Discriminating Between Isolates of PSbMV Using Nucleotide Sequence Polymorphisms in the HC-Pro Coding Region

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ABSTRACT

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The molecular diversity of 14 isolates of *Pea seed-borne mosaic virus* (PSbMV) from southern Australia, 13 previously described isolates from Pakistan, and a reference isolate from the United States have been studied to determine whether a relatively simple molecular diagnostic assay and classification scheme could be developed for this virus. The Australian isolates were placed into either pathotype P1 or pathotype P4 by bioassay on differential genotypes of *Pisum sativum*. The Pakistani isolates represented pathotypes P1, P4, U1, and U2, and an undetermined pathotype. The reference US isolate was pathotype P1. A reverse transcription–polymerase chain reaction (RT-PCR) assay based on an amplicon from the variable HC-Pro coding region of potyviruses was shown to distinguish PSbMV from seven other legume infecting potyviruses. Restriction fragment length polymorphisms (RFLPs) generated from the HC-Pro RT-PCR products of all 28 isolates using seven restriction endonucleases placed them into eight groups. A phylogenetic tree based on a Bray-Curtis similarity comparison placed the groups into three clusters. The groups and clusters had no clear association with either pathotype or geographic source. It is concluded that within the range of viruses and isolates tested, the RT-PCR-RFLP method will both specifically identify PSbMV and provide a simple, qualitative, and rapid means for placing PSbMV isolates into groups. Applications could include mapping and tracking isolates in space and time.

Additional keywords: biological pathotyping

Pea seed-borne mosaic virus (PSbMV) is an economically important seed-transmitted potyvirus (family *Potyviridae*: genus *Potyvirus*) infecting pea (*Pisum sativum* L). It has a worldwide distribution (2,16,19,20,30,31) and is common in pea germ plasm collections. Control is based on eliminating PSbMV from germ plasm (8), as secondary spread from primary seedborne infections by aphid vectors can lead to high disease incidence. Yield losses are in the range of 11 to 82%, varying with pea cultivar and the PSbMV isolate (3,19). Infection delays flowering, pod formation, and plant maturation (3) and reduces seed weight (19). Necrosis of the testa may occur, reducing seed quality (3,9,14).

Rates of seed transmission range from 0.3 to 100%, depending on the pea cultivar and strain of the virus (3,8,13,15,19,29). PSbMV is seedborne in a number of leguminous hosts, including *Lens culinaris* (10,11,15,18), *Vicia faba*, *Cicer arietinum* (15,18), *V. articulata*, *V. narbonensis*, *V. pannonica*, *V. sativa*, *Lathyrus ochrus,* and *L. sativa* (9,15,19). The mechanism of seed infection has been described by Wang and Maule (29).

PSbMV isolates have been grouped into three pathotypes (P1, P4, and P2) according to their ability to infect specific pea genotypes (1). This is a functional classification because genetic resistance (immunity) to PSbMV in *P. sativum* is based on four homozygous recessive genes: *sbm*1, *sbm*2, *sbm*3, and *sbm*4 (22,23). Thus, *sbm*1, *sbm*3, and *sbm*4 (linkage group IV located on chromosome 6 of *P. sativum*) confer resistance to PSbMV pathotypes P1, P2, and P4, respectively (6,24,25,28); and *sbm*2 (linkage group II located on chromosome 2 of *P. sativum*) also confers resistance to pathotype P2 (24). Identification of the pathotype of a prevalent isolate of PSbMV will therefore allow breeders to identify which genes should be incorporated into germ plasm to confer resistance. This pathotype classification is inadequate, however, to describe the variation within the PSbMV species because isolates within pathotypes vary in virulence (24), and tentative additional pathotypes, U1 and U2 (2) and Pi and Pv (12), have also been described which differ from P1, P2, and P4 in their reaction with the specific differential genotypes (1).

PSbMV was first reported in Australia in 1978 in pea germ plasm imported from Sweden (20), then in Queensland and South Australia in field pea crops (16). It is now widely disseminated in pea, chickpea, lentil, faba bean, and other legumes (14,15). Describing the diversity that now exists among Australian PSbMV isolates is important because it may indicate the number and sources of virus incursions, and may also suggest pathways of distribution into and within the country. In the course of determining the pathotypes of isolates collected in southern Australia, we found that there was considerable biological diversity among them. These biological properties were not considered to be reliable descriptors of the isolates, and we therefore sought a reproducible and rapid molecular method for describing and classifying them. The HC-Pro coding sequence comprising about 1,292 nt has been shown by ribonuclease protection assay (RPA) to have a greater level of variation in nucleotide sequence than the coat protein (CP) region for PSbMV (4). We have targeted this region as a way to discriminate between isolates and report here the development of a reverse transcription–polymerase chain reaction (RT-PCR) assay both for distinguishing PSbMV from other legume-infecting potyviruses and, in conjunction with restriction fragment length polymorphisms (RFLPs), for placing isolates in groups. The method was tested with a collection of 28 Australian and foreign isolates of PSbMV. The possible application of this scheme to determining virus epidemiology is discussed.

MATERIALS AND METHODS

Virus isolates. The 14 Australian isolates of PSbMV used in this study are listed in Table 1. Single lesion cultures were prepared by three passages in *Chenopodium quinoa* or *C. amaranticolor* leaves. Thirteen isolates were collected previously from Pakistan, including PSbMV, PK1-PK12 (2), and PK13 (A. Ali, *unpublished*). Type isolates were US for pathotype P1 and S6 for pathotype P4 (16). All isolates were maintained by mechanical passage in *P. sativum* cv. Dundale at 6- to 8-week intervals in an insect-free glasshouse (21 to 24°C).

Bean yellow mosaic virus (BYMV-S), *Pea mosaic virus* (PMV), and *Tobacco mosaic virus* (TMV) were provided from the Waite Campus plant virus collection. *Bean common mosaic virus* (BCMV iso-

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late 295) and *Peanut mottle virus* (PeMoV isolate 133-E) were provided by J. Thomas, Department of Primary Industries, Indooroopilly, Queensland, Australia. *Clover yellow vein virus* (ClYVV) and *Lettuce mosaic virus* (LMV) were provided by D. Graetz, Field Crops Pathology Unit, South Australian Research and Development Institute, South Australia, Australia. *Turnip mosaic virus* (TuMV) was provided by J. F. Laliberte, INRS-Institut Armand-Frappier, Université du Québec, Québec, Canada. All virus isolates were maintained in the glasshouse by mechanical inoculation on the following host species: *P. sativum* cv. Dundale (PMV and BYMV-S), *C. quinoa* (TuMV, LMV, and ClYVV), *Phaseolus vulgaris* cv. Hawkesbury Wonder (BCMV 295 and PeMoV 113-E), and *Nicotiana tabacum* cv. Samsun (TMV).

Determination of pathotype. Two to five seedlings of each of the four pea differential genotypes (Table 2) were mechanically inoculated at the four-leaf stage with the Australian isolates. The reference isolates were US and S6 (see above). Susceptibility to the PSbMV isolates was determined by symptomatology and dot immuno-binding assay (DIBA) using polyclonal antiserum to PSbMV-US (16) at 2 to 3 weeks postinoculation and again 1 week later.

Total nucleic acid extraction. Total nucleic acids were extracted by a modification of the method of Doyle and Doyle (5). Fresh leaf tissue (0.5 g) was crushed in 5 volumes of CTAB buffer (1.4 M NaCl, 0.1 M Tris HCl, pH 8.0, 2% *N*-cetyl-*N,N,N*-trimethyl-ammoniumbromide, 20 mM EDTA) containing 0.2% 2-mercaptoethanol and incubated at 60°C for 30 min. An equal volume of chloroform:isoamyl alcohol (24:1) was added and mixed, then centrifuged at 13,000 × *g*. The aqueous phase was collected and precipitated with 0.9 volume of isopropanol in the presence of 0.3 M sodium acetate, pH 5.2, and incubated at –20°C for 16 h. Nucleic acids were pelleted by centrifugation at 13,000 × *g* for 30 min, washed with 70% ethanol, dried in a SpeedVac (Savant Instruments Inc., Farmingdale, NY), and resuspended in 200 µl of sterile water.

RT-PCR. RT was primed with VT04 (5′-CTCCAAAACCATGCTTCACTCTTGA-3′), which targeted the P3 coding region of PSbMV corresponding to position 3297-3273 of PSbMV DPD1 (GenBank accession number D10930). PCR was primed with VT02 (5′-GCAGTTGCTACATCCA TCATTGTTGGCCAT-3′, position 2500-2471) and VT03 (5′-GTGTTGGAGGAA TCACACCAGAAGAATGTG-3′, position 1417-1446) within the HC-Pro coding region of PSbMV DPD1.

Other potyviruses were tested with the generic potyvirus RT-PCR primers of Mackenzie et al. (17). PV1/SP6 (5′-GATTTAGGTGACACTATAG$(T)_{17}$(A/G/C) -3′) corresponding to position 9936-9919 of PSbMV DPD1 was used as both the RT and reverse PCR primer. PV2I/T7 (5′-TAATACGACTCACTATAGGGIAA(C/T) AA(C/T)AG(C/T)GGICA(A/G)CC-3′) corresponding to position 8250-8266 of PSbMV DPD1 was the forward PCR primer.

The reverse transcription mixture (10 µl) contained 1 µl of nucleic acid extract, RT primer, 25 mM Tris-HCl, pH 8.3, 50 mM KCl, 5 mM $MgCl_2$, 2 mM DTT, 0.8 mM dNTPs, 1 U/µl RNase Inhibitor (GeneWorks, Australia), and 5 U AMV Reverse transcriptase (GeneWorks, Australia). For PSbMV, the primer was 0.4 µM VT04, and for potyvirus detection it was 0.6 µM PV1/SP6. Test nucleic acids and primers (5 µl) were preincubated at 70°C for 10 min, quenched on ice, and the remaining RT components were added before incubation at 42°C for 40 min.

The PCR reaction mixture (10 µl) contained 1 µl of cDNA from the RT step, reverse and forward primers, 1.5 mM $MgCl_2$, 50 mM KCl, 10 mM Tris HCl, pH 9.0, 0.1% Triton X-100, 0.8 mM dNTPs, and 1 U *Taq* DNA polymerase (Promega). PSbMV-specific PCR mixes contained 0.4 µM each of the VT02 and VT03 primers, and the potyvirus-specific PCR mixes contained 0.3 µM PV1/SP6 primer and 0.6 µM PV2I/T7 primer.

Thermal cycling was done in a GeneAmp PCR System 2400 (Perkin Elmer). For PSbMV-specific PCR, mixtures were initially incubated at 94°C for 3 min followed by 20 cycles consisting of: denaturation at 94°C for 30 s, annealing at 68°C for 30 s, and extension at 72°C for 30 s, with a final extension step of 72°C for 2 min. For the generic potyvirus PCR assay, mixtures were initially incubated at 94°C for 3 min followed by 30 cycles consisting of: denaturation at 94°C for 30 s, annealing at 58°C for 1 min, and extension at 72°C for 3 min, with a final extension step of 72°C for 8 min. RT-PCR products were analyzed by agarose (1%) gel electrophoresis.

RFLP analysis of PSbMV RT-PCR amplicons. Amplicons from all PSbMV isolates were digested with seven 4-bp specific restriction endonucleases: *Alu*I, *Hha*I, *Tru*91 (Boehringer Mannheim, Germany); *Hae*III, *Rsa*I, *Sau*3AI, (Promega); and *Taq*$^{\alpha}$I (New England BioLabs) according to the manufacturer's instructions. Restriction products were analyzed by agarose (2.5 to 3%) gel electrophoresis.

Phylogenetic analysis based on RFLP data. RFLP patterns were compared by tabulating the presence (1) or absence (0) of bands. A Bray-Curtis similarity matrix was calculated and a dendrogram produced using "cluster" in Primer-5 v5.2.9 (Primer-E Ltd., Plymouth, UK).

RESULTS

Sources of Australian PSbMV isolates. As shown in Table 1, PSbMV was recovered from pea breeders' germ plasm, pea and faba bean field trials, and commercially available pea cultivars in Western Australia, South Australia, and Victoria during the period 1985 to 1997.

Differential reactions and pathotyping of isolates. All PSbMV isolates induced chlorotic or necrotic local lesions on *C. quinoa* and *C. amaranticolor* leaves. In addition, isolates S4 and S6 induced systemic vein chlorosis and leaf distortion.

Table 1. Sources of the Australian *Pea seed-borne mosaic virus* (PSbMV) isolates

PSbMV isolate	Source[a]	Tissue	Geographic region	Year isolated
S4	D. Cartwright[b]	Virus culture	South Australia	1985
S6	D. Cartwright[b]	Virus culture	South Australia	1985
UK4	Commercial cultivar	Leaf	South Australia	1995
2(5)	Breeder's line	Seed	South Australia	1995
3(6)	Breeder's line	Seed	South Australia	1995
19(7)	Breeder's line	Seed	South Australia	1995
19(1)	Field trial	Leaf	Strathalbyn, South Australia	1995
21(1)	Commercial cultivar	Leaf	Strathalbyn, South Australia	1995
43(1)	Field trial	Leaf	Strathalbyn, South Australia	1995
VIDA	Breeder's line	Seed	Horsham, Victoria	1996
N 20-5	Breeder's line	Seed	Turretfield, South Australia	1996
FFD	*Vicia faba*	Leaf	Perth, Western Australia	1997
M257	Breeder's line	Seed	South Australia	1997
P515	Breeder's line	Seed	South Australia	1997

[a] Pea, unless otherwise indicated.
[b] Department of Agriculture, South Australia.

Table 2. Pea differential genotypes and the scheme for defining pathotypes P1, P4, and P2 (1,26)[a]

Pea differential	Pathotype P1	Pathotype P4	Pathotype P2
PI 174319	S (necrosis)	S (moderate)	S (stunting)
PI 193586	R	R	R
PI 193835	R	R	R
PI 269774	R	S	R

[a] R = resistant, S = susceptible.

Table 3 shows the range of symptoms induced by the Australian PSbMV isolates on four pea differential genotypes and places them into pathotypes from their infectivity profile. PI 174319 was susceptible to all PSbMV isolates tested, but symptoms varied among isolates. Plants inoculated with isolates 2(5) and 43(1) showed either whole plant necrosis or leaf rolling, whereas all other PSbMV isolates expressed uniform symptoms on all inoculated PI 174319 plants.

PI 193586 and PI 193835 were immune to all isolates tested as confirmed by DIBA. PI 269774 differentiated the isolates into P4 or P1/P2. Resistance and susceptibility of each group of test plants was confirmed by DIBA. DIBA was more sensitive than symptomatology alone, as all symptomatic plants were positive by DIBA, whereas not all DIBA-positive plants were symptomatic (data not shown). Not all the PI 269774 test plants inoculated with PSbMV M257 and 19(1) showed symptoms (Table 3), yet all these test plants were DIBA positive.

Specificity of the RT-PCR for PSbMV. Figure 1A shows that the RT-PCR for PSbMV was specific. None of the other potyviruses tested, or TMV, showed the single amplicon ca. 1,085 bp in size obtained with the primers designed for this assay. Supplementation of each reaction with purified PSbMV RNA confirmed (Fig. 1B) the negative results in Figure 1A for viruses other than PSbMV. An amplicon about 1,085 bp in size was obtained for all of the PSbMV isolates compared in this study.

Figure 2 shows that the generic potyvirus RT-PCR assay with the redundant PV1/SP6 and PV2I/T7 primers (17) produced amplicons 1.6 to 2.0 kb in size for all of the potyviruses tested. The faint amplicon observed for the TMV sample was estimated to be 1.4 to 1.5 kb in size. Additional amplicons smaller than 1.0 kb were produced from RNA isolated from PSbMV, BYMV, PMV, PeMoV, BCMV, and LMV infected plants.

RFLP analysis of the 1,085-bp HC-Pro amplicon from PSbMV. The 1,085-bp amplicons (Fig. 3a) from the HC-Pro coding region of all of the 28 PSbMV isolates showed a range of RFLP patterns. *Alu*I generated four distinct patterns (Fig. 3b), *Hae*III, *Hha*I, *Rsa*I, and *Sau*3AI each generated three (Fig. 3c, d, e, f), *Taq*$^{\alpha}$I generated two (Fig. 3g), and *Tru*91 generated six (Fig. 3h). These patterns were collated for each isolate as shown in Table 4, where the letter code for each enzyme pattern corresponds with the RFLP pattern shown in Figure 3. When the isolates were compared by the RFLP patterns obtained with all seven restriction enzymes, eight groups were identified (Table 4). Some groups contained only one isolate, such as groups 1, 4, 7, and 8. Group 3 was the largest with 15 isolates.

Figure 4 shows the clustering of the 28 isolates as a dendrogram based on the similarities of patterns obtained by RFLP analysis. The type US isolate and an undetermined Pakistani isolate were in separate monotypic groups. Two groups contained only Australian P4 isolates. Four groups contained only Pakistani isolates, and the larger two of these had more than one pathotype. The largest of the groups contained almost equal numbers of Australian and Pakistani isolates, and all pathotypes were found in this group. Each of the three clusters contained isolates from more than one country and more than one pathotype.

DISCUSSION

PSbMV has been identified in Western Australia, South Australia, and Victoria from pea and faba bean field trial material, pea breeder's germ plasm, and in seed from commercially available pea cultivars. These isolates were serologically indistinguishable.

The diversity of origins of the isolates compared in this study (Table 1) showed that PSbMV is more widely distributed in Australia than previously reported (14,15), but its incidence was low at the time of the surveys. The 14 isolates were placed into

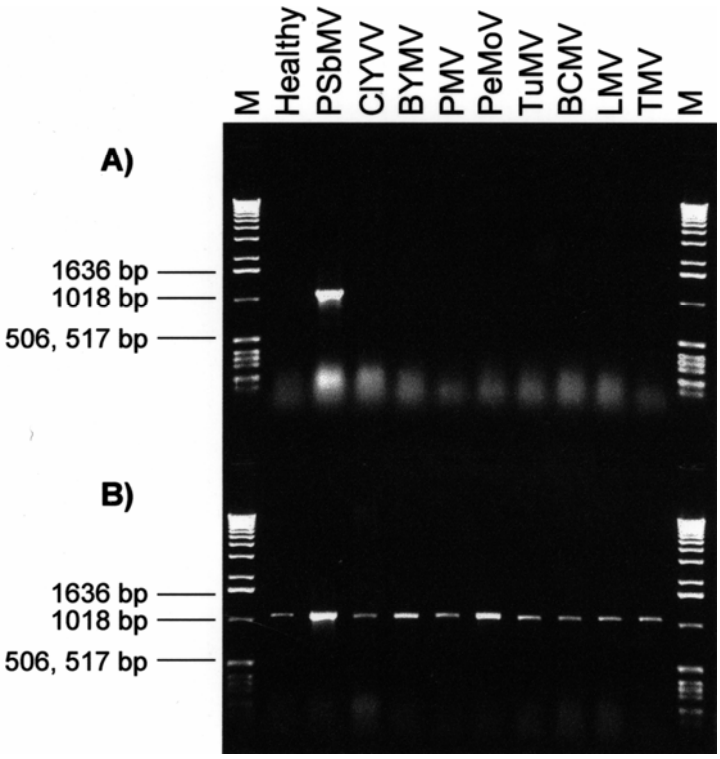

Fig. 1. Agarose gel electrophoresis of *Pea seed-borne mosaic virus* (PSbMV) specific reverse transcription–polymerase chain reaction (RT-PCR) amplicons **A,** obtained from total nucleic acid extracts of healthy pea and a range of virus-infected plants, and **B,** a set of positive controls where the same nucleic acid series was supplemented with 900 pg of PSbMV RNA. M, 1-kb DNA Plus ladder (GIBCO BRL Life Technologies). Abbreviations of virus names are in the text.

Table 3. Symptoms expressed by pea differential lines inoculated with Australian *Pea seed-borne mosaic virus* (PSbMV) isolates[a,b]

Pea genotype	PSbMV isolates														
	US	S6	2(5)	N_{20-5}	M257	3(6)	43(1)	VIDA	19(1)	21(1)	19(7)	UK4	FFD	P515	S4
PI 174319	LR M$^{(4/4)}$	LR M s$^{(5/5)}$	LR s$^{(2/5)}$ N$^{(3/5)}$	LR M$^{(5/5)}$	N$^{(5/5)}$	LR s$^{(2/2)}$	lr$^{(1/2)}$ N$^{(1/2)}$	lr VC$^{(5/5)}$	lr$^{(5/5)}$	N$^{(2/2)}$	N$^{(5/5)}$	N$^{(4/4)}$	N$^{(2/2)}$	LR VC$^{(5/5)}$	LR$^{(5/5)}$
PI 193586	-$^{(2/2)}$	-$^{(3/3)}$	-$^{(3/3)}$	-$^{(3/3)}$	-$^{(3/3)}$	NT	NT	-$^{(3/3)}$	-$^{(3/3)}$	NT	-$^{(3/3)}$	-$^{(2/2)}$	NT	-$^{(3/3)}$	-$^{(3/3)}$
PI 193835	-$^{(4/4)}$	-$^{(5/5)}$	-$^{(5/5)}$	-$^{(5/5)}$	-$^{(5/5)}$	-$^{(2/2)}$	-$^{(2/2)}$	-$^{(5/5)}$	-$^{(5/5)}$	-$^{(2/2)}$	-$^{(5/5)}$	-$^{(4/4)}$	-$^{(2/2)}$	-$^{(5/5)}$	-$^{(5/5)}$
PI 269774	-$^{(4/4)}$	M$^{(5/5)}$	-$^{(3/5)}$ VC$^{(2/5)}$	lr m$^{(5/5)}$	-$^{(3/5)}$ lr m$^{(2/5)}$	M$^{(2/2)}$	-$^{(2/2)}$	M$^{(5/5)}$	-$^{(2/5)}$ m VC$^{(3/5)}$	-$^{(2/2)}$	-$^{(4/4)}$	M$^{(4/4)}$	-$^{(2/2)}$	M$^{(5/5)}$	M$^{(4/4)}$
Pathotype	P1	P4	P4	P4	P4	P4	P1	P4	P4	P1	P1	P4	P1	P4	P4

[a] LR (severe leafroll); lr (mild leafroll); M (severe mosaic); m (mild mosaic); VC (vein clearing); N (whole plant necrosis); - (symptomless); s (stunting).
[b] Numbers in parentheses indicate the proportion of plants within the test group displaying the observed symptoms. NT: not tested.

pathotypes P1 and P4 using differential pea genotypes. None were classified as P2, but the unavailability of a reliable biological indicator and a known P2 type isolate precluded us from confirming that no isolates belonged to this pathotype. Isolates were biologically diverse as shown by the range of symptoms they induced on the differential genotypes, implying that a number of variants may be present, even though the virus has a known history of approximately 20 years in Australia.

A difficulty was noted in allocating isolates to pathotypes. Only four differential genotypes were regarded as reliable. We tested three other differential genotypes (PI 193836, PI 347329, and PI 347422) (1,26) against these isolates, but the reactions suggested that the lines were not isogenic, and we did not use these results for classification into pathotypes. Variations in susceptibility within these and other pea differentials to PSbMV pathotypes have been reported elsewhere (7,21,26,28).

Within pathotypes there was biological diversity. On the two pea genotypes that showed symptoms, some isolates showed reactions such as leaf rolling, mosaic, vein clearing, stunting, and whole plant necrosis either singly or in combination. On the basis of this limited symptomatology, 11 of the 14 isolates were different, and three (21(1), 19(7), and FFD) were the same. The latter three were also grouped together by RT-PCR-RFLP analysis. It was noted that two sets of symptoms were produced with isolates 2(5) and 43(1) on PI 174319, suggesting that they may have comprised a mixture of isolates. Although these bioassays distinguished isolates from each other and grouped them into pathotypes, we suggest that maintaining and testing the trueness of test plant genotypes, providing sufficient plants for assays, and standardizing growing conditions across time and space limits their value for characterizing and comparing isolates. The bioassays do not necessarily distinguish PSbMV from other legume-infecting potyviruses, and so additional serological or biological assays are needed to identify the virus.

The development of reliable molecular descriptors for PSbMV isolates required, firstly, proving of the previously described generic RT-PCR method for identifying potyviruses, secondly, the development of primers that would distinguish PSbMV from other potyviruses, and thirdly, testing of a range of restriction endonucleases that would identify distinctive polymorphisms.

The generic potyvirus RT-PCR assay (17) amplified a distinctive 1.6- to 2-kb fragment from total nucleic acid extracts of plants infected with the pea-infecting potyviruses PSbMV, ClYVV, BYMV, PMV, PeMoV, BCMV, and LMV. This is the first report that this assay can be used to identify these potyviruses. The temperature used with the generic potyvirus primer set of Mackenzie et. al. (17) to amplify diagnostic PCR fragments for the eight potyviruses also produced nonspecific products for TMV. This illustrates the importance of using amplicon size as a diagnostic criterion for this method.

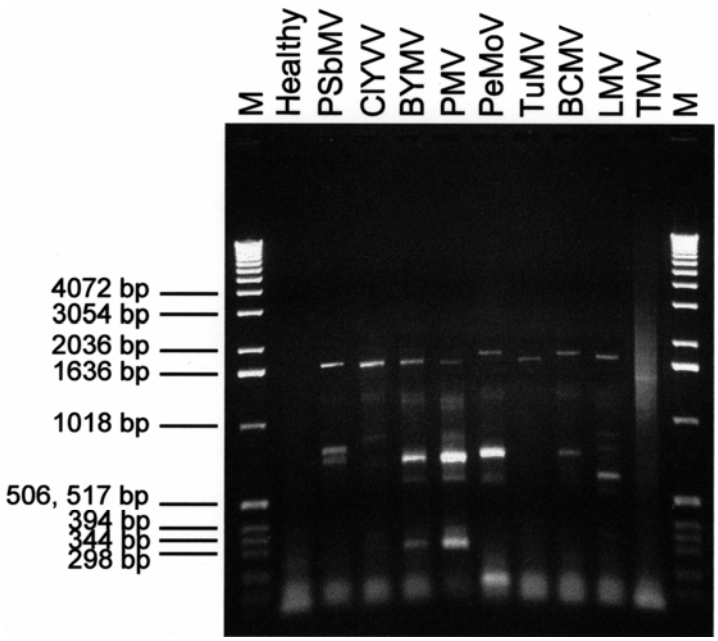

Fig. 2. Agarose gel electrophoresis of reverse transcription–polymerase chain reaction (RT-PCR) products with generic redundant potyvirus primers. Abbreviations of virus names are in the text.

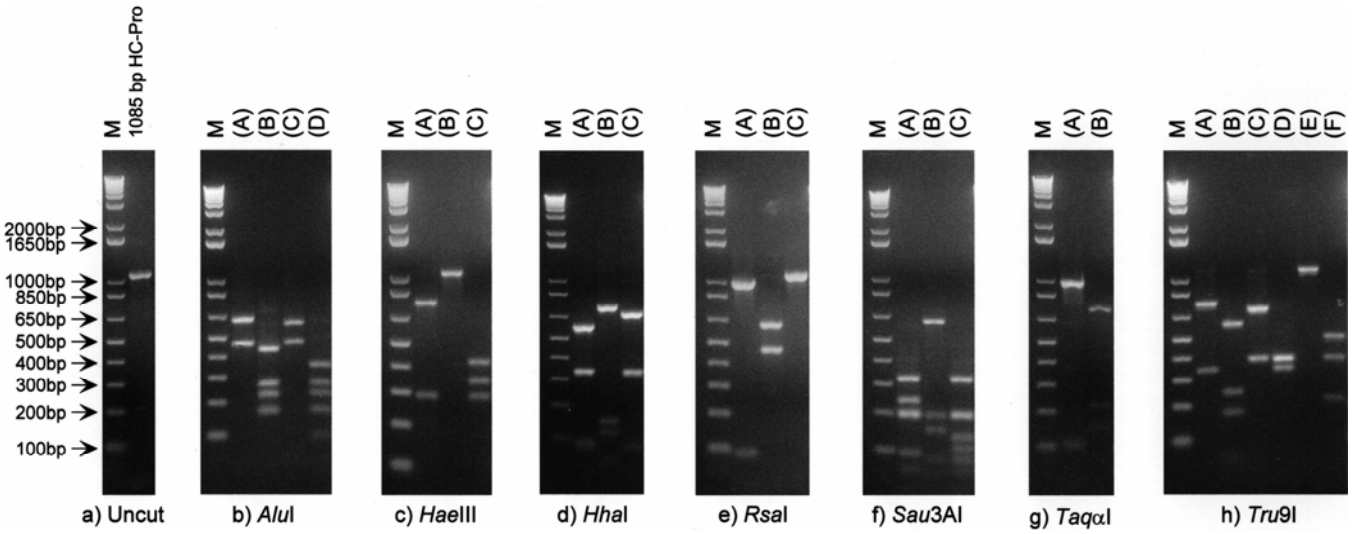

Fig. 3. Examples of restriction fragment length polymorphism (RFLP) patterns generated from the reverse transcription–polymerase chain reaction (RT-PCR) 1,085-bp HC-Pro amplicon of 28 *Pea seed-borne mosaic virus* (PSbMV) isolates. (M) 1-kb DNA Plus ladder (GIBCO BRL Life Technologies). (a) Shows the unrestricted RT-PCR generated amplicon from the HC-Pro region of PSbMV. (b-h) Summarize the different RFLP patterns observed from 28 PSbMV isolates with each restriction endonuclease (RE). Each of the RFLP patterns produced with each enzyme is identified alphabetically, but each letter is independent of that assigned to another RE. The RFLP patterns described here are the same as those presented in Table 4.

Table 4. Restriction fragment length polymorphism (RFLP) analysis of HC-Pro coding regions of 28 *Pea seed-borne mosaic virus* (PSbMV) isolates from the United States, Australia, and Pakistan

PSbMV isolates	RFLP patterns with restriction endonucleases																						
	*Alu*I*[a]				*Hae*III*			*Hha*I*			*Rsa*I*			*Sau*3AI*			*Taq*$^{\alpha}$I *		*Tru*91*					
	A	B	C	D	A	B	C	A	B	C	A	B	C	A	B	C	A	B	A	B	C	D	E	F
United States																								
US pathotype P1	1[b]				1			1			1			1			1		1					
Australia																								
S6 pathotype P4		2				2			2			2			2			2		2				
3(6)		2				2			2			2			2			2		2				
N_{20-5}		2				2			2			2			2			2		2				
VIDA		2				2			2			2			2			2		2				
S4			3				3			3	3					3	3				3			
M257			3				3			3	3					3	3				3			
P515			3				3			3	3					3	3				3			
43(1)			3				3			3	3					3	3				3			
19(7)			3				3			3	3					3	3				3			
2(5)			3				3			3	3					3	3				3			
21(1)			3				3			3	3					3	3				3			
FFD			3				3			3	3					3	3				3			
UK4			3				3			3	3					3	3				3			
19(1)				4		4			4				4		4			4		4				
Pakistani																								
PK3	5				5			5			5			5			5					5		
PK5	5				5			5			5			5			5					5		
PK7	5				5			5			5			5			5					5		
PK6		6				6			6				6		6			6		6				
PK8		6				6			6				6		6			6		6				
PK1			3				3			3	3					3	3				3			
PK2			3				3			3	3					3	3				3			
PK4			3				3			3	3					3	3				3			
PK9			3				3			3	3					3	3				3			
PK11			3				3			3	3					3	3				3			
PK12			3				3			3	3					3	3				3			
PK10			7				7			7	7					7	7						7	
PK13			8				8			8	8					8	8							8

[a] *, RFLP patterns observed for each restriction endonuclease (RE) are identified alphabetically and directly correlate with those described in Figure 3.
[b] PSbMV isolates were placed into numerical groups (1 to 8) based on the different combinations of RFLP patterns observed with the seven different REs.

The PSbMV RT-PCR was shown to be species specific by amplifying a 1,085-bp region of the HC-Pro from all of the 28 PSbMV isolates but not from that of the other pea-infecting potyviruses tested above, or the tobamovirus TMV. Controls in which extracts from other potyviruses were supplemented with PSbMV US RNA to produce a 1,085-bp amplicon demonstrated that the negative results for other viruses were genuine.

RFLP analysis of the 1,085-bp PSbMV-specific amplicon subdivided the 28 PSbMV isolates into eight groups. The dendrogram constructed from the RFLP data showed that two groups contained only Australian PSbMV isolates, four groups contained only Pakistani PSbMV isolates, and the PSbMV isolate from the United States formed its own group. Group 3 was the largest group, containing 15 Australian and Pakistani PSbMV isolates, and including isolates from all pathotypes represented in both countries, that is, P1, P4, U1, and U2. Overall, the eight groups formed three clusters, comprising groups 3, 7, and 8; groups 1 and 5; and groups 6, 4, and 2. No correlation was apparent between the groups and the pathotype.

A previous comparison of the same Pakistani isolates by RPA showed that they could be placed in three groups using a probe from the NIb/CP/UTR coding region (4). Much higher variation was detected between the isolates when an HC-Pro probe was used, and the sequence diversity appeared to be too great for the isolates to be grouped with this probe. Using the RFLP analysis of the same region, the Pakistani isolates could be differentiated into five groups. We conclude that the RFLP method described here is more convenient for routine use than the RPA, as it has the advantages of speed and avoidance of radioactivity.

Although only limited conclusions can be drawn because of the small number of isolates compared, it is worth noting that Australian PSbMV isolates are only represented in three of the eight groups, and two of the three clusters. This would be consistent with the view that a limited number of PSbMV isolates have been introduced into Australia and spread over a period of about 20 years. In comparison, the isolates from Pakistan, the center of origin of peas and where the virus is more diverse as indicated by the presence of a wider range of pathotypes (2,4), were placed in five groups and in all three clusters.

Of the three Australian PSbMV isolates in group 2, two were isolated from South Australian breeders' seed lines in 1995 and 1996 and one from Victorian breeders' seed lines in 1996. Of the nine Australian PSbMV isolates in group 3; four were isolated from South Australian breeders' seed lines between 1995 and 1997, one from pea field trial material in 1995, two from commercially available pea cultivars in 1995, one from a PSbMV virus culture collected in 1985, and one from faba bean field trial material from Western Australia in 1997. The one representative of group 4 was collected from South Australian pea field trial material in 1995 and may have remained localized.

In summary, this study has shown that PSbMV is widespread in southern Australia and that isolates are variable by both biological and molecular criteria. This is the first collection of PSbMV isolates made in an attempt to characterize strains, pathotypes, and molecular variability present among PSbMV isolates in Australia. In a previous Australian survey for the distribution and incidence of PSbMV, it was shown that PSbMV is able to infect several other crop legume and pasture or forage legume species and is seed transmitted in some of these crops (15). These results show that despite its earlier status as a quarantinable virus in Australia, containment of PSbMV has been unsuccessful. It is currently listed as

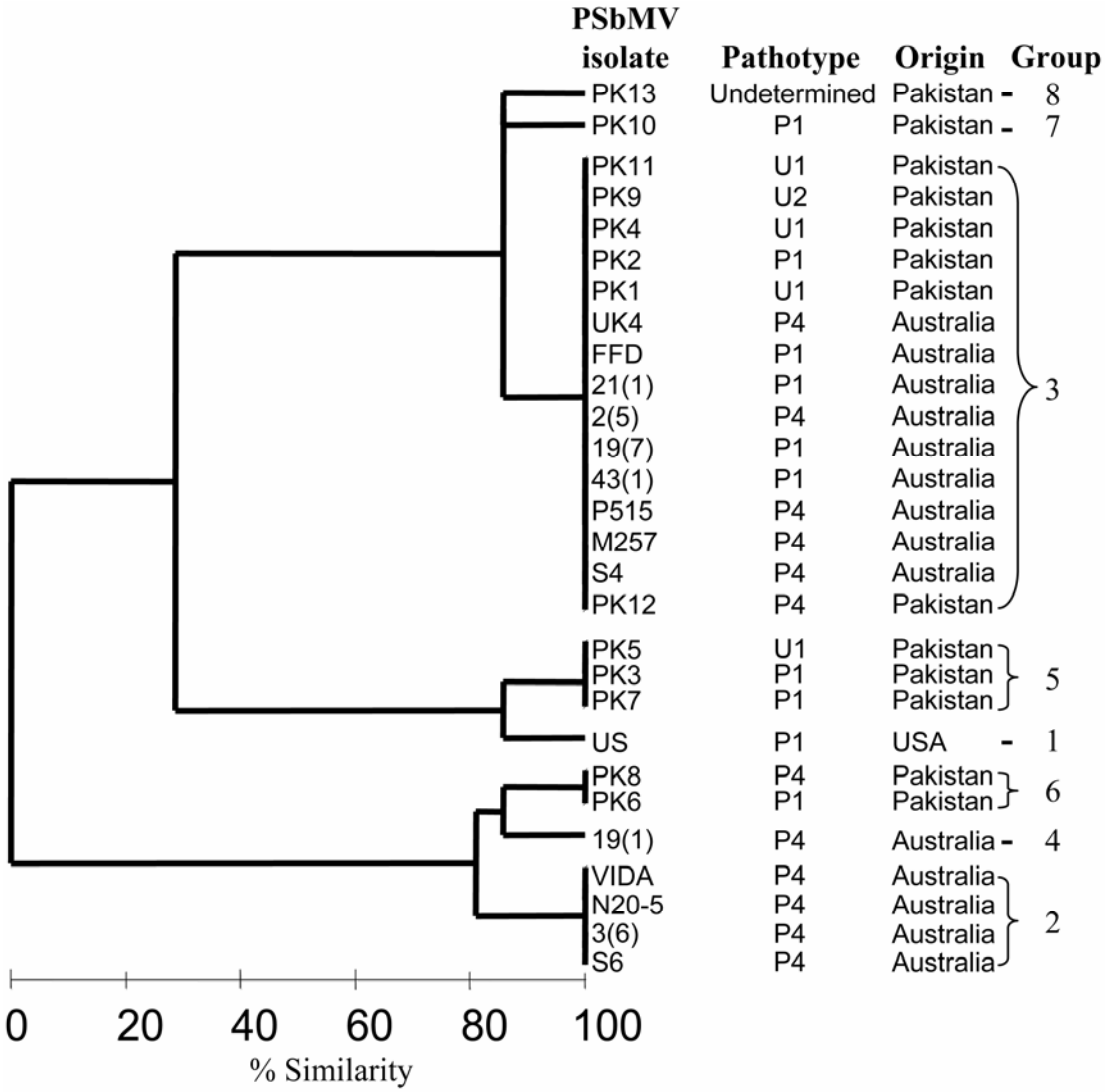

Fig. 4. Clustering of *Pea seed-borne mosaic virus* (PSbMV) isolates based on Bray-Curtis similarity matrix of restriction fragment length polymorphism (RFLP) patterns.

a pest of quarantine concern by Biosecurity Australia (M. Nasir, *personal communication*). The prevalence of PSbMV has serious implications for Australian pea breeding programs, and concerns have been raised of the possible introduction of more severe virus strains in seed from overseas (14). Therefore, the sensitive and virus species specific RT-PCR assay developed for the detection of PSbMV, which in conjunction with RFLP analysis enables differentiation and comparison of PSbMV isolates at the molecular level, will be a valuable tool for the detection and characterization of new isolates. Molecular discrimination of isolates will aid diagnosis, epidemiological surveys of incidence and distribution (27), and evaluation of breeders' lines for virus freedom prior to release. It will have application to identifying and intercepting potentially damaging strains of PSbMV in imported germ plasm at quarantine borders. It will be available for pea breeders to test lines for resistance to a range of PSbMV isolates.

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ERRATUM

This article was corrected on May 30, 2007. On page 491, column 1, under the heading "RT-PCR," the second sentence now correctly reads "PCR was primed with VT02 (5′-GCAGTTGCTACATCCATCATTGTTGGCCAT-3′, position 2500-2471) and VT03 (5′-GTGTTGGAGGAATCACACCAGAAGAATGTG-3′, position 1417-1446) within the HC-Pro coding region of PSbMV DPD1."