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Biological and molecular characterization of Potato yellow blotch virus, a new species of the genus *Potyvirus*

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A new species of the genus *Potyvirus* infecting potatoes, with the proposed name Potato yellow blotch virus (PYBV), was discovered in a breeding line 99m-022-026 in Scotland. The infected plants show isolated yellow blotches on the leaves. The genome of PYBV contains a large open reading frame encoding a single polyprotein of 3054 amino acids. Sequence analysis shows that PYBV is closely related to *Potato virus A* (PVA), with an overall 72% identity at the nucleotide level for the whole genome. The least conserved P1 protease gene shares only 50% nucleotide identity with PVA. The host range of PYBV was comparable to PVA on solanaceaous and non-solanaceous indicator plant species with the exception of *Solanum demissum* A and Y. Different symptoms were also observed for PYBV and PVA in *Nicotiana benthamiana*, *Nicotiana hesperis* and *Nicotiana occidentalis* P1. The susceptibility of potato (*Solanum tuberosum*) cultivars to PYBV and PVA was similar. In over 5 years of investigation, PYBV has not been found in commercial seed and ware potato crops in Scotland.

Keywords: diagnosis, host range, incidence, new potyvirus, potato, Potato yellow blotch virus

Introduction

Viruses have co-evolved with their hosts in order to adapt to many ecological niches including wild and agricultural ecosystems (Alexander *et al.*, 2014). While the centre of origin and cultivation of the potato (*Solanum tuberosum*) is primarily located in the Andean region of South America, this region is also believed to be the origin of most of the viruses that cause economic losses in potato (Salazar, 1996). At least 40 viruses in 20 genera, including the genus *Potyvirus*, have been reported worldwide to infect potato (Jeffries *et al.*, 2006). Although many potyviruses infect potatoes experimentally, only four of them, *Potato virus A* (PVA), *Potato virus V* (PVV), *Potato virus Y* (PVY) and *Wild potato mosaic virus* (WPMV) are currently known to infect potato naturally (Jeffries, 1998). While PVY is considered to be the most important virus infecting potato crops worldwide (Valkonen, 2007), yield losses caused by PVA can also be significant, reaching up to 40% in extreme cases, with greater losses in mixed infection with *Potato virus X* (PVX; Hooker, 1981).

The genus *Potyvirus* (family *Potyviridae*) has a plus-sense single-strand (ss)RNA polyadenylated genome encapsidated in flexuous filamentous particles of 680–900 × 11–13 nm (Hull, 2001). The genome organization of PVY (type member of the genus) is composed of two open reading frames (ORFs), a major ORF encoding a single large polyprotein (*c.* 340–370 kDa) cleaved into 10 functional proteins (Danci *et al.*, 2009) and an additional protein (PIPO) generated by a +2 frameshift within the P3 ORF (Olspert *et al.*, 2015). Potyviruses are transmitted by aphids in a nonpersistent manner and maintained by vegetative/tuber propagation in potato (reviewed in Katis *et al.*, 2007).

Cultivated and wild potato species display various levels of resistance and susceptibility towards different potyvirus species and strains (Valkonen, 1994; Rajamäki *et al.*, 1998). Within cultivated potatoes, it is widely acknowledged that the emergence of PVY^{N} and PVY recombinant strains, such as PVY^{NTN}, worldwide is in part due to their ability to overcome host resistance mechanisms, such as the resistance genes *Nc* and Ny_{tbr} mediating a hypersensitive response (HR) to nonrecombinant PVY^{C} and PVY^{O} (Karasev & Gray, 2013). The biological properties of other closely related potyviruses, such as PVA, based on their symptomatology on potato cultivars are not well documented. Field inspection surveys undertaken in the UK indicate that PVY and PVA are the two most prevalent viruses infecting potato, accounting for 50–60% and 10–20% of all viruses identified in virus-infected plants, respectively (Fenton *et al.*, 2012; Davie *et al.*, 2017). PVY infects a large number of commonly grown potato cultivars but only a few cultivars such as Cabaret, Désirée, Estima and Hermes are found to be naturally infected by PVA. Cabaret accounts for the majority of crops infected with PVA, but Estima crops have the highest incidence (Fenton *et al.*, 2012).

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In 2008 a breeding line (99m-022-026) growing in the UK (Scotland), displaying unusual yellow symptoms on the foliage that could have been mistaken for *Potato aucuba mosaic virus* (PAMV), *Potato mop top virus* (PMTV) or *Tobacco rattle virus* (TRV), was further investigated to identify the causal agent, referred to as HG12 (Nisbet *et al.*, 2013). This paper describes the characterization of this agent as a new potyvirus with the proposed name Potato yellow blotch virus (PYBV).

Materials and methods

Virus isolates

PYBV-infected microplants of potato breeding line 99m-022-026 (SASA isolate reference QV276) originating from a breeding station in England, and PVA (QV282; GenBank accession KF539821) were used for the host range studies.

Serological detection

The breeding line was tested using double antibody sandwich (DAS)-ELISA for 19 viruses covering both quarantine (EPPO A1 and A2 list) and regulated non-quarantine viruses. All antibodies used were from SASA (mostly monoclonal antibodies (Mabs) unless specified otherwise: *Andean potato latent virus* (APLV) and *Andean potato mottle virus* (APMV; both polyclonal antibodies (Pabs); Prime Diagnostics), *Arracacha virus B*-oca (AVB-O), PAMV (Pab;), *Potato black ringspot virus* (PBRV), *Potato latent virus* (PotLV), *Potato leafroll virus* (PLRV, Pab), PMTV, PVA (mix of Mab cell lines 58/0+58/6), *Potato virus M* (PVM), *Potato virus P* (PVP, Pab; INRA), *Potato virus S* (PVS, Pab and Mab), *Potato virus T* (PVT), PVV, PVX, PVY, Potato yellowing virus (PYV, Pab; CIP), *Tomato black ring virus* (TBRV) and *Tomato spotted wilt virus* (TSWV, Pab; LOEWE). Additionally, a PVA Pab (Prime Diagnostics) and a PVA + PYBV Mab (mix of Mab cell lines 58/0+58/6+58/7 raised against PVA) were evaluated.

Molecular analysis and detection

The breeding line was tested by real-time reverse transcription (RT)-PCR for TRV (Mumford *et al.*, 2000) and conventional RT-PCR with generic primers to detect the following genera: *Carlavirus* (Badge *et al.*, 1996), *Potexvirus* (van der Vlugt & Berendsen, 2002) and *Potyvirus* (Gibbs & Mackenzie, 1997). Amplicons obtained by the latter assay were partially sequenced using generic primers designed to amplify a conserved region of the coat protein (CP; Gibbs & Mackenzie, 1997). To generate a full-length genome sequence of the new potyvirus, specific sequential primers were designed to the PVA genome (GenBank CAC17411) to give a series of overlapping amplicons (*c.* 800 bp each). At least two products were produced that covered each region of the viral genome. The 5′-end of the genome was obtained using a 5′-RACE kit (Invitrogen).

Phylogenetic analysis of the full-length genome sequences of the new virus and other related potyviruses was performed using the CLUSTALW algorithm (Thompson *et al.*, 1994) in MEGA v. 5 (Tamura *et al.*, 2011). Phylogenetic trees were constructed using the neighbour-joining method (replicas bootstrapped to 1000, cut-off set to 60%).

In order to confirm data obtained by conventional techniques and whether only one virus was present, PYBV-infected microplants of the breeding line were analysed using high-throughput sequencing. Small RNAs were extracted from infected plants using TRIzol reagent (Life Technologies) (Kreuze *et al.*, 2009), and the library was prepared according to Chen *et al.* (2012). Sequencing was performed by Argonne National Laboratory (Ilinois, USA) on an Illumina HiSeq2500 system. Bioinformatic analysis was performed with the VIRUSDETECT software package (Zheng *et al.*, 2017).

Primers and probes for specific detection of PYBV and PVA in real-time RT-PCR were designed using PRIMER EXPRESS v. 1 software (Applied Biosystems).

Immunosorbent electron microscopy

Immunosorbent electron microscopy was carried out to measure virus particles using a PVA Pab (Prime Diagnostics) diluted 1:500 in 70 mM Sörensen's phosphate buffer (pH 6.5) and incubated at room temperature for 1 h. After washing, drops of sap (two-fold dilution in the above buffer) were placed on carbon-coated grids and incubated overnight at 4 °C. Grids were washed with deionized water and stained with 1% uranyl acetate. Virus particles were examined using a Zeiss 900 transmission electron microscope and measured using ANALYSIS v. 3.0 soft imaging system software (Olympus).

Host range

Potato cultivars and herbaceous indicator plants (Jeffries, 1998) were grown with a 16 h photoperiod at *c.* 20 °C (day)/15 °C (night). Thirty-five of the most widely grown potato cultivars in Scotland by seed acreage were grown from pathogen-free *in vitro* plantlets obtained from the SASA potato nuclear stock collection. Plants were inoculated mechanically, as previously described (Browning, 2009) with sap extract from PYBV isolate QV276 and PVA isolate QV282 propagated in *Nicotiana benthamiana* (1:5 w/v in water). Three or four potato plants per cultivar (six leaves per plant) were inoculated for each virus, and two plants per cultivar were inoculated with sap from healthy potato (cv. Atlantic) as a control. For the herbaceous indicators, at least four plants of each species (two leaves per plant) were inoculated. Symptoms were recorded at weekly intervals and virus infection was confirmed 6 weeks after inoculation using DAS-ELISA. To assess virus transmission to the tubers, six tubers from each of 20 cultivars were sprouted, and the sprouts were tested using ELISA (some confirmed by real-time RT-PCR). Tubers from five of these cultivars were planted and inspected for symptoms of PYBV.

Monitoring of viruses in potato crops

Leaf samples from 1884 seed crops and 30 ware crops displaying a range of virus symptoms (e.g. mild mosaic, severe mosaic, leaf roll, stunting, rugosity, chlorotic/yellow blotches) submitted from official growing crop inspections in Scotland in 2012–16 as part of the Seed Potato Certification Scheme (SPCS) were tested for 10 viruses (PLRV, PMTV, PVA, PVM, PVS, PVV, PVX, PVY, PYBV and TBRV) using DAS-ELISA as previously described, and for TRV by real-time RT-PCR (Lacomme *et al.*, 2015). In addition, tubers of Estima (susceptible to PYBV), tested annually to ensure compliance with SPCS statutory tolerances for PVA, PVV and PVY in the direct progeny, were also tested for PYBV over a 5-year period together with tubers of 16 other cultivars submitted for virus testing. Detection was either

Table 1 List of oligonucleotides and probes used for PYBV and PVA detection.

Primer	Sequence (5′–3′)	Region amplified	Method
PYBV-NIb F	AGACGACTTGTTGTTGGCAATAGA	PYBV NIb region, 7989–8060 bp	Real-time RT-PCR PYBV detection
PYBV-NIb R	TCGGCAAAGAATTCAGAGAACTT		
PYBV-NIb P	FAM-CCCACCCACCATGAGTGCTTG-BHQ1		
PYBV-CP F	GATGACTCAAAGAAGCAGGATGTC	PYBV CP region, 7358–7433 bp	Real-time RT-PCR PYBV detection (survey of potato)
PYBV-CP R	GAGTCCCAGTTGTACCCAAGTCTAC		
PYBV-CP P	FAM-CAGTGGCGGCAACCAAGCAACCT-BHQ1		
PVA-F	AGCAGCAGTTGGTGCATTGTA	PVA NIb region (NC_004039), 7358–7433 bp	Real-time RT-PCR PVA detection
PVA-R	GTGTTCTCTCTCACTAGGGCTCAC		
PVA-P	FAM-AGAGGGAAGAAGCGCGATTACTTTGACG-BHQ1		

NIb, nuclear inclusion b protein; CP, coat protein; RT, reverse transcription.

by testing eye-plug plants grown from 120 tubers from each crop (i.e. from a single field) by ELISA, or by direct testing of the tubers using real-time RT-PCR with specific primers/probes for PYBV (Table 1), PVA, PVV and PVY according to Lacomme *et al.* (2015).

Results

Symptoms on potato plants

PYBV was originally found in the potato breeding line 99m-022-026, where symptoms of yellow blotches were observed (Fig. 1). Yellow blotches were obvious on older leaves, while newly developed leaves displayed mild or no symptoms. Blotches were visible along veins, often on the distal part of the leaf.

Serological detection of PYBV

Leaves from the breeding line 99m-022-026 displaying yellowing were negative for the 19 potato viruses described previously when tested using DAS-ELISA, including use of a Mab for PVA (mix of Mab cell lines 58/0+58/6). Evaluation of a further SASA PVA Mab (58/7) originating from a different cell line found that it detected both PYBV and PVA. Additional tests

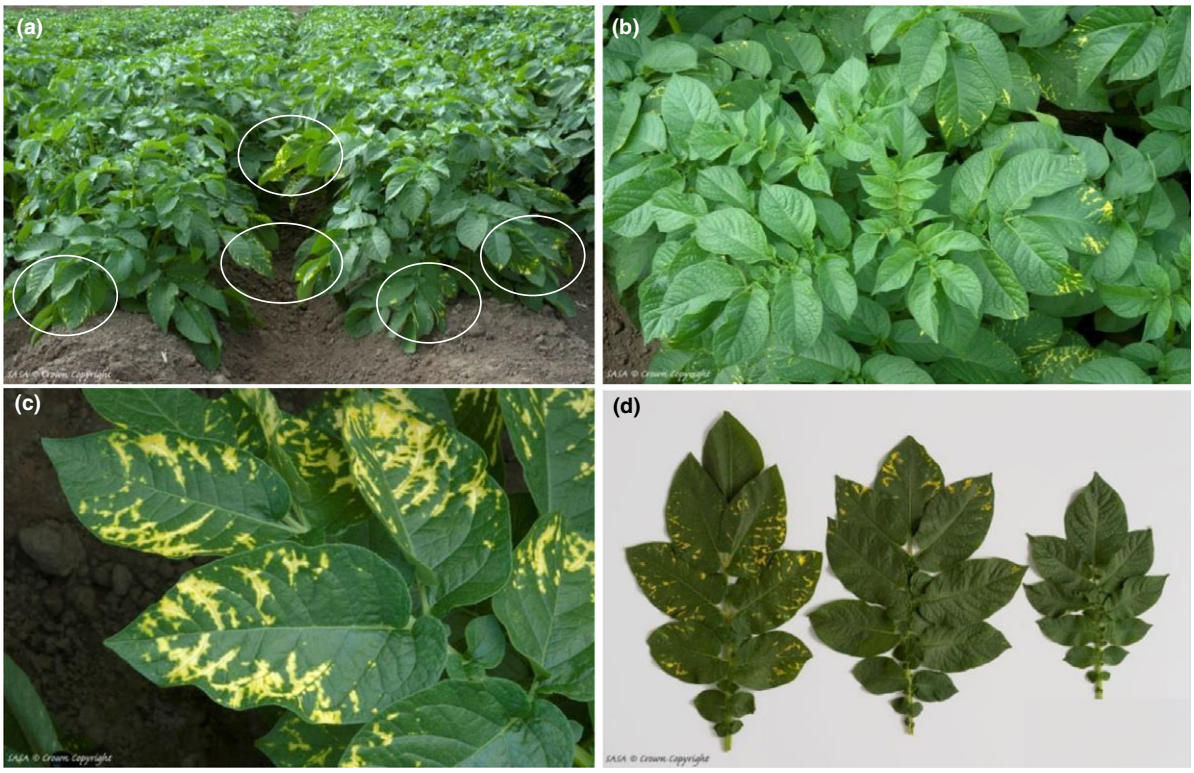
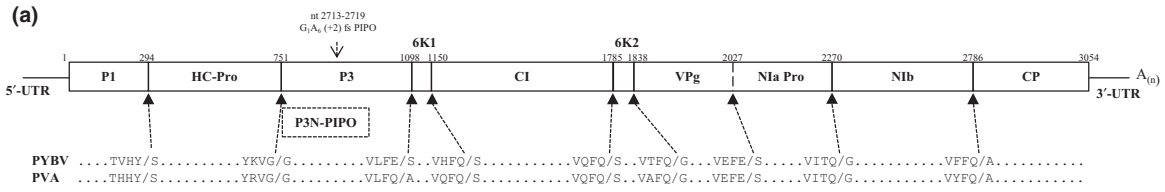


Figure 1 PYBV symptoms on field-grown potato breeding line 99m-022-026. Blotches, circled in (a), close-ups in (b) and (c), are more obvious on older leaves (d, left and centre), while younger leaves (d, right) do not display symptoms.



(b)

	Whole genome	5′-UTR	P1	HC-Pro	P3	6K1	CI	6K2	VPg	NIaPro	NIb	CP	3′-UTR
AA	77.4 / 77.6	n.a.	40.6 / 41.0	84.3 / 85.6	74.9 / 77.2	88.5 / 90.4	85.7 / 86.8	69.7 / 71.7	78.8 / 82.5	77.4 / 80.2	73.3 / 76.2	82.1 / 83.6	n.a.
Nt	71.5 / 71.9	67.2 /71.9	49.4 /50.6	74.4 / 75.2	72.7 / 74.0	71.8 / 76.9	75.0 / 76.3	66.0 / 69.2	73.2 / 73.7	72.7 / 74.5	68.2 / 69.8	77.0 / 77.8	76.8 / 79.2
Cut-off	n.a.	n.a.	**58**	**76**	**74**	n.a.	**78**	n.a.	**76**	**76.5**	**75**	**76**	**76**

Figure 2 Genome organization of PYBV and its comparison with PVA isolates. (a) The PYBV genome (not to scale) is represented with predicted protein cleavage sites and associated translation product P3N-PIPO. (b) Percentages of the nucleotide (Nt) and deduced protein sequence (AA) identities between PYBV and PVA (eight isolates in NCBI database: Z21670, AF543212, AF543709, AJ131400, AJ131401, AJ131402, AJ131403 and KF977085). Each value represents the lowest and highest percentage of identity for each gene between PYBV and all the PVA isolates analysed. For each individual gene and noncoding region, the nucleotide identity cut-off value (%) for species demarcation (Wylie *et al.*, 2017) is indicated.

performed using a commercially available PVA Pab (data not shown) and SASA PVA + PYBV (mix of Mab cell lines 58/0+58/6+58/7) showed reproducible detection of PYBV in PYBV-infected plants including the breeding line and the mechanically inoculated indicator plants *N. benthamiana* and *Nicotiana tabacum* 'White Burley', whereas SASA PVA (mix of Mab cell lines 58/0+58/6) only detected PVA (Fig. S1).

Genome structure of PYBV and phylogeny

The complete genome of PYBV was determined to be of 9518 nucleotides excluding a polyA tail (GenBank accession AFS28882), containing a single large ORF coding for a polyprotein of 3054 amino acids with an initiation codon at position 130–132 and a UAA stop codon at position 9292–9294. The consensus cleavage site for each protein (Adams *et al.*, 2005) is presented in Figure 2a. In addition to the 10 predicted proteins from the cleavage of the polyprotein, the putative P3N-PIPO (Chung *et al.*, 2008) protein is 232 amino acids in length with a calculated molecular weight of 26.1 kDa. The conserved nucleotide sequence G_1A_6 introducing a +2 reading frame shift in the P3 gene was identified at position 2842–2848. The PYBV genome has the cognate domains associated with aphid-transmitted viruses,

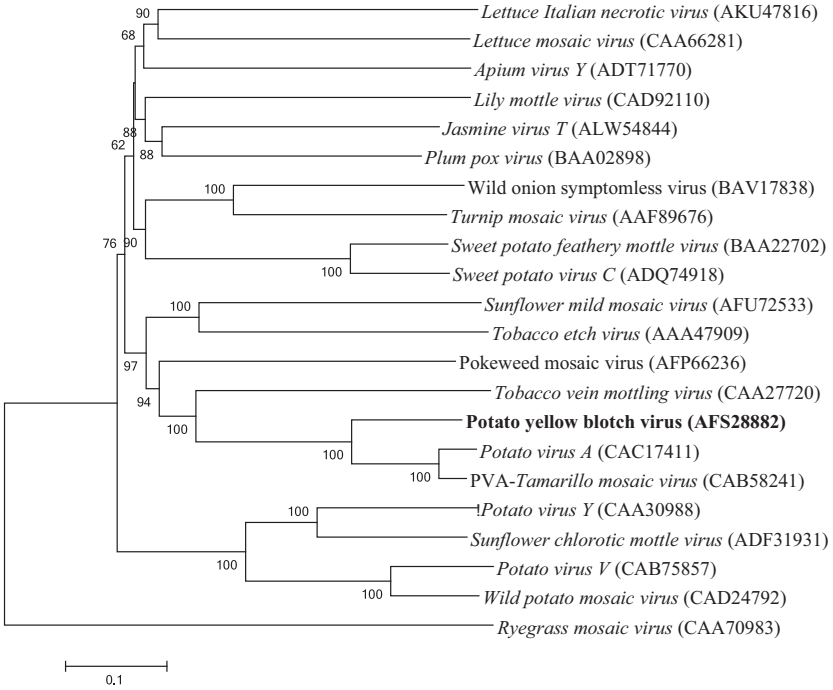


Figure 3 Phylogenetic relationship of PYBV and other potyviruses. The phylogenetic tree was generated from an alignment of complete polyprotein sequences of PYBV with related potyviruses. *Ryegrass mosaic virus* was used as the out-group.

including the DAG motif located in the CP at amino acid positions 2790–2792 and the KITC motif located in the HC-Pro at amino acid positions 344–347 (Table S1 and references therein). A BLASTX search of the NCBI database identified PVA as the closest relative (Fig. 3). Phylogenetic analysis of the PYBV genome together with related viruses within the genus *Potyvirus* shows that it is a member of the *Tobacco etch virus* group, with PVA as the closest relative (Fig. 3). The analysis also showed the dissimilarity with the other potato-infecting potyviruses, i.e. PVV, PVY and WPMV, that fell into more distant clades.

Sequence comparison with other potyviruses

Sequence comparison was performed using the complete genome nucleotide sequence and deduced amino acid sequences of PYBV and eight PVA isolates (GenBank accessions Z21670, AF543212, AF543709, AJ131400, AJ131401, AJ131402, AJ131403 and KF977085; Fig. 2b). PYBV had less than 72% identity with other PVA isolates at the nucleotide level for the whole genome, which is well below the 76% identity cut-off for species demarcation (Wylie *et al.*, 2017). The PVA-TamMV isolate shared 84% nucleotide sequence identity with the other PVA isolates and 71.5% with PYBV. Identities lower than the individual protein cut-off demarcation values, supporting classification of PYBV as a distinct species, were obtained for the P1, HC-Pro, CI, VPg, NIaPro and NIb; while the P3 and CP had sequence identities above the species demarcation criteria as did the 3′-UTR. The lowest sequence identity between PYBV and PVA was found with the P1 protein, 41% at the amino acid sequence level. Other differences between PYBV and PVA were found also in the length of the coding and untranslated regions. The biggest difference was seen with the 5′-UTR where PYBV was considerably shorter (129 nt) than for Z21670 (161 nt) and AJ131403 (169 nt). Other differences were seen with the P1 protein, which is composed of only 293 aa for PYBV compared to 298 aa for other PVA isolates, while the PYBV CP (268 aa) is similar to all previously mentioned PVA CP (269 aa) and PVA-TamMV CP (AJ131403, 268 aa) isolates. The 3′-UTR is also slightly shorter in PYBV (224 nt) compared to all mentioned PVA (227 nt) and PVA-TamMV (229 nt) isolates. Comparison of the cleavage sites of the polyprotein of PYBV and PVY showed a difference between the P3 and 6K1 proteins with a predicted E/S cleavage site for PYBV and Q/A site for PVA.

Molecular detection of PYBV

Primers and probes designed for the detection of PYBV (Table 1) were specific for its detection when tested against a range of plant species infected with other potyviruses (PVA, PVV, PVY^{C}, PVY^{NTN} PVY^{O}; Table S2).

Virion morphology

Electron microscopy on preparations of sap from PYBV-infected *N. benthamiana* plants showed flexuous filamentous particles (Fig. 4a). From the measurements of 98

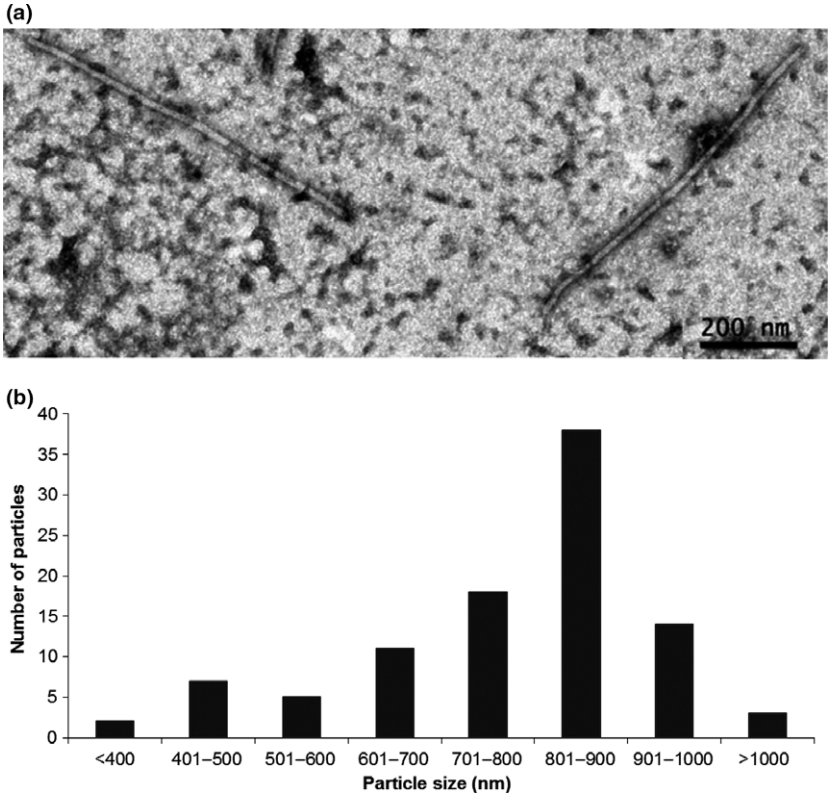


Figure 4 PYBV virions and length distribution. (a) Electron micrograph of negatively stained PYBV viral particles from immunosorbent electron microscopy. (b) Length distribution of PYBV particles in plant sap.

particles, 70 particles were in the length range of 701–900 nm (average 848 nm) The largest group of virions (38 particles) had a size distribution of 801–900 nm (average 853 nm; Fig. 4b).

Host range and symptoms

For all plant species inoculated, similar results were obtained for PYBV and PVA with the exception of *Solanum demissum* A and Y, *Nicotiana hesperis* and *N. benthamiana*. In *S. demissum* A and Y, PYBV caused a symptomless infection whereas PVA caused a HR with necrotic spots on the inoculated leaves, for which the ELISA result was negative (Table 2). In *N. benthamiana*, PYBV induced systemic symptoms of leaf curling and vein clearing, but PVA infection was symptomless. The only other indicator plants to produce symptoms were *N. hesperis* (systemic leaf curling and vein clearing by PYBV and local chlorotic spots and systemic leaf distortion, vein clearing and stunting by PVA) and *Nicotiana occidentalis* P1 (local necrotic lesions and systemic vein clearing by both PYBV and PVA).

Susceptibility of potato cultivars

PYBV and PVA were not detected in the foliage of 19 potato cultivars after mechanical inoculation: Atlantic, Burren, Cara, Charlotte, Fambo, Harmony, King Edward, Lady Rosetta, Maris Peer, Maris Piper, Pentland Crown, Pentland Dell, Rooster, Saturna, Saxon, Shepody, Slaney, Valor and Wilja (Table 3). Both viruses were detected in 15 cultivars: Banba, Cabaret, Casablanca, Désirée, Estima, Hermes, Kennebec, Marfona, Maris Bard, Markies, Melody, Russet Burbank, Vales Sovereign, Winston and Yukon Gold. In contrast, Fontane was susceptible to PYBV and resistant to PVA, with no observed symptoms in both cases.

In 19 out of 20 potato cultivars tested, PYBV infection or noninfection of tuber sprouts corresponded to whether the mother plant was infected. However, for Fontane, PYBV was only detected in the foliage, not in tuber sprouts. PVA was detected in 18 out of 20 cultivars, with Kennebec and Yukon Gold showing infection of the foliage but not tuber sprouts.

Of the cultivars Estima, Hermes, Russet Burbank and Yukon Gold, testing positive for PYBV and planted to assess for secondary symptoms, only Estima and Russet Burbank developed symptoms. The yellow blotches were observed in younger plants and remained on older leaves, while new emerging leaves did not display symptoms (data not shown). However, in Estima the localized chlorotic symptoms reappeared on new growth. The symptoms produced were not as severe as those seen in the field for breeding line 99m-022-026 (data not shown).

Table 2 Susceptibility of indicator plant species to mechanical inoculation with PYBV isolate QV276 and PVA isolate QV282, as determined by the symptoms produced and ELISA.

	PYBV		PVA	
Indicator plant species	Symptoms[a]	ELISA[b]	Symptoms[a]	ELISA[b]
Chenopodium amaranticolor	–	–	–	–
Chenopodium murale	o	L, +	o	L, +
Chenopodium quinoa	o	L, +	o	L&S, +
Datura innoxia	o	L&S, +	o	L&S, +
Datura metel	o	L&S, +	o	L&S, +
Datura stramonium	–	–	–	–
Nicandra physaloides	o	L&S, +	o	L&S, +
Nicotiana benthamiana	lc, vc	L&S, +	o	L&S, +
Nicotiana bigelovii	o	L&S, +	o	L&S, +
Nicotiana clevelandii	o	L&S, +	o	L&S, +
Nicotiana debneyi	o	L&S, +	o	L&S, +
Nicotiana edwardsonii	o	L&S, +	o	L&S, +
Nicotiana glutinosa	o	L, (+)	o	L, +
Nicotiana hesperis	lc, vc	L&S, +	cs, ld, st, vc	L&S, +
Nicotiana occidentalis P1	ns, vc	L&S, +	ns, vc	L&S, +
Nicotiana rustica	o	L&S, +	o	L&S, +
Nicotiana tabacum 'Samsun'	o	L&S, +	o	L&S, +
N. tabacum 'White Burley'	o	L&S, +	o	L&S, +
N. tabacum 'Xanthi'	o	L&S, +	o	L&S, +
Solanum demissum A	o	L&S, +	ns	–
S. demissum Y	o	L&S, +	ns	–
Solanum lycopersicum 'Moneymaker'	o	L&S, +	o	L&S, +

[a](–) no symptoms and negative by ELISA; (o) symptomless infection confirmed by ELISA. Local symptoms: necrotic spots (ns), chlorotic spots (cs); systemic symptoms: leaf curling (lc), leaf distortion (ld), stunting (st), vein clearing (vc).

[b]All plants were tested by ELISA: inoculated leaves (local infection, L); inoculated and uninoculated leaves (local and systemic infection, L&S); + all plants infected, (+) occasional plant infected, – plants not infected.

Table 3 Infectivity of PYBV (isolate QV276) and PVA (isolate QV282) on a range of potato cultivars determined by ELISA.

Cultivar	PYBV		PVA		PVA resistance rating[c]
	Leaf[a]	Tuber[b]	Leaf[a]	Tuber[b]	
Atlantic	–	nt	–	nt	H
Banba	++	nt	+	nt	
Burren	–	–	–	–	
Cabaret	+	nt	+	nt	
Cara	–	nt	–	nt	L-H
Casablanca	+++	nt	+++	nt	
Charlotte	–	nt	–	nt	H
Désirée	+++	nt	+++	nt	M-H
Estima	+++	+	+++	+	L-H
Fambo	–	nt	–	nt	H
Fontane	+	–	–	–	
Harmony	–	–	–	–	
Hermes	+++	nt	+++	nt	H
Kennebec	+++	+	+++	–	H
King Edward	–	–	–	–	H
Lady Rosetta	–	–	–	–	
Marfona	+++	nt	+++	nt	H
Maris Bard	+++	nt	+++	nt	L
Maris Peer	–	–	–	–	H
Maris Piper	–	–	–	–	H
Markies	+	nt	+	nt	H
Melody	+++	+	+	+	
Pentland Crown	–	nt	–	nt	
Pentland Dell	–	–	–	–	H
Rooster	–	–	–	–	
Russet Burbank	+++	+	+++	+	L
Saturna	–	–	–	–	L-H
Saxon	–	–	–	–	
Shepody	–	–	–	–	H
Slaney	–	–	–	–	
Vales Sovereign	+	nt	+++	nt	
Valor	–	–	–	–	
Wilja	–	–	–	–	
Winston	+++	nt	+	nt	
Yukon Gold	+	+	+++	–	

[a]+++, most or all plants infected; +, occasional plant infected; –, plants not infected (determined by ELISA).
[b]+, tuber infected; –, tuber not infected; nt, not tested.
[c]H, high; M, medium; L, low. Resistance ratings entered only if available on database (https://www.europotato.org/menu.php).

For PVA, the resistance ratings of 10 cultivars matched those on the European Cultivated Potato Database (ECPD; https://www.europotato.org/menu.php), with high resistance ratings for Atlantic, Charlotte, Fambo, King Edward, Maris Peer, Maris Piper, Pentland Dell and Shepody, and low resistance ratings for Maris Bard and Russet Burbank. Similarly these cultivars corresponded to resistant or susceptible to PYBV infection in the current work. PVA resistance ratings for other cultivars did not correspond with those on the ECPD.

Incidence of PYBV in field-grown potatoes

PYBV was not detected in leaf samples showing various symptoms including yellow blotches taken from commercial seed and ware potato crops and tubers representing 2591 virus cases (i.e. virus species found in a crop) and more than 95 potato cultivars, including those susceptible to PYBV. Leaf symptoms and virus cases in tubers were attributable to other virus species (Table 4).

Discussion

A new potyvirus species was identified in a potato breeding line displaying distinct yellow symptoms. ELISAs used for detecting 19 potato-infecting viruses were negative and electron microscopic analysis of infected tissues showed flexuous filamentous particles in the 800–900 nm size range typical of a potyvirus. RT-PCR using *Potyvirus* genus-specific primers and subsequent sequencing identified the infectious agent as a potentially new virus species, PYBV, belonging to the genus *Potyvirus*. Further genome sequence analysis of PYBV and phylogenetic analysis with other viruses confirmed that PYBV belongs to the genus *Potyvirus*. The molecular criteria for species discrimination within the *Potyvirus* genus have been established (Wylie *et al*., 2017). The species demarcation criteria, based upon the large ORF or its protein product, are generally accepted as <76% nucleotide identity and <82% amino acid identity. The corresponding thresholds for species demarcation using nucleotide identity values for other coding regions range from <58% (P1 coding region) to <74–78% (other regions), although these ranges are exceeded in some cases. Pairwise homology studies of individual virus genes were undertaken between PYBV and its closest related potyvirus, PVA. While PYBV has a CP amino acid identity of 83% and nucleotide of 77% with PVA, slightly above the species demarcation criteria, most of the differences in the CP sequence were at its 5′-end. Further studies should identify whether nucleotide differences are due to potential recombination events between related potyviruses. In addition a different polypeptide cleavage site was identified in the PYBV genome between the P3 and 6K1 proteins. Low identity levels that support species demarcation were found for the P1, HC-Pro, CI, VPg, NIa and NIb genes. As expected, the lowest sequence identity between PYBV and PVA was found with the P1 protein, which is the least conserved protein in the *Potyviridae* genome (Tordo *et al*., 1995), with only 40% of identity at the amino acid level. PYBV therefore meets the molecular criteria to be considered as a new virus species.

The 5′ sequence of PYBV was determined using the 5′-RACE system used to ensure completeness of 5′-end of the genome, and was performed four times from two independent RNA extractions. The PYBV genome sequence (GenBank accession AFS28882) was reported as containing five adenine residues at its 5′-end, differing in this respect from the four adenine residues reported for the PVA isolates analysed.

PYBV's relatedness to PVA was confirmed by its detection in ELISA using polyclonal antibodies to PVA and a

Table 4 Monitoring of PYBV and other potato-infecting viruses in field-grown crops.

Nature of material[b]	No. crops sampled	No. potato cultivars tested	No. samples tested	No. samples tested negative	Virus cases[a]	
					PYBV	Other viruses[c]
Leaves of seed crops with symptoms	1884	>95	3254	952	0	2302
Leaves of ware crops with symptoms	30	18	93	0	0	93
Leaves of breeding lines with symptoms	95	NA	114	58	1	55
Tubers from seed and ware crops (bulks of tubers)	100	17[d]	1694	1553	0	141

[a]A' virus case' is a single leaf sample of a plant with symptoms or a single bulk of tubers found positive for one virus species per individual crop. A single sample may be infected with more than one virus species. For leaves with symptoms, the number of samples (i.e. plants with symptoms tested per crop) ranges from 1 to 8 plants per crop.

[b]Leaf samples with symptoms were from Scotland (Dumfries, Galashiels, Inverness, Inverurie, Kirkwall, Perth and Thurso); tubers were from Scotland (as above) and England (Yorkshire).

[c]Other viruses detected include: PMTV, PLRV, PVA, PVM, PVS, PVV, PVX, PVY^O, PVY^C, PVY^N, TBRV and TRV. NA: not applicable.

[d]Potato cultivars tested by random sampling of tubers including those known to be susceptible to PYBV (in italics, Table 3): Accord, Brooke, *Estima*, *Fontane*, *Hermes*, King Edward, La Norma, Lady Claire, *Marfona*, Maris Bard, Maris Peer, *Melody*, Nectar, Rudolph, Safari, Slaney, *Yukon Gold*.

mix of monoclonal antibodies raised to PVA (SASA PVA + PYBV Mabs 58/0+58/6+58/7).

PYBV isolate QV276 and PVA isolate QV282 both infected the same indicator plant species after mechanical inoculation, with the exception of *S. demissum* A and *S. demissum* Y. With these indicator plants, although PYBV was confirmed by ELISA no symptoms were produced. In contrast, PVA elicited an HR and no virus was detected when tested using ELISA. Therefore *S. demissum* A and Y may be used to discriminate PYBV from PVA. Previously, *S. demissum* A has been used as a test plant to distinguish PVA (local necrotic lesions) from PVY (no symptoms; Bartels, 1971) and *S. demissum* Y to detect both PVA and PVY (local necrotic lesions; Chrzanowska & Waś, 1974). Because PYBV systemically infects these plants but does not elicit an HR on *S. demissum* A and Y, it suggests that PYBV overcomes Ny_{dms} resistance (Solomon-Blackburn & Barker, 2001). *N. benthamiana* may also be used for discrimination, with PYBV causing systemic symptoms of leaf and vein clearing and leaf curling whereas PVA causes latent infection. Additionally *N. benthamiana* may be used as a propagation host for PYBV. The only other indicator plants to produce symptoms were *N. hesperis* (systemic leaf curling and vein clearing for PYBV and local chlorotic spots and systemic leaf distortion and vein clearing for PVA) and *N. occidentalis* P1 (local necrotic lesions and systemic vein clearing for both PYBV and PVA).

A total of 35 commonly grown cultivars of potato were mechanically inoculated to assess their susceptibility to PYBV and PVA. Results for PVA are consistent with those previously reported for some cultivars such as King Edward, Maris Piper and Pentland Dell, known to harbour the Na_{tbr} gene mediating HR to PVA (Rajamäki *et al.*, 1998). Because PYBV did not infect these cultivars, it indicates that PYBV also does not overcome Na_{tbr}-mediated resistance (Solomon-Blackburn & Barker, 2001). For these cultivars and some other cultivars, resistance to PVA is listed on the ECPD, and for seven cultivars these resistance ratings were confirmed in the current work. These cultivars were also found to be resistant to PYBV. For other cultivars, resistance ratings for PVA on the ECPD did not agree with the ratings obtained in this paper indicating that the methods used to determine resistance, i.e. mechanical inoculation in this study but both mechanical inoculation and aphid transmission for ECPD results, may have affected the resistance rating. However, in most cases PYBV closely matched the PVA results, further indicating that the resistance mechanism is probably similar for both viruses, although Banba, Melody and Winston appeared to be slightly more susceptible to PYBV than PVA while Vales Sovereign and Yukon Gold were less susceptible. The main varietal difference was found with Fontane and Yukon Gold. PYBV was detected in the foliage but not in the tubers of Fontane, which was resistant to PVA. With Yukon Gold, PYBV was detected in both leaves and progeny tubers, whereas PVA was detected in leaves but not in progeny tubers. This may indicate that virus translocation to the tubers is inhibited in Fontane for both PVA and PYBV and Yukon Gold for PVA while PYBV is translocated to tubers.

In glasshouse experiments, primary symptoms were not observed for PYBV and PVA and secondary symptoms were only observed for PYBV in breeding line 99m-022-026, Estima and Russet Burbank. However, this may underestimate cultivars able to produce symptoms, because symptoms were more distinct in the field for 99m-022-026. Similarly for PVA, although no symptoms were observed in the glasshouse for Cabaret, Casablanca, Désirée, Estima, Hermes, they are likely to be found in the field (SASA, 2018).

While potyviruses are the main cause of virus diseases in Scotland (Fenton *et al.*, 2012; Davie *et al.*, 2017), PYBV was not detected in over 5 years of monitoring commercial cultivated potato crops by inspection of growing crops and post-harvest testing of tubers. The monitoring of commercial cultivated potato crops

undertaken in this study by inspection, supported by post-harvest testing of tubers, suggests that PYBV is unlikely to be present in these stocks.

Although further studies are required to fully understand the epidemiology of PYBV, it is proposed that because PYBV is very rare, is closely related to PVA and cultivar resistance is comparable to that for PVA, PYBV will be expected to have a low impact on potato production. Worldwide, PYBV might have been misidentified as PVA by certification authorities if ELISA tests are performed using polyclonal PVA antibodies. However, there are no reports in the literature attributing yellowing symptoms to PVA, which typically has symptoms of mild mosaic, roughness of surface and wavy leaf margin, or no symptoms depending on cultivar (Jeffries, 1998). PYBV can be detected using SASA Mab 58/0+58/6+58/7 (which detects both PVA and PYBV) and can be identified by deduction if SASA Mab 58/0+58/6 is also used (which only detects PVA). Alternatively conventional and real-time RT-PCR assays reported in this paper may be used for detection and identification.

As for all potyviruses, PYBV appears to have retained all genetic determinants required for aphid transmission, i.e. KITC-PTK residues in the HC-Pro (Atreya & Pirone, 1993) and DAG residues in the CP (Atreya *et al.*, 1990). However, studies are required to determine if aphid transmission of PYBV occurs and whether aphid transmission efficiency is similar to other potyviruses such as PVY and PVA (Verbeek *et al.*, 2010; Fox *et al.*, 2016).

Viruses species that have been found naturally infecting potato and *Solanum* tuber-forming or stolon-forming species are increasing. This is partly due to the fact that potatoes are grown in new areas and are exposed to viruses that infect other plant species (Jeffries & Lacomme, 2018), or these other plant species are grown in closer association with potato. In addition, virus species are being discovered by investigating the cause of new or unusual symptoms, and by recharacterizing current species. Additionally the use of high-throughput sequencing for plant virus metagenomics (Boonham *et al.*, 2014) increases the prospect of the discovery of as yet unknown viruses (Kreuze *et al.*, 2009). Recent new additions to the list of potato-infecting viruses are *Potato virus H*, a new carlavirus species found to be widespread in several potato-growing areas in China (Li *et al.*, 2013) and a new pomovirus *Colombian potato soil-borne virus* (Gil *et al.*, 2016) that was found to coinfect potato with the pomovirus PMTV. Known viruses, *Cherry leaf roll virus* in *Solanum acaule* from Peru (Crosslin *et al.*, 2010) and *Tomato chlorosis virus*, have been reported to infect potatoes in Spain and Brazil (Fortes & Navas-Castillo, 2012; Freitas *et al.*, 2012). Additionally molecular analysis has shown *Andean potato mild mosaic virus* to be a distinct virus species, when it was previously considered to be the Hu serotype of APLV (Kreuze *et al.*, 2013). To this list of potato-infecting viruses can now be added Potato yellow blotch virus.

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Supporting Information

Additional Supporting Information may be found in the online version of this article at the publisher's web-site.

Figure S1. DAS-ELISA assays using PVA (mix of Mab cell lines 58/0+58/6) and PVA + PYBV (mix of Mab cell lines 58/0+58/6+58/7) performed on a range of PYBV-infected, PVA-infected and noninfected 'healthy' plants (colour-coded). The plant samples tested are indicated on the right hand side of the graph: potato breeding line 99m-022-026 (PYBV-infected) samples labelled '99m-022-026-sap' and 'Orchard field 2010' (PYBV-infected breeding line 99m-022-026 field-grown in 2010); noninfected (healthy) Glen Almond potato plant; 8 individual PYBV-infected *Nicotiana benthamiana* plants (N.benth1–8); 10 individual PYBV-infected *Nicotiana tabacum* 'White Burley' (White Burley 1–10) and their corresponding healthy plants. 'PVA positive control': PVA-infected potato; 'Negative control': healthy (noninfected) potato plant. A sample is reported as positive for $A_{405\ nm}$ value above the set threshold (2× the $A_{405\ nm}$ value of a healthy noninfected plant) represented as a horizontal lines for each mix of monoclonal antibodies.

Table S1. Main conserved motifs in PYBV and potyvirus genomes. 5′-UTR = 5′-untranslated region; P1 = protein 1; HC-Pro = helper component proteinase; P3 = protein 3; PIPO = pretty interesting *Potyviridae* ORF; CI = cylindrical inclusion protein; VPg = viral genome-linked protein; NIa-Pro = nuclear inclusion a (proteinase); NIb = nuclear inclusion b (viral replicase); CP = coat protein.

Table S2. C_t values for real-time reverse transcription (RT)-PCR assays for PVA and PYBV-coat protein performed on potato plants infected by either PYBV or PVA.