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Phylogenetic relationships, strain diversity and biogeography of tritimoviruses

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North American and Eurasian isolates of *Wheat streak mosaic virus* (WSMV; genus *Tritimovirus*) and *Oat necrotic mottle virus* (ONMV; genus *Rymovirus*) were examined. Nine WSMV isolates differentially infected oat, barley, inbred maize line SDp2 and sorghum line KS56. The WSMV isolates clustered into groups based on phylogenetic analyses of the capsid protein (CP) cistron and flanking regions. WSMV isolates from the United States (US) and Turkey were closely related, suggesting recent movement between continents. Although more divergent, WSMV from Iran (WSMV-I) also shared a most recent common ancestor with the US and Turkish isolates. Another group of WSMV isolates from central Europe and Russia may represent a distinct Eurasian population. Complete genome sequences of WSMV from the Czech Republic (WSMV-CZ) and Turkey (WSMV-TK1) were determined and comparisons based on complete sequences yielded relationships similar to those based on partial sequences. ONMV-Pp recovered from blue grass (*Poa pratensis* L.) in Germany displayed the same narrow host range as ONMV-Type from Canada. Western blots revealed a heterologous relationship among CP of WSMV and ONMV. Phylogenetic analyses of the capsid protein cistron and flanking genomic regions indicated that WSMV and ONMV are related species sharing 74.2–76.2% (nucleotide) and 79.2–81.0% (amino acid) identity. Thus, ONMV should be removed from the genus *Rymovirus* and designated a definitive member of the genus *Tritimovirus*. Phylogenetic analyses further suggest that *Sugarcane streak mosaic virus* is not a tritimovirus, and may represent a new genus within the family *Potyviridae*.

Introduction

Species of the family *Potyviridae* with monopartite genomes, infecting monocotyledonous hosts and known, or suspected, to be transmitted by eriophyid mites were placed into the genus *Rymovirus*, with *Ryegrass mosaic virus* (RGMV) designated as the type species (Zagula *et al.*, 1992; Van Regenmortel *et al.*, 2000). However, when the genus *Rymovirus* was established, sequence data were available (Niblett *et al.*, 1991) only for one virus species, *Wheat streak mosaic virus* (WSMV). Later phylogenetic studies (Salm *et al.*, 1996a, b; Hall *et al.*, 1998; Stenger *et al.*, 1998) revealed that virus transmission by eriophyid mites is a paraphyletic trait within the family. Consequently, WSMV and *Brome streak mosaic virus* (BrSMV)

were removed from the genus *Rymovirus* and placed in the new genus *Tritimovirus*, with WSMV designated as the type species (Stenger *et al.*, 1998; Mayo, 1999). RGMV, *Hordeum mosaic virus* (HoMV) and *Agropyron mosaic virus* (AgMV) are more closely related to aphid-transmitted species of the genus *Potyvirus* (Schubert *et al.*, 1995, 1999; Götz *et al.*, 1995; Salm *et al.*, 1996a, b) and have been retained as definitive members of the genus *Rymovirus* (Van Regenmortel *et al.*, 2000). The recently described *Sugarcane streak mosaic virus* (SCSMV) from Pakistan (Hall *et al.*, 1998) and India (Hema *et al.*, 1999) is a novel and unclassified species for which no vector has been identified and which resembles tritimoviruses and ipomoviruses more than other members of the family (Hall *et al.*, 1998).

Oat necrotic mottle virus (ONMV) is currently a definitive member of the genus *Rymovirus* (Shukla *et al.*, 1998; Van Regenmortel *et al.*, 2000) and may be distinguished from other

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rymo- and tritimoviruses on the basis of a narrow host range within the Poaceae (Gill, 1976a, b). However, assignment of ONMV to the genus *Rymovirus* is questionable. Gill (1976b) reported that the ONMV-Type capsid protein (CP) reacted weakly to WSMV antiserum in a microprecipitin assay. Remah (1993) determined that the ONMV cylindrical inclusion (CI) protein is serologically related to the WSMV CI protein. However, a CP sequence purported to be derived from ONMV-Type shares high sequence identity with BrSMV, leading Chen *et al.* (2001) to propose that ONMV is a synonym of BrSMV, serological and host range differences notwithstanding.

Three strains of WSMV have been completely sequenced (Stenger *et al.*, 1998; Choi *et al.*, 2001). The Sidney 81 and Type strains of WSMV were isolated from wheat (*Triticum aestivum* L.) in the American Great Plains and are closely related (97.6% nucleotide sequence identity) to each other (Choi *et al.*, 2001) and to other WSMV isolates from the United States (US) and Canada (Chenault *et al.*, 1996; Niblett *et al.*, 1991). The divergent El Batán 3 strain isolated from wheat in the Central Highlands of Mexico (Sánchez-Sánchez *et al.*, 2001) shares only ~ 79% nucleotide sequence identity with Type or Sidney 81 (Choi *et al.*, 2001). All three completely sequenced strains of WSMV are vectored by the wheat curl mite, *Aceria tosichella* (Keifer) (Brakke, 1958; Choi *et al.*, 1999; Sánchez-Sánchez *et al.*, 2001; Hall *et al.*, 2001a).

There are numerous reports of WSMV outside of North America. Slykhuus & Bell (1963) examined WSMV isolates from Romania and Jordan that were distinguishable by host range from the only other mite-transmitted viruses known at that time (AgMV and RGMV). Djembiev (1956) cites an unverified report of WSMV-like symptoms on wheat in Kazakhstan in 1949. Viruses similar to WSMV also have been found in Turkey (Bremer, 1971), the former Yugoslavia (Juretic, 1979), China (Xie *et al.*, 1982), Iran (Foulad & Izadpanah, 1986), Hungary (Nyitrai & Gaborjanyi, 1988), Ukraine (Reshetnik *et al.*, 1996), Syria (Makkouk & Kumari, 1997), Italy (Credi *et al.*, 1997) and Poland (Jezewska & Wiczorek, 1998). However, identification of these Eurasian isolates as WSMV was based mainly on biological properties such as host range, symptom expression and/or transmission by eriophyid mites. Because BrSMV also is transmitted by eriophyid mites, causes a similar disease on many of the same hosts as WSMV, and occurs in Europe (Milicic *et al.*, 1980, 1982; Huth *et al.*, 1995; Götz & Maiss, 1995; Schubert & Rabenstein, 1995), biological properties alone are not sufficient to authenticate Eurasian isolates as WSMV. In fact, one putative WSMV isolate from Germany (Rabenstein *et al.*, 1982) was later shown by sequencing to be BrSMV (Schubert & Rabenstein, 1995).

Given the economic importance of tritimoviruses as pathogens of cereals, a better understanding of phylogeny, strain diversity and biogeography is desirable and has significant implications for disease management in cereal crops worldwide. In this report we examine host range, serological

relationships and nucleotide sequences of WSMV and ONMV isolates from North America and Eurasia. Through phylogenetic analyses, we define both within- and between-species variation in the genus *Tritimovirus* and propose several taxonomic revisions of the family *Potyviriidae*.

Methods

■ **Virus isolates and experimental host range.** The three North American strains of WSMV (Type, Sidney 81 and El Batán 3) have been characterized previously (Brakke, 1958; Brakke *et al.*, 1990; Stenger *et al.*, 1998; Choi *et al.*, 1999, 2001; Sánchez-Sánchez *et al.*, 2001). Six Eurasian isolates of WSMV from the Czech Republic (WSMV-CZ), Hungary (WSMV-HU), Turkey (WSMV-TK1 and WSMV-TK2), Russia (WSMV-R) and Iran (WSMV-I) were maintained in wheat cvs Centurk, Tomahawk or Alcedo by mechanical transmission. ONMV-Type from Canada, originally described by Gill (1976a, b), was obtained from the ATCC as PV-107. A second isolate of ONMV (ONMV-Pp) was recovered from a vegetatively propagated clone of blue grass (*Poa pratensis* L.) from a germplasm collection at Gatersleben, Germany. Both ONMV isolates were maintained in oat (*Avena sativum* L.) cv. Bruno by mechanical transmission. BrSMV-Hm (Schubert & Rabenstein, 1995), BrSMV-11-Cal (Huth *et al.*, 1995; Götz & Maiss, 1995) and AgMV-Asl (Schubert & Rabenstein, 1995) were maintained by mechanical transmission in wheat cv. Alcedo.

Isolates of WSMV, ONMV and BrSMV were mechanically inoculated to 7–10-day-old seedlings of wheat (cvs Centurk or Alcedo), barley (*Hordeum vulgare* L. cvs Black Hulless or Maris Otter), oat (cvs Bruno or Coast Black), maize (line SDp2) and sorghum (line KS56). Inoculated plants (8–20 per replicate per host species) were maintained under ambient greenhouse conditions and assayed for virus infection 3–5 weeks post-inoculation. Infection status was determined by double antibody sandwich enzyme-linked immunosorbent assay (DAS-ELISA) using antiserum to WSMV-Type, ONMV-Type or BrSMV-11-Cal. For some WSMV isolates, infection status was determined by RT-PCR (Hall *et al.*, 2001a).

■ **Antisera and Western blotting.** Virions of AgMV-Asl, BrSMV-11-Cal, BrSMV-Hm, WSMV-Type, WSMV-CZ and ONMV-Type were purified (Schubert & Rabenstein, 1995) from infected plants 3–4 weeks post-inoculation. Polyclonal antisera were raised in rabbits using the immunization schedule of Richter (1992). Plant samples for polyacrylamide gel electrophoresis were prepared by grinding leaf tissue in water (1:3, w/v) and, after a brief centrifugation, the supernatant was mixed with an equal volume of Laemmli buffer (Laemmli, 1970). The plant samples were boiled for 3 min and proteins separated by electrophoresis on SDS–10% polyacrylamide gels. Separated proteins were transferred to Hybond C nitrocellulose membranes and probed with antibodies (Richter *et al.*, 1994). Western blots were developed by incubation with alkaline phosphatase-conjugated goat anti-rabbit IgG (Dianova) and nitroblue tetrazolium chloride/5-bromo-4-chloro-3-indoyl phosphate (Loewe Biochemica) as a substrate. Additional serological assays were performed using DAS-ELISA.

■ **Cloning and sequencing of the 3' terminus of WSMV and ONMV.** Virions were partially purified (Schubert & Rabenstein, 1995) from wheat infected with WSMV-CZ, -HU, -I and -R and immunoprecipitated with antiserum to WSMV-Type. Virions of WSMV-TK1 and -TK2 also were partially purified from infected wheat using a slightly different procedure (Brakke *et al.*, 1990) but not immunoprecipitated. ONMV-Type and -Pp virions were partially purified (Schubert & Rabenstein, 1995) from infected oat and immunoprecipitated with antiserum to ONMV-Type.

Table 1. Taxa of the family *Potyviridae* compared in phylogenetic analyses

Virus	Acronym	GenBank accession no.
<i>Wheat streak mosaic virus</i> -Sidney 81	WSMV-Sidney 81	AF057533
<i>Wheat streak mosaic virus</i> -Type	WSMV-Type	AF285169
<i>Wheat streak mosaic virus</i> -El Batán 3	WSMV-El Batán 3	AF285170
<i>Wheat streak mosaic virus</i> -Czech	WSMV-CZ	AF454454
<i>Wheat streak mosaic virus</i> -Turkey 1	WSMV-TK1	AF454455
<i>Wheat streak mosaic virus</i> -Hungary	WSMV-HU	AF454456
<i>Wheat streak mosaic virus</i> -Turkey 2	WSMV-TK2	AF454457
<i>Wheat streak mosaic virus</i> -Iran	WSMV-I	AF454458
<i>Wheat streak mosaic virus</i> -Russia	WSMV-R	AF454459
<i>Oat necrotic mottle virus</i> -Type*	ONMV-Type	AF454460
<i>Oat necrotic mottle virus</i> - <i>Poa pratensis</i> *	ONMV-Pp	AF4544561
<i>Brome streak mosaic virus</i> -A†	BrSMV-A	AJ271086
<i>Brome streak mosaic virus</i> -11-Cal	BrSMV-11-Cal	Z48506
<i>Brome streak mosaic virus</i> - <i>Hordeum murinum</i>	BrSMV-Hm	X78485
<i>Sugarcane streak mosaic virus</i> -Pakistan	SCSMV-Pk	U75456
<i>Sweet potato mild mottle virus</i>	SPMMV	Z73124
<i>Cucumber vein yellowing virus</i>	CVYV	AF233429
<i>Agropyron mosaic virus</i>	AgMV	U30615
<i>Hordeum mosaic virus</i>	HoMV	U30616
<i>Ryegrass mosaic virus</i> -South Africa	RgMV-SA	U27383
<i>Ryegrass mosaic virus</i> -Danish	RgMV-D	Y09854
<i>Tobacco etch virus</i>	TEV	M11458
<i>Potato virus Y</i> -necrotic	PVY-N	X12456
<i>Johnsongrass mosaic virus</i>	JGMV	Z26920
<i>Wheat yellow mosaic virus</i>	WYMV	D86634

* Currently classified in the genus *Rymovirus*

† Referred to as ONMV-Type in Chen *et al.* (2001).

RNA extracted from virions was reverse transcribed (RT) with primer RCF1 (5' AGCTGGATCCTTTTTTTTTTTT 3') (McNeil *et al.*, 1996). Approximately 2 kb of the 3' terminus of each cDNA was amplified by PCR with *Taq* DNA polymerase (Roche) using primer RCF1 and one of two degenerate primers (Poty4 or Poty4.1). WSMV-CZ, -HU, ONMV-Type and -Pp were amplified using Poty4; all other cDNAs were amplified using Poty4.1. Both Poty4 (5' GCGGGATCCGTNTGYGTN-GAYGAYTTYAAAYAA 3') and Poty4.1 (5' CGGGATCCGGICARCC-IWCIACNGTNGT 3') primers anneal to conserved regions within the N1b cistron, and may be viewed as 'universal' potyvirus primers (Zebrini *et al.*, 1995; Salm *et al.*, 1996a; Hall *et al.*, 1998). PCR with Poty 4.0 was performed under the regime: 1 min at 94 °C, 1 min at 41 °C, 3 min ramp to 72 °C and 2 min at 72 °C for 35 cycles followed by a final extension incubation of 10 min at 72 °C. PCR with Poty4.1 was performed under the regime: 1 min at 94 °C, 15 s at 41 °C, 2 min at 72 °C for 30 cycles, followed by a final extension incubation of 10 min at 72 °C.

PCR products were ligated to pGEM-T or pGEM-Teasy (Promega) and then used to transform *E. coli* DH5 α . For each virus isolate, three cloned inserts were completely sequenced on both strands using a combination of universal and custom primers. All nucleotide sequencing was performed at the DNA Sequencing Facility, Iowa State University, Ames, IA. RT-PCR errors were eliminated by compiling consensus sequences derived from three independent clones for each virus isolate.

■ **Cloning and sequencing of the complete genomes of WSMV-CZ and -TK1.** To ensure complete coverage of the WSMV-CZ and -TK1 genomes, RT was performed with both RCF1 and random

hexamer primers (Choi *et al.*, 2001). Primers used to generate overlapping PCR products derived from WSMV-TK1 cDNA were based on the WSMV-Sidney 81 sequence. The genomic 5' terminus to the 3'-proximal end of P3 was amplified using the primers 5' GTGTGTCGACCGATT-AGGTGACACTATAGAAATTAACCAACCCAAATCG 3' and 5' CCGGATCCTATTGGTATTCAACCAATTC 3'. The 5'-proximal end of P3 to the 3'-proximal end of N1a was amplified with the primers 5' GCGGATCCGCGGGTTCCAAGAGACTGTT 3' and 5' GACTTCTA-GATCATTGCCAACTAACCAAG 3'. The 5'-proximal end of N1a to the 3'-proximal end of N1b was amplified with the primers 5' GTCTAAGCTTGGGCAAAGCAGCACGCA 3' and 5' GGGGATCC-TCATTCGTACACGCAGTATTG 3'.

Primers for generating overlapping PCR products of WSMV-CZ cDNA were based on the WSMV-Sidney 81 sequence or, when necessary, on partial nucleotide sequence of WSMV-CZ. The 5'-proximal end of P1 to the 3'-proximal end of P3 was amplified using the primers 5' CCGGATCCCAATGGCAACAGCGAATTGT 3' and 5' CCGGAT-CCTATTGGTATTCAACCAATTC 3'. A second PCR amplified a region encompassing the 3'-proximal region of P3 to the 3'-proximal region of N1b using the primers 5' GAACGTCTTGCAAGTTACT-CATAG-3' and 5'-AGCCATCAGCATCTATGAACC 3'. To obtain the genomic 5' terminus of WSMV-CZ, the 5'/3'RACE kit (Promega) was employed (Stenger *et al.*, 1998).

PCR products of the WSMV-CZ and -TK1 genomes were ligated to pGEM-T and then used to transform *E. coli* DH5 α . For each region of the WSMV-CZ and -TK1 genomes amplified by PCR, three independent

clones were sequenced on both strands using universal and custom primers. For both WSMV-CZ and WSMV-TK1, the 3'-terminal sequences were determined from clones described above. Complete genome sequences of WSMV-CZ and -TK1 were compiled as consensus sequences to eliminate RT-PCR errors.

■ **Phylogenetic analyses.** Potyviral taxa and corresponding GenBank accession numbers are presented in Table 1. Nucleotide sequences were aligned using CLUSTAL X (Thompson *et al.*, 1997), with the output adjusted using the Sequence Alignment Editor (Version 1.0 alpha 1, Copyright © 1996; A. Rambaut, Department of Zoology, University of Oxford, UK) such that gaps did not occur within codons. The 5'-terminal regions were trimmed to that available for all taxa such that the final alignment compared nt 7618–9384 (coordinates relative to WSMV-Sidney 81). A neighbour-joining majority-rule consensus phylogram based on 1000 bootstrap replicates was generated by CLUSTAL X. A Markov Chain Monte Carlo (MCMC) maximum likelihood phylogram was generated using the Mr Bayes 2.0 computer program (Huelsenbeck & Ronquist, 2001) with six base-substitution categories (A ↔ G, C ↔ T, A ↔ C, C ↔ A, G ↔ T, T ↔ G) and three substitution rate categories (1st, 2nd and 3rd codon positions), with substitution rates within a category fitted to a gamma distribution. Four chains of 30 000 steps each were computed starting from different random trees to confirm that the (MCMC) procedure was converging on a single most-probable tree. Parameter optimization (1·1 million cycles) was performed starting with a random tree. After 100 000 cycles, every thousandth tree was sampled (1000 trees sampled) and used to generate a majority-rule consensus tree using RadCon (Thorley & Page, 2000). Neighbour-joining and maximum likelihood majority-rule consensus phylograms were visualized using TREEVIEW 1.5.3 (Page, 1996), with the bymovirus *Wheat yellow mosaic virus* (WYMV) designated as the outgroup. Nucleotide or amino acid sequence alignments based only on WSMV, BrSMV, ONMV and SCSMV were used by CLUSTAL X to generate genetic distance matrices, with the output converted to percent identity. A third set of alignments was used to calculate percent identity among complete genomes (nucleotide) or polyproteins (amino acid) of five WSMV isolates. The BrSMV, WSMV and ONMV populations were analysed using the SITES program (Hey & Wakely, 1997) and DnaSP v.3.0 (Rozas & Rozas, 1999).

Relative rates of locus-wide mutation (μ) and selection (γ) were estimated using the Poisson Random Field (PRF) model (Hartl *et al.*, 1994; Sawyer, 1997). Nucleotide substitution polymorphisms within the Nib-CP region among nine WSMV isolates were separated into folded allelic classes (i.e. those base substitutions at the same position which occurred 1-, 2-, 3- or 4-times in the sample of nine sequences – see Sawyer, 1997), and characterized with respect to nonsynonymous versus synonymous substitutions. The observed allelic class distribution was fitted to the PRF model (equation 2 in Hartl *et al.*, 1994) by determining values of μ and γ that maximize a likelihood function (equation 3 in Hartl *et al.*, 1994) for comparing predicted PRF distributions and the allelic class distribution actually observed. The significance of the values obtained for synonymous and nonsynonymous substitutions was obtained by a likelihood ratio test with $\gamma = 0$ as the null hypothesis (Hartl *et al.*, 1994; Sawyer, 1997).

Results

Host range properties of WSMV, ONMV and BrSMV isolates

ONMV-Pp was isolated from Kentucky bluegrass displaying systemic mosaic and chlorotic streaking similar to that reported by Gill (1976a) for ONMV-Type on this host. The

experimental host ranges of ONMV-Type and ONMV-Pp were identical. Both isolates produced systemic infection of oat and SDp2 maize, but not wheat, barley or sorghum KS56. In contrast, BrSMV-11-Cal and -Hm infected wheat, barley and oat, but not maize SDp2 or sorghum KS56. Of the five hosts tested, oat was the only common host of ONMV and BrSMV.

Among the WSMV isolates, several differences in experimental host range were noted. Although all nine WSMV isolates systemically infected wheat, only some of the isolates systemically infected barley, oat, SDp2 maize or sorghum KS56. SDp2 maize is susceptible to WSMV-Sidney 81 but not to WSMV-Type (Choi *et al.*, 1999). With the exception of El Batán 3, the remaining WSMV isolates systemically infected SDp2 maize. Although oat and barley are considered universally susceptible to WSMV (Brakke, 1971), two Eurasian isolates (-R and -I) did not infect oat and WSMV-R did not infect barley. Some WSMV isolates infect certain sorghum genotypes (Seifers *et al.*, 1996). The two Turkish isolates -TK1 and -TK2 systemically infected sorghum KS56, whereas the seven other isolates of WSMV did not, demonstrating additional isolate × cultivar interactions on this host.

WSMV and ONMV capsid proteins are serologically related

Western blots of WSMV-Type, ONMV-Type, AgMV-Asl and BrSMV-Hm probed with antibodies raised to each virus are presented in Fig. 1. Each antibody reacted with the homologous antigen and did not react to a total protein extract from healthy oat. Reciprocal cross-reactivity of WSMV-Type and ONMV-Type antibodies with ONMV-Type or WSMV-Type CP was observed in Western blots (Fig. 1) and DAS-ELISA (data not shown), albeit at levels lower than that of homologous antibody-antigen combinations. WSMV-Type and ONMV-Type antibodies did not cross-react with CP of AgMV-Asl or BrSMV-Hm. No cross-reactivity with heterologous antigens was observed for antibodies raised to AgMV-Asl or BrSMV-Hm.

Serological relationships among WSMV and ONMV isolates are presented in Fig. 2. Antibodies raised to WSMV-CZ reacted strongly to the CP of WSMV-Type, -I, -R, -HU and -CZ, and weakly to the CP of ONMV-Pp and -Type. Antibodies raised to ONMV-Type reacted to the CP of both ONMV isolates and weakly to the CP of all five WSMV isolates tested. Neither antibody reacted to BrSMV-11-Cal CP or total soluble protein extracted from healthy oat. A serological relationship among the CP of WSMV-Type, -Sidney 81 and -El Batán 3 has been demonstrated (Choi *et al.*, 2001). WSMV-TK1 and -TK2 also reacted strongly to WSMV-Sidney 81 antiserum in DAS-ELISA tests (data not shown).

Sequence comparisons and phylogenetic relationships.

Taxa representative of five genera within the family *Potyviridae* were compared and included all known or suspected

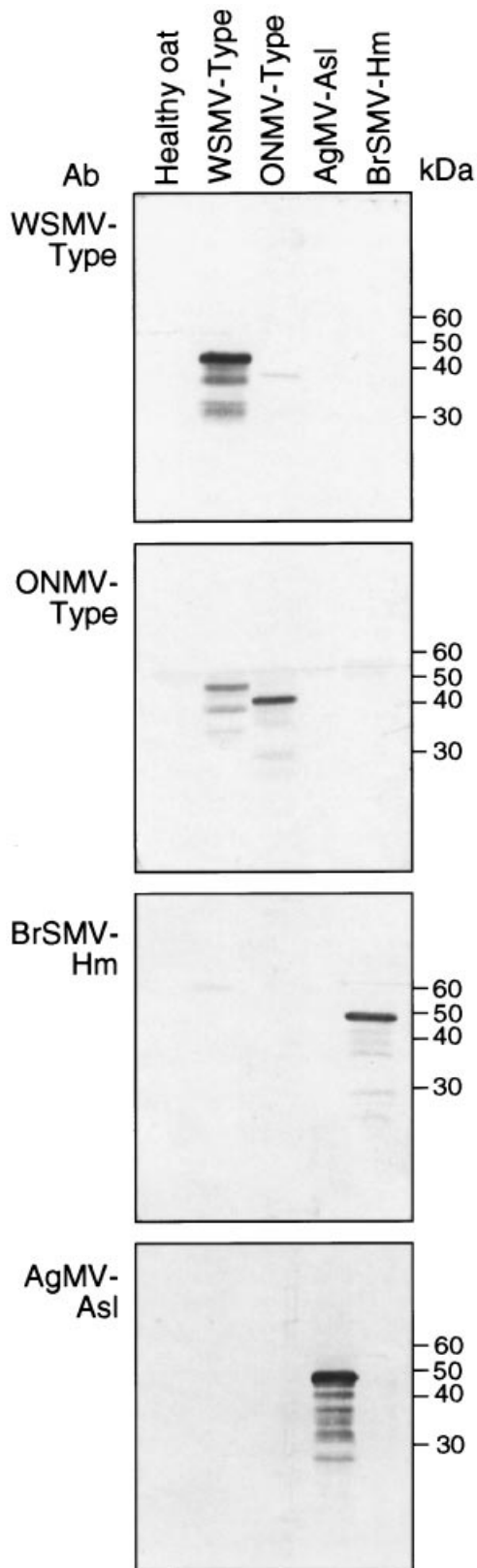


Fig. 1. Serological relationships among capsid proteins (CP) of four species of the family *Potyviridae*. Presented are Western blots of total soluble protein extracts of virus-infected tissue or healthy oat. Antibodies

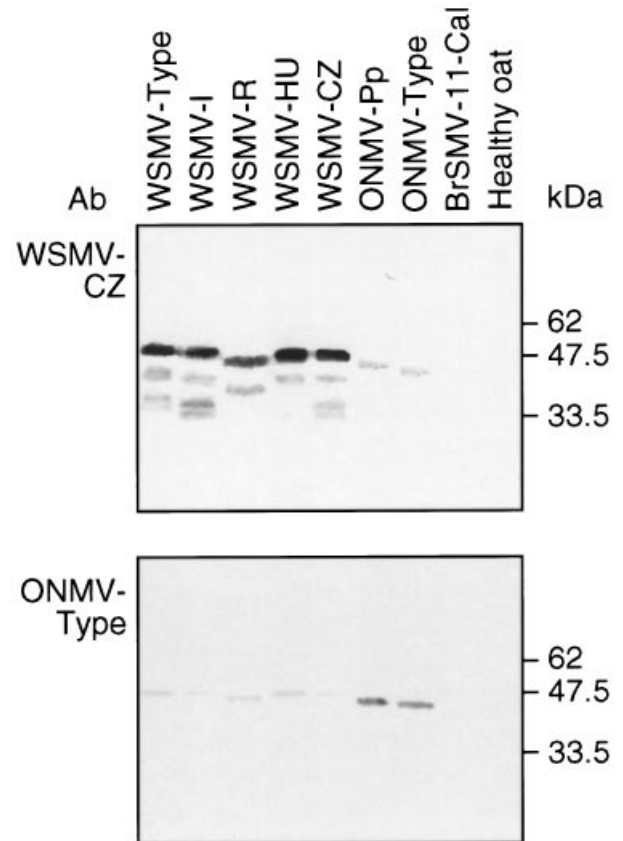


Fig. 2. Serological relationships among capsid proteins (CP) of selected strains of *Wheat streak mosaic virus* (WSMV) and *Oat necrotic mottle virus* (ONMV). Presented are Western blots of total soluble protein extracts of virus infected tissue or healthy oat. Antibodies (Ab) used to probe each Western blot are indicated on the left. The sizes and mobility of protein standards are indicated on the right in kDa. Minor bands of less than full-length represent degradation products of the CP commonly observed during infection (Brakke *et al.*, 1990). Note that all strains of WSMV and ONMV reacted strongly to the respective homologous antibody, and less strongly to the heterologous antibody. Neither antibody cross-reacted with *Brome streak mosaic virus-11-Cal* (BrSMV-11-Cal).

tritimoviruses (Table 1). Species of the genus *Macluravirus* were excluded from the analysis, as these taxa are most closely related to bymoviruses (Hall *et al.*, 1998), used here as the outgroup.

Phylograms depicting relationships among potyvirus taxa based on neighbour-joining (Fig. 3A) or a maximum likelihood model (Fig. 3B) produced similar, but not identical topologies. All WSMV isolates formed a clade and shared a most recent common ancestor. Within the WSMV clade, three groups were

(Ab) used to probe each Western blot are indicated on the left. Virus CP tested were *Wheat streak mosaic virus-Type* (WSMV-Type), *Oat necrotic mottle virus-Type* (ONMV-Type), *Brome streak mosaic virus-Hordeum murinum* (BrSMV-Hm) and *Agropyron mosaic virus-Aschersleben* (AgMV-Asl). The sizes and mobility of protein standards are indicated on the right in kDa. Minor bands of less than full-length represent degradation products of the CP commonly observed during infection (Brakke *et al.*, 1990). Note the reciprocal cross-reactivity of WSMV and ONMV.

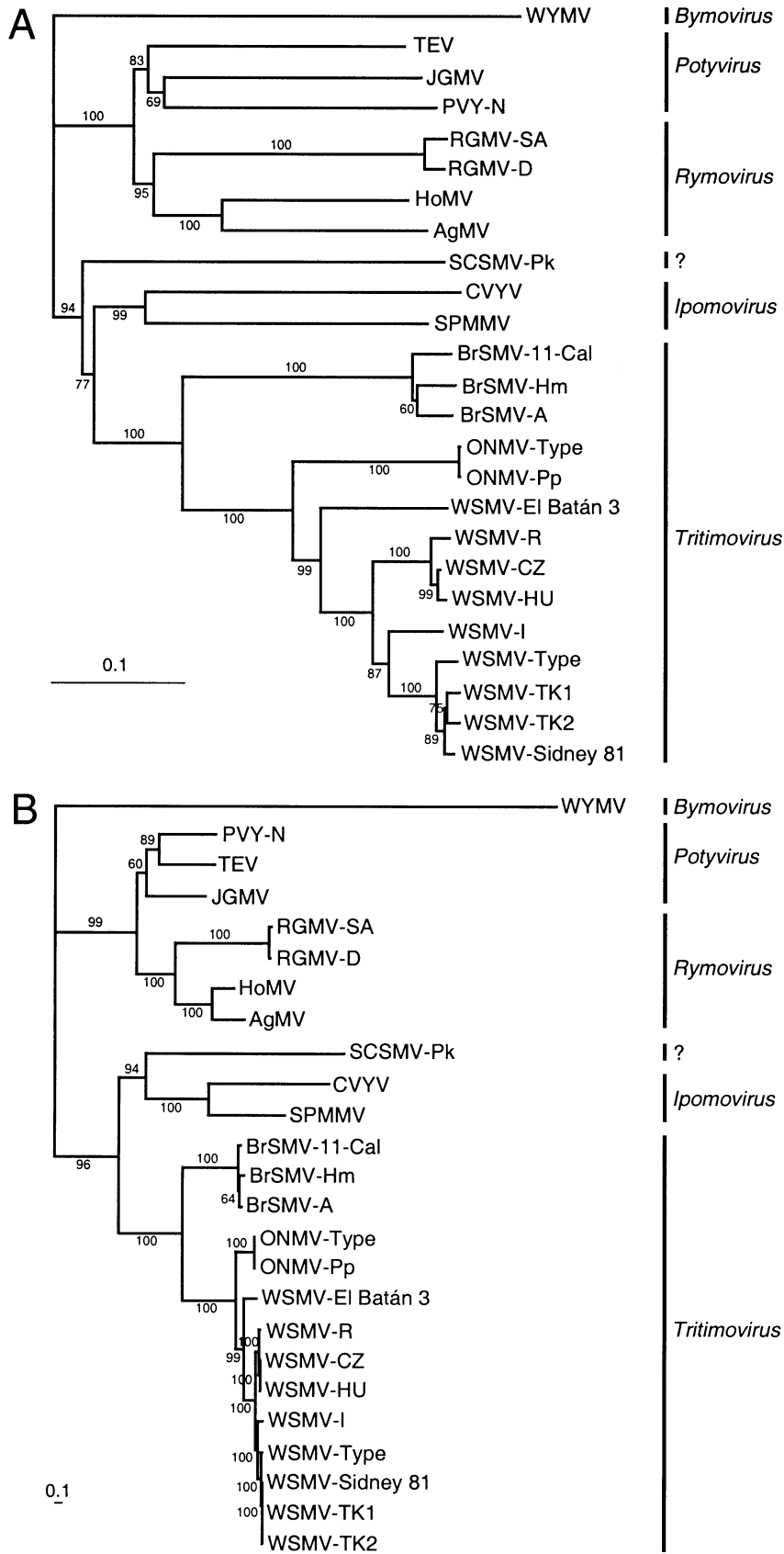


Fig. 3. Phylogenetic relationships among select species and strains of the family *Potyviridae*. Presented are majority-rule consensus phylograms derived from (A) neighbour-joining or (B) maximum likelihood analyses based on a nucleotide sequence alignment corresponding to *Wheat streak mosaic virus*-Sidney 81 nt 7618–9384. Support values for branches are indicated as percentages. Proposed affiliation of taxa within genera of the family *Potyviridae* are indicated on the right for each phylogram.

Table 2. Mean and 95% confidence intervals of each likelihood model parameter of 1000 trees sampled from 10^6 Markov Chain Monte Carlo steps

Parameter	Mean	(95% confidence interval)
Relative substitution rates (reversible)		
G ↔ U	1.000	(1.000–1.000)
C ↔ U	6.145	(4.785–7.896)
C ↔ G	1.979	(1.501–2.721)
A ↔ U	1.624	(1.241–2.176)
A ↔ G	4.129	(3.298–5.315)
A ↔ C	2.867	(2.219–3.724)
Nucleotide frequencies		
π A	0.31	(0.30–0.32)
π C	0.21	(0.21–0.22)
π G	0.25	(0.24–0.25)
π U	0.23	(0.22–0.24)
Shape parameter		
alpha	1.216	(1.043–1.410)
Codon position		
First	0.445	(0.375–0.509)
Second	0.322	(0.268–0.372)
Third	2.233	(2.139–2.333)
Tree log-likelihoods	–26240.7	(–26255.2 to –26228.9)

apparent in both analyses (Fig. 3). One group contained only El Batán 3, the most divergent WSMV isolate; the second included Sidney 81, Type, TK1 and TK2; and the third comprised CZ, HU and R. WSMV-I was nearly equally divergent in sequence relative to isolates of the second and third groups, but shared a common node with the US and Turkish isolates in both analyses. ONMV-Type and -Pp were included in a clade also containing the two definitive tritimovirus species, WSMV and BrSMV. Placement of SCSMV-Pk varied depending on the analytical method employed; however, in both cases SCSMV did not share a most recent common ancestor with a clade composed of only tritimoviruses.

The evolutionary model parameters (and their 95% confidence intervals) used to generate the phylogenetic tree in Fig. 3(B) are given in Table 2. There was a clear bias in the base composition of the model with $A > G > U > C$. This compositional bias was consistent among all lineages of the tree depicted in Fig. 3(B). Average transitions were 2.75 times more likely than transversions. First and second codon positions were 5- to 7-fold less likely to vary than the third codon position. The substitution rate heterogeneity parameter alpha can vary from 0 to infinity, with large alpha indicating little or no rate heterogeneity. The estimated alpha for the phylogeny was 1.2, suggesting that there is substantial variation of evolutionary rates from codon to codon. MCMC maximum likelihood trees using a more complex model with 12 base substitution rates had the same overall topology and es-

entially the same average tree likelihood score (data not shown). There are few examples of evolutionary models applied to phylogenetic analysis of RNA virus families. Bollback & Huelsenbech (2001) examined the family *Leviviridae* and found transition/transversion ratios and variation in substitution rates among codon positions that were nearly identical to those of the *Potyviridae* described here.

Genetic distances among tritimovirus species (*sensu* this study) and strains are presented in Table 3. Not surprisingly, between-species differences were greater than within-species differences. ONMV and WSMV shared 74.2–76.2% (nucleotide) and 79.2–81.0% (amino acid) identity. BrSMV exhibited considerable divergence from WSMV (56.4–59.8% nucleotide and 48.1–49.9% amino acid identity) and ONMV (54.9–55.5% nucleotide and 43.3–48.3% amino acid identity). SCSMV-Pk was the most divergent virus among known or suspected tritimoviruses, sharing no more than 49.7% nucleotide or 35.9% amino acid identity with WSMV, ONMV or BrSMV. Among the latter three viruses, the ratio of synonymous versus nonsynonymous substitution rates (ds/dn) was 2.1, 2.2 and 5.1 between BrSMV and WSMV, BrSMV and ONMV, and ONMV and WSMV, respectively. The ds rate among all viruses was about 75%, suggesting complete saturation of substitutions at silent sites. Lastly, average ds among the nine WSMV isolates was 39% and the ds/dn ratio was 9.3.

The PRF model provides a theoretical framework for estimating parameters proportional to mutation and selection

Table 3. Percent nucleotide (above diagonal) and amino acid (below diagonal) identity among WSMV, ONMV, BrSMV and SCSMV strains and isolates

Identities are based on alignment of nucleotides 7618–9384 or amino acid residues 2496–3035 (WSMV-Sidney 81 coordinates).

Strain/ isolate	WSMV*						ONMV†			BrSMV‡			SCSMV§		
	Sidney 81	Type	El Batán 3	Hungary	Czech	Russia	Turkey 1	Turkey 2	Iran	Type	Pp	A	11-Cal	Hm	Pk
Sidney 81	–	97.4	81.5	91.0	91.3	90.5	98.6	98.4	92.0	75.2	75.1	59.8	58.6	58.9	49.2
Type	97.8	–	81.0	91.0	91.1	90.3	96.8	96.8	92.3	75.5	75.4	59.5	58.4	58.8	49.4
El Batán 3	86.5	85.7	–	81.4	81.3	81.0	81.0	81.4	81.3	75.6	75.6	57.4	57.5	56.4	48.3
Hungary	97.2	97.0	86.8	–	99.0	98.0	90.6	90.5	91.3	75.9	76.0	58.8	58.6	59.7	49.4
Czech	97.2	96.8	86.8	99.1	–	98.2	90.8	90.7	91.8	75.7	75.8	59.0	58.7	59.7	49.5
Russia	97.4	97.0	87.0	99.4	99.3	–	90.9	90.7	91.8	76.1	76.2	58.0	57.9	58.6	49.4
Turkey 1	99.4	97.8	86.3	97.0	97.0	97.2	–	98.4	92.3	75.5	75.5	59.0	58.0	58.5	49.6
Turkey 2	98.7	96.8	86.1	96.3	96.3	96.5	98.9	–	92.3	75.0	74.9	59.2	58.2	58.5	49.1
Iran	96.3	95.9	86.5	97.2	97.2	97.4	96.5	95.7	–	74.3	74.2	58.7	58.1	57.7	49.7
ONMV-Type	80.2	79.8	79.8	80.8	80.6	81.0	80.2	79.2	80.0	–	99.8	55.3	55.5	54.9	47.6
ONMV-Pp	80.2	79.8	79.8	80.8	80.6	81.0	80.2	79.2	80.0	100	–	55.2	55.5	54.9	47.5
BrSMV-A	49.7	49.7	49.3	49.7	49.7	49.7	49.3	49.3	49.1	48.1	48.1	–	87.4	84.3	44.2
BrSMV-11-Cal	49.9	49.5	49.3	49.9	49.9	49.9	49.5	49.5	49.3	48.3	48.3	98.4	–	81.2	44.5
BrSMV-Hm	44.9	44.9	44.3	44.9	44.9	44.9	44.5	44.7	44.5	43.3	43.3	88.8	88.2	–	43.9
SCSMV-Pk	35.5	35.5	35.5	35.9	35.9	35.9	35.3	35.7	35.5	35.7	35.7	33.2	33.2	31.0	–

* Type species of genus *Tritimovirus*.† Currently classified in the genus *Rymovirus*.‡ Definitive species of genus *Tritimovirus*.

§ Currently unclassified.

Table 4. Percent nucleotide (above diagonal) and amino acid (below diagonal) identity among complete sequences of five WSMV strains

WSMV strain	Sidney 81	Type	Turkey 1	Czech	El Batán 3
Sidney 81	–	97.6	98.3	88.9	79.4
Type	98.8	–	97.4	88.7	79.3
Turkey 1	99.6	98.7	–	88.7	79.5
Czech	98.3	97.6	98.1	–	79.9
El Batán 3	90.4	90.2	90.2	90.3	–

rates from allele frequency data. Here, 539 aligned codons (corresponding to Sidney 81 nt 7619–9235) of nine WSMV isolates were examined in which an allele is defined by a difference from the consensus sequence. Such differences may occur in sequences of one, two, three or four of nine isolates. The observed allele frequency distribution for silent substitutions was class 1: 230; class 2: 25; class 3: 39; and class 4: 94. PRF maximum likelihood estimate for μ , the mutation parameter, was 41.4 ± 4.8 and for γ , the selection parameter, was 1.00 ± 1.71 . The latter parameter was not significantly different from $\gamma = 0$ ($P = 0.103$) by the log ratio test. The allele frequency distribution for replacement substitutions was class

1: 70; class 2: 7; class 3: 4; and class 4: 10. The estimate for μ (replacement) was 51.2 ± 10.5 and the γ (replacement) estimate was -3.81 ± 1.48 which is significantly different from the null hypothesis of $\gamma = 0$ ($P < 10^{-8}$).

Sequences examined include the N1b–CP junction cleaved by N1a proteinase, which for tritimoviruses has been experimentally determined only for WSMV (Choi *et al.*, 2000). The N1b–CP junction N1a proteinase cleavage site (QYCVYE/S), corresponding to Sidney 81 amino acid residues 2681–2686, was identical among all nine WSMV isolates. The deduced N1b–CP junction (KYCVYE/S) for ONMV was very similar to that of WSMV, whereas the deduced N1b–CP

junction (DVCKFE/S) of BrSMV contained multiple substitutions.

Comparison of full-length WSMV sequences

The complete genomes of WSMV-CZ and WSMV-TK1 were 9381 and 9384 nt, respectively, exclusive of the polyadenylated 3' terminus. The TK1 genome contained no insertions or deletions relative to Sidney 81 or Type, whereas the CZ genome lacked a single codon within the CP cistron (Sidney 81 nt 8411–8413) that also was absent in the partial sequences of the HU and R isolates. Both WSMV-CZ and -TK1 encoded a polyprotein as a single open reading frame, as is typical for species of the family with monopartite genomes. A genetic distance matrix, expressed as percent identities of entire genome (nucleotide) or entire polyprotein (amino acid) sequences, for five WSMV complete sequences is presented in Table 4. The percent identities calculated for complete sequences were very similar to those for partial sequences (Table 3), suggesting that relationships based on partial sequences of the 3' terminus were reasonable estimates of overall relatedness.

Discussion

ONMV is a distinct virus species and definitive member of the genus *Tritimovirus*

Host range and Western blotting authenticated two ONMV isolates. ONMV-Type is the same isolate examined by Gill (1976a, b) and purported to have been cloned and sequenced by Chen *et al.* (2001). The second isolate, ONMV-Pp, was identified as a strain of ONMV based on a narrow experimental host range identical to that of ONMV-Type, and by strong cross-reactivity in Western blots probed with ONMV-Type antiserum. The nucleotide sequences of ONMV-Type and -Pp were nearly identical, and differed by only two silent transitions in the coding region and two transitions in the 3'-untranslated region. Although maize was listed as a non-host of ONMV-Type by Gill (1976a), both ONMV isolates systemically infected SDp2 maize. This lone difference in host range for ONMV reported by Gill (1976a) and here most likely reflects genetic differences among maize lines.

Western blots demonstrated that ONMV is related to WSMV. Reciprocal cross-reactivity of both WSMV and ONMV antisera with heterologous CP in Western blots confirms and extends the findings of Gill (1976b), who first reported a serological relationship between ONMV and WSMV. The lack of reactivity of ONMV or BrSMV antisera in heterologous combinations indicates that ONMV and BrSMV are not synonymous.

Comparison of nucleotide and amino acid sequences of ONMV-Type and -Pp with other members of the family *Potyviridae* (Table 3, Fig. 3) further demonstrate that ONMV is

most similar to WSMV and not to BrSMV. Because the sequence designated as ONMV-Type by Chen *et al.* (2001) clearly represents a strain of BrSMV (here referred to as BrSMV-A), they proposed that ONMV is not a distinct virus, but rather a synonym of BrSMV. Chen *et al.* (2001) unfortunately did not authenticate their isolate. DAS-ELISA tests performed with lyophilized infected tissue of the ONMV-Type culture from the source used by Chen *et al.* (2001) confirmed mixed infection by BrSMV and ONMV (data not shown). An earlier passage of this same ONMV-Type culture used by us tested positive for ONMV but negative for BrSMV by DAS-ELISA (data not shown) and Western blots (Fig. 1). Thus, the simplest explanation is contamination of the ONMV-Type culture with BrSMV prior to analysis by Chen *et al.* (2001), who inadvertently cloned and sequenced BrSMV after PCR amplification with degenerate primers.

Collectively, our data indicate that ONMV is a distinct species of the family *Potyviridae* and is currently misclassified as a definitive member of the genus *Rymovirus*. The serological and nucleotide sequence relationships presented here indicate that ONMV is a tritimovirus and not a rymovirus. Thus, it is proposed that ONMV be removed from the genus *Rymovirus* and instead designated as a definitive member of the genus *Tritimovirus*.

Does SCSMV represent a new genus?

SCSMV-Pk is quite divergent from other viruses of the family *Potyviridae*, and shares similar levels of sequence identity with both tritimoviruses and ipomoviruses (Hall *et al.*, 1998). Since both neighbour joining and maximum likelihood methods did not place SCSMV in a clade composed exclusively of tritimoviruses, designation of SCSMV as a tritimovirus species is not warranted. The maximum likelihood phylogram (Fig. 3B) has a node common to SCSMV-Pk and the two ipomovirus species, but these taxa are not closely related as exemplified by long branch lengths in both phylograms (Fig. 3). Thus, we propose that SCSMV represents a new and separate genus of the family *Potyviridae*.

Strain diversity within tritimovirus species

In addition to the nine isolates of WSMV compared here, partial CP sequences of nine additional US isolates of WSMV have been reported (Niblett *et al.*, 1991; Chenault *et al.*, 1996). All US isolates of WSMV are closely related. Surprisingly, the two Turkish isolates were more closely related to the US isolates than to other Eurasian isolates of WSMV. Collectively, these data suggest that the US and Turkish isolates have diverged only recently, relative to WSMV from Mexico, central Europe, Russia and Iran. Previous analyses of the complete genomes of Sidney 81, Type and El Batán 3 demonstrated that much of the divergence among WSMV strains may be explained by genetic drift (Choi *et al.*, 2001).

Several genetic isolation mechanisms foster divergence among sympatric WSMV genotypes (Hall *et al.*, 2001a) that may contribute to consensus sequence drift upon passage (Hall *et al.*, 2001b). The distribution of nucleotide substitutions among the nine WSMV isolates supports the genetic drift hypothesis for divergence within WSMV since the PRF model estimate for selection at synonymous sites was close to zero. Not unexpectedly, however, there was a 'footprint' of negative selection in the distribution of nonsynonymous substitutions. Within BrSMV, sequence comparisons indicated that most nucleotide sequence polymorphisms within the coding region examined for BrSMV-11-Cal and -A are silent (Table 3), whereas BrSMV-Hm has diverged considerably from the other two BrSMV sequences. In this regard, the extent of divergence within BrSMV is similar to that within WSMV. In contrast, the two ONMV isolates were nearly identical in sequence, suggesting a very recent common ancestor.

Biogeography of tritimoviruses

WSMV clearly occurs on both sides of the Atlantic. Nonetheless, it appears that there are at least two resident populations each in North America and Eurasia. McNeil *et al.* (1996) hypothesized that WSMV in the American Great Plains may constitute a single population. WSMV is encountered only infrequently in Mexico (Sánchez-Sánchez *et al.*, 2001), but the divergent genome of El Batán 3 suggest that the Mexican population has been genetically isolated from the Great Plains population for some time (Choi *et al.*, 2001). The three WSMV isolates from central Europe and Russia share a most recent common ancestor, and may be representative of a third allopatric population separate from the WSMV population of Asia Minor. The extent of divergence of WSMV-I from the US and Turkish isolates could be viewed as evidence of yet another WSMV population in Eurasia.

High sequence identity of US and Turkish genotypes suggests recent and, most likely, human-assisted movement of WSMV across the Atlantic. Although the direction and number of introductions cannot be ascertained, we do note that hard red winter wheat culture in South Dakota, Nebraska and Kansas was initiated during the 1880s by immigrants from the Crimea (Reitz & Heyne, 1944; Ross, 1969). These introduced 'Turkey wheats' were so successful that during the past century germplasm has been extensively exchanged between the Great Plains and the Black Sea region. Given that wheat curl mites are difficult to detect with the naked eye and if WSMV is seed-borne at low efficiency in wheat as in maize (Hill *et al.*, 1974), both virus and vector could have passaged the Atlantic in grain shipments.

Less is known about the geographical distribution of other tritimoviruses. BrSMV is widespread in Europe, but has not been found outside continental Europe. ONMV has been isolated in Canada only twice (Gill, 1976a; Remah, 1993). This study represents the first report of ONMV in Europe.

However, because ONMV-Pp was recovered from a vegetatively propagated Kentucky blue grass accession, it is possible that ONMV-Pp originated in North America and was recently transported to Germany in an infected plant. The high sequence identity shared by ONMV-Type and -Pp supports this hypothesis.

We have defined relationships among and within species of the genus *Tritimovirus* and propose taxonomic revision within the family *Potyviridae*. WSMV and ONMV occur on both sides of the Atlantic, most probably due to human activities. Given the scope of world trade, it is likely that additional transoceanic introductions have occurred or will occur in the future. Thus, there is a need to have a global perspective such that control strategies are designed to address the full range of diversity within a viral species.

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