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Molecular evidence that the whitefly-transmitted sweetpotato mild mottle virus belongs to a distinct genus of the *Potyviridae*

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Summary. Complementary DNA representing 2 108 nucleotides at the 3′ end of the genomic RNA of the whitefly-transmitted sweetpotato mild mottle virus (SPMMV) was cloned after PCR. Sequence analysis revealed an open reading frame of 1 797 nucleotides which codes for a protein of 599 amino acids, followed by a 3′ non-coding region of 311 nucleotides. Alignment of the deduced amino acid sequence with corresponding sequences of other members of the *Potyviridae* demonstrated that part of the presumptive RNA-dependent RNA polymerase and the coat protein coding regions of SPMMV are found at the 3′ end of its genome, in that order. Alignment of the amino acid sequence of the core of SPMMV coat protein with those of selected members of the *Potyviridae* showed limited identity, thus demonstrating – with phylogenetic analysis – that SPMMV belongs to a distinct genus of the family *Potyviridae*.

Introduction

The taxonomic family *Potyviridae*, which is the largest family of plant viruses, has been divided into three recognised genera (*Potyvirus*, *Rymovirus*, and *Bymovirus*) on the basis of coat protein amino acid and genomic RNA sequences [31]. Potyviruses, rymoviruses and bymoviruses are transmitted, respectively, by aphids, eriophyid mites and the fungus *Polymyxa graminis* [31]. The *Potyviridae* genome consists of a single-stranded positive-sense RNA. Potyviruses and rymoviruses have a single RNA molecule of approximately 10 000 nucleotides, while the genome of bymoviruses consists of two molecules of RNA [25].

The *Potyviridae* also contains some unassigned viruses, among which the whiteflytransmitted sweetpotato mild mottle virus (SPMMV) was tentatively classified in a fourth possible genus, Ipomovirus [2, 31]. However, this genus would require validation based on coat protein sequence data.

SPMMV was isolated in East Africa from sweetpotatoes showing leaf mottling, veinal chlorosis, dwarfing and poor growth [12]. SPMMV has not been characterised so far, but the morphologies of virions and cytoplasmic inclusions are similar to those of potyviruses [19]. The virus particle is filamentous and contains one coat protein of 37.7 kDa [12], which shares epitopes with those of other potyviruses [29].

The PCR DNA amplification procedure using degenerate primers has been shown to be a powerful approach for obtaining virus sequence information in the potyvirus genus [4, 5, 17, 21, 23]. To unequivocally assign SPMMV to the family *Potyviridae*, we used cDNA amplification by PCR to obtain information on part of its genome organisation and sequence. The 3′ terminus of the SPMMV genome was analysed and its coat protein core amino acid sequence was aligned with those of selected members of the *Potyviridae*.

Materials and methods

RNA extraction, cDNA synthesis and amplification with degenerate primers

SPMMV was received from Dr. Salazar (International Centre of Potato, Lima, Peru) and propagated in *Nicotiana benthamiana*. Total RNA was extracted from symptomatic leaves by the method of Chirgwin et al. [3]. Purification of polyA-containing RNA was performed with Oligotex-dT (Qiagen). Oligo(dT)-primed single-stranded cDNA was synthesised using the Gibco/BRL Superscript Preamplification System. Amplification of oligo(dT)-primed cDNA with Pot8 and Pot9 degenerate primers (Fig. 1) was performed for 35 cycles (Biometra cycler): template denaturation at 94 °C for 30 sec, primer annealing at 37 °C (cycles 1–5) or 50 °C (cycles 6–35) for 1 min and DNA synthesis at 72 °C for 1 min. After electrophoresis, the amplified fragment was directionally cloned into pBluescript [28].

cDNA synthesis and nested amplification of cDNA 3′ end

Oligo(dT_{17})-adapter [9] primed single-stranded cDNA was synthesised from total RNA. Nested amplification of the cDNA was performed with 0.1 μg each of adapter primer [8] and specific primer (MVCP1 in the primary amplification, MVCP2 in the secondary nested amplification, Fig. 1). After denaturation for 5 min at 94 °C, primer annealing for 1 min at 50 °C and elongation for 10 min at 72 °C, the following thermal cycling scheme was used: template denaturation at 94 °C for 30 sec annealing at 50 °C (cycles 1–5) or 55 °C (cycles 6–35) for 1 min and elongation at 72 °C for 2 min. The second nested amplification was performed on 1 μl from a 1000-fold dilution of the first PCR reaction. The amplified fragment was cloned into pCRII using the TA-Cloning Kit from Invitrogen.

Sequence analysis of the amplified fragments

The nucleotide sequence of each amplified fragment was obtained from three independent clones either by cleavage with restriction enzymes or by specific primers derived from the sequence. Double-stranded DNA sequencing by the dideoxy chain termination method was performed using T7 DNA polymerase (Pharmacia) according to the manufacturer's instructions.

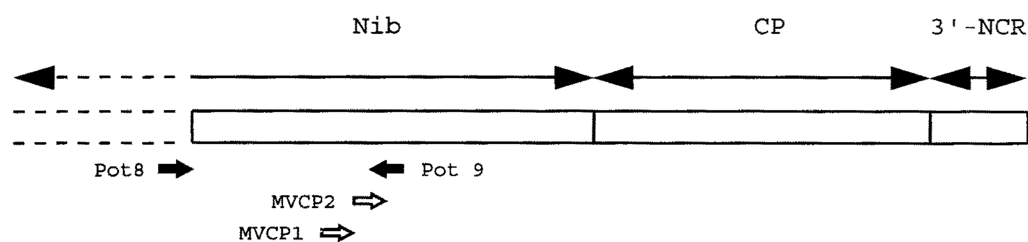
Multiple sequence alignments were done using ClustalV [10, 11] which produced a dendrogram of sequence relationship and calculated a phylogenetic neighbor-joining tree. Groupings were considered significantly supported when they were found in at least 95% of

1000 bootstrap replicates. The following members of the *Potyviridae* were used for sequence comparisons: barley yellow mosaic virus (BaYMV) [15], plum pox virus (PPV) [18], potato virus Y (PVY) [26], tobacco etch virus (TEV) [1], tobacco vein mottling virus (TVMV) [6] and wheat streak mosaic virus (WSMV) [20].

Results

Design of the degenerate PCR primers

The amino acid sequences of the polyproteins of BaYMV, Johnsongrass mosaic virus (JGMV) [9], papaya ringspot virus (PRSV) [34], PPV, pea seed-borne mosaic virus (PSbMV) [14], PVY, soybean mosaic virus (SbMV) [13], TEV, turnip mosaic virus (TuMV) [22], TVMV and the amino acid sequence of the C-terminal part of the polyprotein of WSMV were compared. Two conserved regions were selected in the RNA-dependent RNA polymerase. One of these regions, corresponding to the Pot9 primer, contained the GDD-box involved in the RNA polymerase function of postive strand RNA viruses [7, 16, 24]. Degenerate primers Pot8 (a 23-mer oligonucleotide with a 32-fold degeneracy)



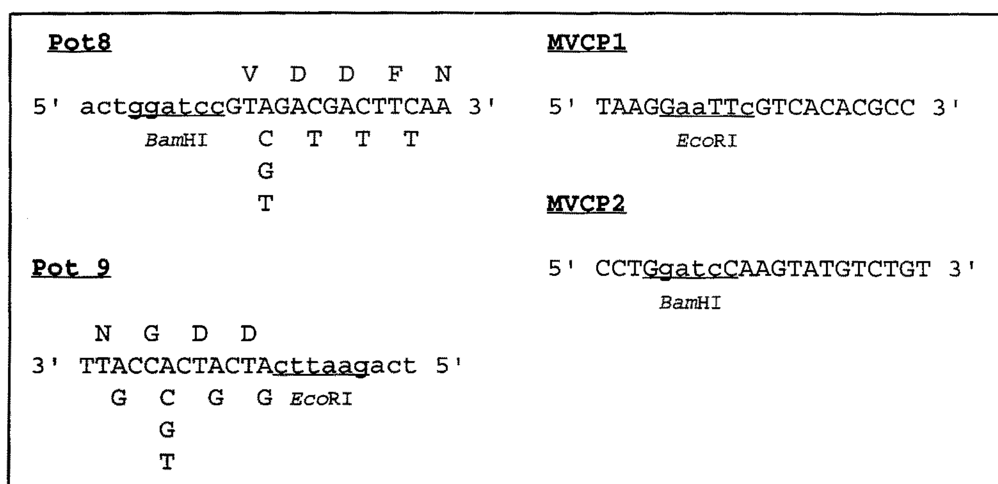


Fig. 1. Genetic map of the 3′ terminal part of SPMMV genome showing the relative positions of the two degenerate primers Pot8 (equivalent to G^{7625} to A^{7638} in PVY) and Pot9 (equivalent to A^{8063} to C^{8074} in PVY) and the two specific primers MVCP1 (Fig. 2, position 372 to 391) and MVCP2 (Fig. 2, position 391 to 411). Lower case letters represent nucleotides not derived from the viral sequences. *Nib* Nuclear inclusion b protein cistron, *CP* coat protein cistron, *3′-NCR* 3′ non-coding region

```
   1  N  Y  F  Y  K  C  H  L  Q  G  P  W  T  V  G  I  N  K  F  N
   1 AATTATTTCTATAAGTGTCATTTGCAAGGACCATGGACAGTTGGGATCAATAAATTCAAT

  21  R  G  W  N  K  L  A  N  Y  F  N  H  D  W  V  F  I  D  C  D
  61 CGAGGGTGGAATAAGTTGGCGAACTATTTCAATCATGATTGGGTTTTCATTGACTGTGAT

  41  G  S  R  F  D  S  S  I  P  P  I  M  F  N  A  V  C  M  L  R
 121 GGGAGTCGGTTTGACAGTTCCATACCCCCAATTATGTTTAATGCAGTTTGCATGTTAAGA

  61  S  V  F  G  D  L  D  P  D  E  N  Q  T  L  S  N  L  Y  T  E
 181 TCAGTTTTTGGTGATTTGGATCCAGATGAAAATCAGACGTTGAGCAATCTTTACACTGAG

  81  I  V  N  T  P  I  L  T  I  E  G  N  I  I  R  K  F  R  G  N
 241 ATTGTGAACACGCCAATACTCACCATTGAAGGGAATATCATCCGGAAATTTAGAGGCAAT

 101  N [S  G  Q  P  S  T  V  V  D  N  T] L  I  L  M  I  A  M  E
 301 AACAGTGGGCAGCCTTCAACAGTTGTTGATAACACGTTGATTCTGATGATTGCTATGGAG

 121  Y  A  I  A  K  V  F  V  T  R  P  D  I  K  Y  V  C  N [G  D
 361 TACGCGATAGCTAAGGTTTTTGTCACACGCCCTGACATCAAGTATGTCTGTAATGGGGAC

 141  D] L  L  I  N  C  P  R  S  T  A  N  A  I  S  E  H  F  K  D
 421 GACCTCTTAATCAATTGTCCAAGGAGCACTGCAAACGCCATTAGTGAGCACTTCAAAGAC

 161  V  F  A  D  L  S  L  N  Y  D  F  D  H  V  C  D  K  I  T  D
 481 GTATTCGCAGACTTAAGCTTGAACTATGATTTTGATCATGTTTGTGATAAAATCACAGAT

 181  V  D  F  M  S  H  S  F  M  W  L  D  T  E  Q  M  Y  I  P  K
 541 GTTGATTTTATGAGTCATAGCTTCATGTGGCTAGATACTGAACAGATGTATATACCAAAG

 201  L  D  K  E  R  I  V  A  I  L  E  W  E  R  S  D  E  Q  F  R
 601 TTGGACAAGGAGCGAATTGTGGCAATTTTGGAATGGGAAAGGAGTGATGAGCAATTCAGA

 221  T  R  S  A  L  N  A  A  Y  I  E  S  F  G  Y  E  D  L  M  T
 661 ACAAGGAGCGCCCTGAATGCTGCTTATATTGAGAGTTTTGGATACGAAGATCTAATGACT

 241  E  I  E  K  F  A  H  F  W  A  K  K  H  G  L  N  D  V  L  M
 721 GAGATCGAGAAATTCGCTCACTTCTGGGCTAAAAAGCATGGTCTCAATGATGTCCTAATG

 261  E  R  E  K  V  R  S  L  Y  V  D  E  N  F  D  A  S  R  F  E
 781 GAGCGGGAAAAGGTTAGAAGCTTGTACGTCGATGAAAATTTTGATGCGTCACGCTTTGAG

 281  K  F  Y  P  E  S  F  S  P  F  D  V  Y  V  E  P  H  A  S  T
 841 AAGTTCTATCCGGAAAGCTTTTCGCCATTTGACGTTTATGTTGAACCACATGCATCAACA

 301  S  K  T  I  E  E  L  Q  Q  E  M  E  D  L  D  A  D  T  T  I
 901 TCCAAAACAATCGAAGAACTGCAGCAAGAAATGGAGGATTTGGACGCAGACACAACAATC

 321  T  V  V  Q  R  E  T  Q  K  A  G  I  R  D  Q  I  E  A  L  R
 961 ACTGTGGTTCAGAGGGAAACACAGAAGGCAGGAATAAGAGATCAAATTGAGGCACTTAGG

 341  A  Q  Q  I  V  R  P  P  E  A  Q  L  Q  P  D  V  T  P  A  Q
1021 GCACAGCAAATTGTGAGGCCTCCTGAGGCACAACTACAGCCTGACGTAACTCCTGCGCAA

 361  I  V  T  F  E  P  P  R  V  T  G  F  G  A  L  W  I  P  R  Q
1081 ATTGTTACGTTTGAACCACCGAGAGTCACTGGATTTGGCGCTCTATGGATTCCGCGCCAA

 381  Q  R  N  Y  M  T  P  S  Y  I  E  K  I  K  A  Y  V  P  H  S
1141 CAAAGGAACTACATGACGCCATCTTACATCGAAAAGATAAAGGCTTATGTTCCACACTCA

 401  N  L  I  E  S  G  L  A  S  E  A  Q  L  T  S  W  F  E  N  T
1201 AACTTGATTGAATCCGGACTAGCTAGTGAAGCTCAATTGACTAGTTGGTTCGAGAACACG

 421  C  R  D  Y  Q  V  S  M  D  V  F  M  S  T  I  L  P  A  W  I
1261 TGCAGAGATTATCAAGTCAGTATGGATGTTTTCATGAGTACAATATTGCCAGCATGGATT

 441  V  N  C  I  I  N  G  T  S  Q  E  R  T  N  E  H  T  W  R  A
1321 GTCAATTGCATAATCAATGGAACGTCTCAGGAGCGCACTAATGAGCATACTTGGAGAGCT

 461  V  I  M  A  N  M  E  D  Q  E  V  L  Y  Y  P  I  K  P  I  I
1381 GTGATTATGGCAAATATGGAAGATCAAGAAGTGCTTTATTATCCCATCAAACCCATAATT
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 481 I  N  A  Q  P  T  L  R  Q  V  M  R  H  F  G  E  Q  A  V  A
1441 ATTAATGCTCAACCAACTTTGAGGCAGGTGATGCGCCACTTTGGCGAGCAAGCCGTTGCC

 501 Q  Y  M  N  S  L  Q  V  G  K  P  F  T  V  K  G  A  V  T  A
1501 CAATACATGAATAGCCTTCAAGTTGGCAAACCTTTCACAGTGAAAGGTGCCGTGACTGCT

 521 G  Y  A  N  V  Q  D  A  W  L  G  I  D  F  L  R  D  T  M  K
1561 GGGTATGCTAATGTTCAGGATGCTTGGCTAGGTATTGACTTTCTTCGAGACACGATGAAG

 541 L  T  T  K  Q  M  E  V  K  H  Q  I  I  A  A  N  V  T  R  R
1621 TTAACAACAAAGCAGATGGAAGTCAAACACCAAATCATCGCAGCGAACGTCACAAGGCGG

 561 K  I  R  V  F  A  L  A  A  P  G  D  G  D  E  L  D  T  E  R
1681 AAAATCCGTGTTTTTGCTCTTGCAGCACCGGGAGATGGCGATGAATTAGACACGGAAAGG

 581 H  V  V  D  D  V  A  R  G  R  H  S  L  R  G  A  Q  L  D
1741 CATGTTGTCGATGACGTAGCTAGAGGCCGTCACAGTCTGAGAGGAGCTCAACTCGATTAA

1801 ATGAGCATGTTTATCTTTACTTTCAACTGCGTGTTTTATTTCACTTACGTTTTATGCTTT

1861 GTGTTTGTGTGTTGTGGCACTTGAACCAGGTACAGCTGGCAAGTGTTTCGGCATGGTGTG

1921 GTTAGACAATTGGTTTGCACCGGTAGTCTAAGAAGCGCTATGTATCACGTGGTTGGTTAA

1981 TTCATGGTTTATGTGGGTTAATCAAGAAGCGTTTATCACCCAAAAGGGTACCAAAAAATG

2041 GTTGCGTCATTCATGGCGTAATCAGTTTTTGGAGTTTAGTTAGGTAACTTGTTGCCTACC

2101 TAAAAGCC(A)n
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Fig. 2. Nucleotide sequence of the 3′ 2108 nucleotides of SPMMV genomic RNA. The predicted amino acid sequence is shown above the nucleotide sequence. The consensus motifs (T/S)GXXXTXXXN(T/S) and GDD characteristic for RNA-dependent RNA polymerases of positive strand RNA viruses are boxed (EMBL accession no. Z48058)

and Pot9 (a 21-mer with a 32-fold degeneracy) were synthesised (Fig. 1). To increase the stability of the primer-template duplex after the first cycles of amplification and to facilitate later cloning of the PCR products, sequences containing *Eco*RI or *Bam*HI restriction sites were added at the 5′ end of each primer, respectively.

Amplification of part of the putative RNA-dependent RNA polymerase of SPMMV

PCR amplification was performed on polyA$^+$ RNA isolated from SPMMV-infected *Nicotiana benthamiana*, using degenerate primers Pot8 and Pot9. A 440 bp fragment was amplified and cloned in pBluescript. Sequence analysis (Fig. 2, nucleotides 1 to 411) revealed the presence of the consensus motif (T/S)GXXXTXXXN(T/S) at position 102–112 of the deduced amino acid sequence. This motif is characteristic of RNA-dependent RNA polymerases of positive-strand RNA viruses [7, 16, 24]. The amino acid sequence deduced from the 440 bp amplified fragment was 43.3% identical to the BaYMV sequence, and 48.9% to 51.9% identical to the sequences of four potyviruses (PPV, PVY, TEV and TVMV).

Amplification of the 3′ terminal region of SPMMV genome

The RACE (Rapid Amplification of cDNA Ends) method [8] was used to amplify a cDNA fragment corresponding to the 3′ terminal region of the SPMMV

genome. Two nested specific primers, MVCP1 and MVCP2 (Fig. 1), were designed from the nucleotide sequence of the 440 bp amplified fragment. Amplification performed with the two nested specific primers and the adapted primer yielded a 1 700 bp fragment; this PCR product was cloned in the pCRII vector.

Sequence analysis of the 3′ terminal part of SPMMV genome

The 1 700 bp PCR product nucleotide sequence (Fig. 2, position 412 to 2108) was combined with that previously determined from the 440 bp fragment amplified with Pot8 and Pot9 degenerate primers. The complete nucleotide sequence of the 3′ terminal part of SPMMV genome is presented in Fig. 2. Computer analysis revealed one large open reading frame (ORF), open at the 5′ end and extending for 1797 nucleotides. The ORF has the capacity to encode 599 amino acids. It terminates in a UAA stop codon and is followed by an untranslated region of 311 nucleotides and a poly (A) tail.

The deduced SPMMV amino acid sequence was aligned with the corresponding sequences of four potyviruses (PPV, PVY, TEV and TVMV), one rymovirus (WSMV) and one bymovirus (BaYMV). This alignment indicated that the putative RNA-dependent RNA polymerase and coat protein coding regions of SPMMV are found consecutively at the 3′ end of the genome, as for other *Potyviridae* (Fig. 3). However, the cleavage site between these two proteins was not determined.

Sequence comparison between the coat protein cores of SPMMV and other members of the Potyviridae

Pairwise percent sequence identities between the coat protein cores of SPMMV (equivalent to Q_{353}-R_{580}) and selected members of the *Potyviridae* are shown in Table 1. The sequence identities between SPMMV and selected potyviruses ranged from 25.7% to 29.9%. The coat protein core sequence from BaYMV displayed 17.6% identity with SPMMV. The coat protein core sequence identity between SPMMV and the rymovirus WSMV was 30.7%. The dendrogram of sequence relationship and the phylogenetic tree generated from the alignment of the coat protein core of SPMMV and those of selected members of the *Potyviridae* are shown in Fig. 4. Grouping of selected polyviruses was supported at 95% of the bootstrap replicates (results not shown).

Discussion

Available sequence data for the family *Potyviridae* permitted the design of two degenerate primers from conserved regions in the RNA-dependent RNA polymerase. The use of these primers and subsequent application of the RACE procedure [9] led to the cloning of 2 108 nucleotides from the 3′ terminus of the whitefly-transmitted SPMMV genome. The amino acid sequence deduced from SPMMV cDNA was aligned with the corresponding sequences of selected members of the *Potyviridae* and revealed that the nucleotide sequence corresponded to the C-terminal region of a polyprotein including part of the putative

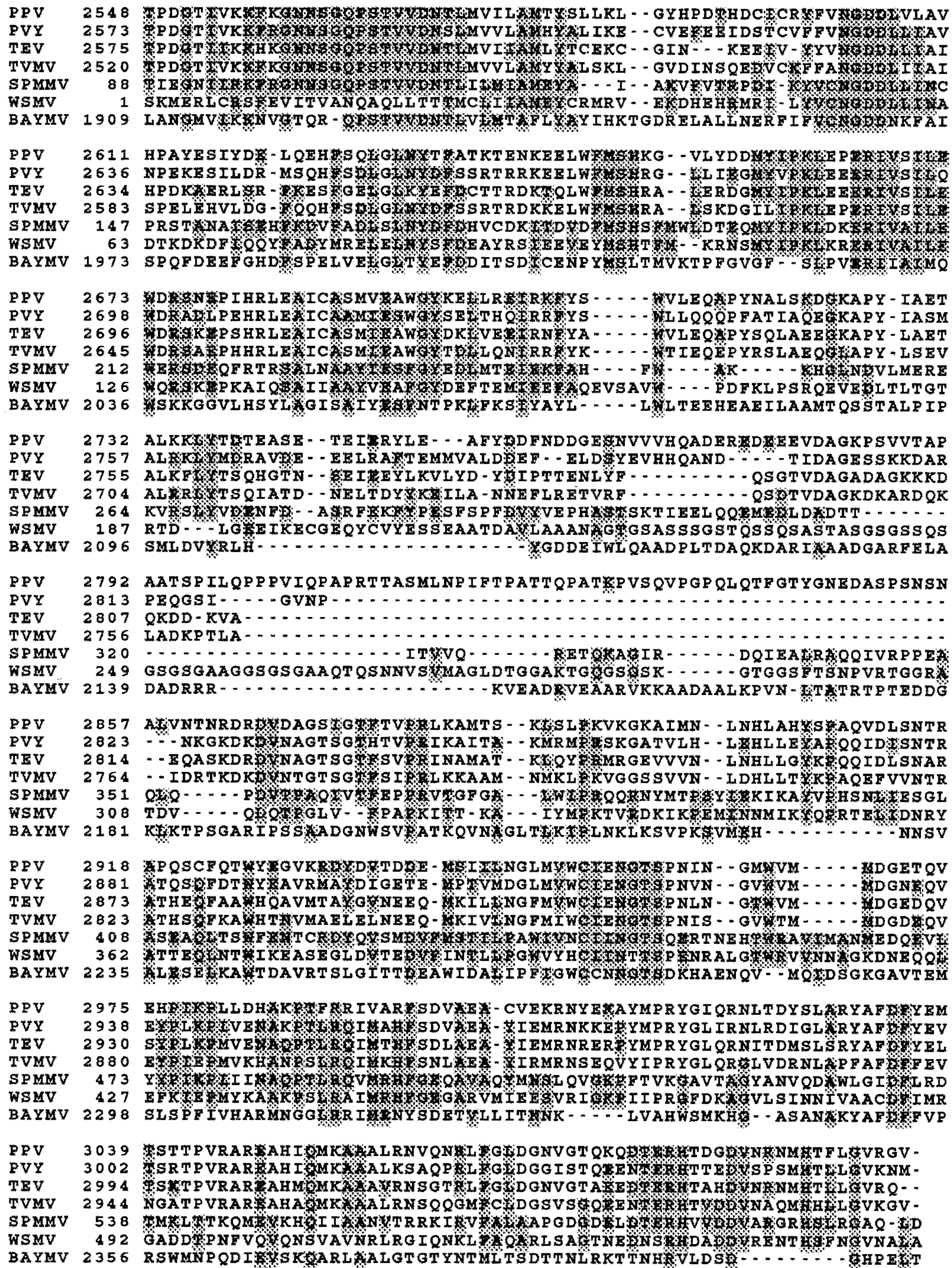

Fig. 3. Multiple alignment of the C-terminal 511 amino acids of SPMMV polyprotein with the corresponding protein sequences of BaYMV, PPV, PVY, TEV, TYMV and WSMV. Residues identical to SPMMV sequence are shadowed. Sequences were aligned using ClustalV

Table 1. Pairwise percent amino acid sequence identities between the core of the coat proteins of SPMMV and other selected members of the *Potyviridae*

	2	3	4	5	6	7
1 SPMMV	29.9	28.5	29.9	25.7	30.7	17.6
2 PPV		68.5	66.2	58.3	28.0	21.5
3 PVY			68.1	59.3	26.2	22.6
4 TEV				63.0	27.1	21.5
5 TVMV					28.5	23.1
6 WSMV						16.6
7 BaYMV						

Pairwise percent sequence identities calculated using ClustalV

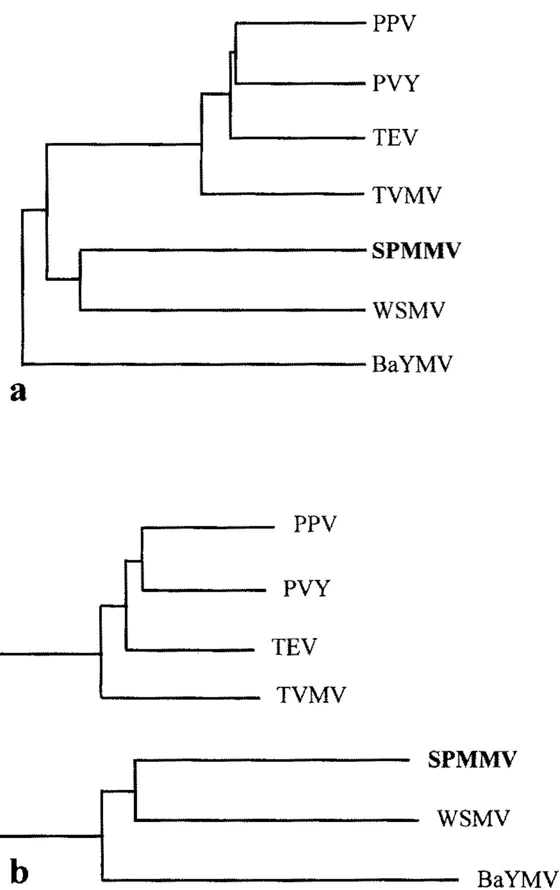

Fig. 4. Taxonomic relationship between the core of the coat protein of SPMMV and those of selected members of the *Potyviridae*. **a** Sequence relationship dendrogram produced using ClustalV. **b** Phylogenetic neighbor-joining tree produced using ClustalV

RNA polymerase and all of the presumptive coat protein. The site of cleavage between these two proteins, generally V-x-x-Q/(A, S, G or V) common to all members of the *Potyviridae* [31], could not be determined. The RNA is polyadenylated and probably has a single large open reading frame typical of the *Potyviridae*.

Amino acid sequence analysis of the coat proteins of members of the *Potyviridae* has been used extensively for establishing the taxonomic status of viruses in this family [27, 30–33]. The conserved core of the coat protein defined four levels of sequence identity [31]. Among these, the lowest correlates with vector specificity and corresponds to genera [31]. The dendrogram of sequence relationship and the phylogenetic tree generated from the alignment of the coat protein core amino acid sequences demonstrated that SPMMV does not belong to any recognised genus in the *Potyviridae*. Coat protein core amino acid sequence identity between SPMMV and four typical potyviruses was 25.7–29.9%, in contrast to the 55–75% range of identities observed between distinct potyviruses [31]. Amino acid sequence identity with the bymovirus was even lower (17.6%). SPMMV seems somewhat closer to WSMV (30.7% coat protein core sequence identity). However, grouping of both viruses is not supported by bootstrapping.

In conclusion, sequence analyses revealed strong similarities between SPMMV and members of the *Potyviridae* in terms of structure and organisation of the genome and homologies in the putative RNA polymerase. However, limited coat protein core sequence identity with potyviruses, bymoviruses and rymoviruses validates the assignment of SPMMV to a distinct genus of the *Potyviridae*. This largest plant virus family would thus comprise four genera based on vector specificity and coat protein sequence data.

Acknowledgements

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