries to establish their importance there. The same applies to PVV's recombinant isolates, one of which, the Iranian recombinant with an Andean parental sequence, has already spread outside the Andes. Furthermore, unless such biological studies with PVV's Andean minor phylogroup isolates and recombinants suggest otherwise, plant biosecurity and quarantine organizations of non-Andean region countries should consider undertaking surveillance to determine their occurrence and adopting precautionary measures to minimize their spread should they become established.

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