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**Complete nucleotide sequence of *Oat necrotic mottle virus*:  
A distinct *Tritimovirus* species (family *Potyviridae*) most  
closely related to *Wheat streak mosaic virus***

Brief Report

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**Summary.** The RNA genome (9346 nucleotides) of *Oat necrotic mottle virus* (ONMV) was cloned and sequenced. Complete genome comparisons indicated that ONMV, currently classified as a rymovirus, was most closely related (~73% nucleotide, ~79% amino acid identity) to the tritimovirus *Wheat streak mosaic virus* (WSMV). ONMV encoded a single polyprotein, with proteinase cleavage sites very similar to those of WSMV. Pairwise comparison of ONMV and WSMV cistrons revealed that P3 was most conserved (~79% nucleotide, ~86% amino acid), whereas HC-Pro was most divergent (~67% nucleotide, ~69% amino acid). In contrast, the ONMV sequence was distantly related (~40% nt, ~26% amino acid identity) to that of the rymovirus *Ryegrass mosaic virus*, with highest sequence conservation noted within the N1b cistron (~47% nucleotide, ~41% amino acid identity). These results firmly establish that ONMV is not a rymovirus but is instead a distinct species of the genus *Tritimovirus*.

\*

*Oat necrotic mottle virus* (ONMV) is a poorly characterized virus of the family *Potyviridae* only known to occur in Manitoba, where it causes a minor disease of oat [2]. ONMV was assigned to the genus *Rymovirus* [20], based on a weak serological relationship with *Wheat streak mosaic virus* (WSMV) [3]. At that time the genus *Rymovirus* included all monocot-infecting potyviruses known, or suspected, to be transmitted by eriophyid mites. It since has been demonstrated that several eriophyid mite transmitted viruses (e.g. WSMV and *Brome streak mosaic virus* [BrSMV]) share an evolutionary history distinct from that of the rymoviruses

*Ryegrass mosaic virus* (RGMV), *Agropyron mosaic virus*, and *Hordeum mosaic virus* [6, 12, 15]. As a result, WSMV and BrSMV were removed from the genus *Rymovirus* and placed within the newly erected genus *Tritimovirus* [18]. More recently, comparison of 3'-terminal nucleotide (nt) sequences coding for CP and part of Nib [10] suggested that ONMV also should be removed from the genus *Rymovirus* and reclassified as a *Tritimovirus* species. However, as taxonomic placement may be most accurately accomplished through analysis of complete genome sequences, the generic affiliation of ONMV remains uncertain. To resolve the taxonomic status of ONMV, we report here the cloning and analysis of the complete ONMV nucleotide sequence.

A culture of the Type isolate of ONMV (ONMV-Type, ATCC PV-107) maintained at the University of Nebraska since ca. 1988 was used in this study, and propagated in mechanically inoculated oat (*Avena sativa* L.) cv. 'Shaw'. This Nebraska culture, designated here as ONMV-Type<sub>NE</sub>, was authenticated by host range and symptom expression: systemic infection of oat with mild mosaic, symptomless systemic infection of Kentucky blue grass (*Poa pratensis* L.), and inability to infect wheat (*Triticum aestivum* L.). The Nebraska culture was further authenticated by comparing sequence of the 3'-terminal 1728 nucleotides, derived from a reverse transcription-polymerase chain reaction (RT-PCR) product, with the corresponding sequence of two isolates of ONMV (ONMV-Type maintained in Aschersleben, Germany [ONMV-Type<sub>A</sub>] and ONMV-*Poa pratensis* [ONMV-Pp]) for which this same region has been sequenced [10]. The RT-PCR product of ONMV-Type<sub>NE</sub> was generated and cloned using the same conditions as reported [10], with the nt sequence used for comparison being a consensus derived from three independent clones. These results indicated that the 1728 nt 3'-terminal sequence of ONMV-Type<sub>NE</sub> shared 99.3% identity with ONMV-Type<sub>A</sub> [AF454460] and 99.1% identity with ONMV-Pp [AF454461].

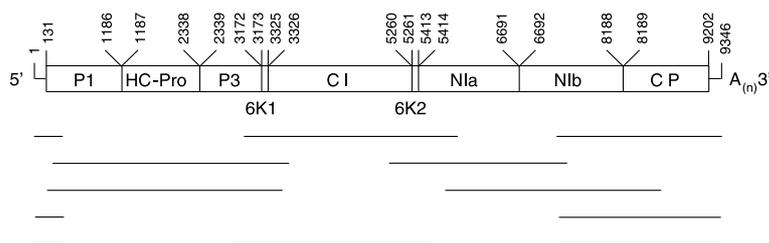
ONMV-Type<sub>NE</sub> virions were purified from systemically infected oat using a protocol modified from Schubert and Rabenstein [13]. Briefly, frozen infected tissue was ground in 1.5 volumes of extraction buffer (0.1 M sodium citrate, pH 7.0) and filtered through Miracloth. The supernatant recovered after centrifugation (20 min., 16,000 × g, 4 °C) was adjusted to 2% triton X-100 and mixed for 5 min. Virions were concentrated by centrifugation (2 hr, 101,000 × g, 4 °C) through a 20% sucrose pad and resuspended in extraction buffer. Virions were further purified by density gradient centrifugation in which resuspended virions were layered onto 28% CsCl and centrifuged in a Beckman SW 55.1 rotor (19 hrs, 35,000 rpm, 10 °C). Virions recovered from gradients were diluted with extraction buffer, concentrated by centrifugation (2 hr, 107,000 × g, 4 °C), and resuspended in a minimum volume of extraction buffer.

ONMV-Type<sub>NE</sub> RNA (~2 µg) extracted from virions was used as template for reverse transcription with oligo dT or random hexamers as primers. Both first strand and second strand cDNA were synthesized using the Universal Ribo Clone cDNA Synthesis System (Promega, Madison, WI, U.S.A.). Double stranded cDNA was made blunt-ended using T4 DNA polymerase and size fractionated on a 1.2% agarose gel. Gel-purified DNA (≥2 kbp) was A-tailed using *Taq* polymerase

(30 min, 70 °C) and ligated to T-tailed pGEM-T Easy (Promega). The ligation products were transformed into *E. coli* DH5 $\alpha$  and clones identified based on plasmid insert size and endonuclease restriction site profiles. To clone the genomic 5'-terminus, the 5'/3' RACE kit (Roche) was employed as described [15]. For each ONMV-Type<sub>NE</sub> cDNA clone, the nucleotide sequence of both strands was obtained by automated sequencing (DNA Sequencing Facility, Iowa State University and Davis Sequencing, Inc., Davis, CA) using a combination of universal and custom primers.

Polyprotein sequences of taxa representative of the family *Potyviridae* were aligned using the T-Coffee computer program [9]. The polyprotein alignment was used as a guide to manually align nucleotide sequences with the Sequence Alignment Editor (Version 1.0 alpha 1, copyright 1996; A. Rambaut, Department of Zoology, University of Oxford, UK). Noncoding nucleotide sequences were aligned with ClustalX [17]. Sequence analyses were done using the computer programs DnaSP [11], MEGA2 [7], and DAMBE [19].

The complete ONMV-Type<sub>NE</sub> consensus sequence (9346 nucleotides, excluding a variable length polyadenylated tail) was assembled from multiple overlapping cDNA clones (Fig. 1) and deposited in GenBank as accession AY377938. As with other monopartite viruses of the family *Potyviridae*, the ONMV-Type<sub>NE</sub> genome encoded a single polyprotein, with an initiation codon beginning at nt 131 and a termination codon ending at nt 9202. Alignment of the ONMV-Type<sub>NE</sub> sequence with that of five sequenced [1, 10, 15] strains of WSMV (Sidney 81 [AF057533], Type [AF285169], TK1 [AF454455], CZ [AF454454], and El Batán 3 [AF285170]) required introduction of gaps within the “hypervariable” region of the CP cistron, in a position similar to that in which the WSMV-El Batán 3 sequence has a 45 nt gap relative to WSMV-Sidney 81 [1]. The alignment also resulted in two additional single nt gaps downstream of the polyprotein termination codon, such that the ONMV-Type<sub>NE</sub> 3'-untranslated region (3'-UTR) was two nucleotides shorter in length relative to WSMV. However, alignment of ONMV-Type<sub>NE</sub> (and the five WSMV strains) with BrSMV-11-Cal (Z48506), two strains (AV and D) of the rymovirus RGMV (AF035818, Y09854), and the N strain of *Potato virus Y* (PVY; NC 001616) required introduction of multiple gaps scattered throughout



**Fig. 1.** Genome organization of *Oat necrotic mottle virus*-Type<sub>NE</sub>. Nucleotide coordinates demarcating individual cistrons are indicated. Relative positions of cDNA clones used to compile the complete nucleotide sequence appear below the genome map. The 3'-terminal nucleotide coordinate (9202) of the CP cistron includes the UAA stop codon

**Table 1.** Predicted *Tritimovirus* polyprotein proteinase cleavage sites

Cleavage site	WSMV <sup>a</sup>	ONMV-Type <sub>NE</sub> <sup>b</sup>	BrSMV-11Cal <sup>b</sup>
P1/HC-Pro	HGLRWY/G <sup>c</sup>	HGLRWY/S	<u>ERIEYY/S</u>
HC-Pro/P3	KDYKIG/G	KDYKIG/G	<u>KEYEIG/G</u>
P3/6K1	ELVEYQ/G	ELVEYQ/S	<u>EVVVFQ/S</u>
6K1/CI	FNCEYQ/S <sup>d,e</sup>	<u>VNCEYQ/S</u>	<u>VGSIYQ/S</u>
CI/6K2	SHVSYQ/A	SHVSYQ/A	<u>AHVMYQ/K</u>
6K2/NIa	RSVKFE/G	<u>HRVKYE/G</u>	<u>HEAKFE/G</u>
NIa/NIb	DLVSWQ/S	<u>ELVNWQ/S</u>	<u>KLVGfQ/N</u>
NIb/CP	QYCVYE/S	<u>KYCVYE/S</u>	<u>DVCKFE/S</u>

<sup>a</sup>Identical for WSMV strains Sidney 81, Type, TK1, CZ, and El Batán 3 unless otherwise noted

<sup>b</sup>Underline denotes amino acid residues different from WSMV

<sup>c</sup>WSMV-CZ cleavage site is HGLRWY/C

<sup>d</sup>Incorrectly reported as FNCEYQ/G in Stenger et al. 1998

<sup>e</sup>WSMV-Type cleavage site is FSCEYQ/S

the genomic sequences. All predicted polyprotein proteinase cleavage sites for ONMV-Type<sub>NE</sub> were located at the same genomic coordinates as that of all five WSMV strains (Fig. 1) and were very similar, or identical, to the corresponding cleavage sites of WSMV (Table 1). In contrast, comparison of ONMV-Type<sub>NE</sub> (or WSMV) with BrSMV-11-Cal [4], revealed additional substitutions at each proteinase cleavage site (Table 1).

Pairwise comparisons of complete genome (nt) or complete polyprotein (amino acid [aa]) sequence identities for ONMV-Type<sub>NE</sub> and two tritimoviruses (5 WSMV strains and BrSMV-11-Cal), the rymovirus RGMV-AV, or the potyvirus PVY-N are listed in Table 2. These data clearly indicate that ONMV is most closely related to WSMV and should be reclassified as a *Tritimovirus*. Although the five WSMV strains vary from one another in complete nucleotide sequence identity by ~2% to 20% [1, 10], ONMV-Type<sub>NE</sub> was essentially equidistant from each WSMV strain in both nt and aa sequence identity when complete genomes or polyproteins were compared. Thus, at ~73% nt sequence identity for the complete genome, ONMV is sufficiently different from WSMV to be a distinct tritimovirus species, as this value is considerably less than the species demarcation threshold (<85% nt sequence identity) established by the International Committee on the Taxonomy of Viruses [18]. ONMV-Type<sub>NE</sub> retained only 52.4% (nt) or 47.0% (aa) sequence identity with the only other known tritimovirus (BrSMV), and these values were similar to that obtained when the complete genomes of BrSMV and WSMV-Sidney 81 were compared (52.7% nt, 46.6% aa). This degree of divergence among species within a genus of the family *Potyviridae* is not uncommon [14]. In contrast, comparison of the ONMV-Type<sub>NE</sub> sequence with RGMV-AV or PVY-N indicated that ONMV is distantly related (>40% nucleotide, >26% amino acid identity) to both rymoviruses and potyviruses (Table 2). A neighbor-joining analysis (based on

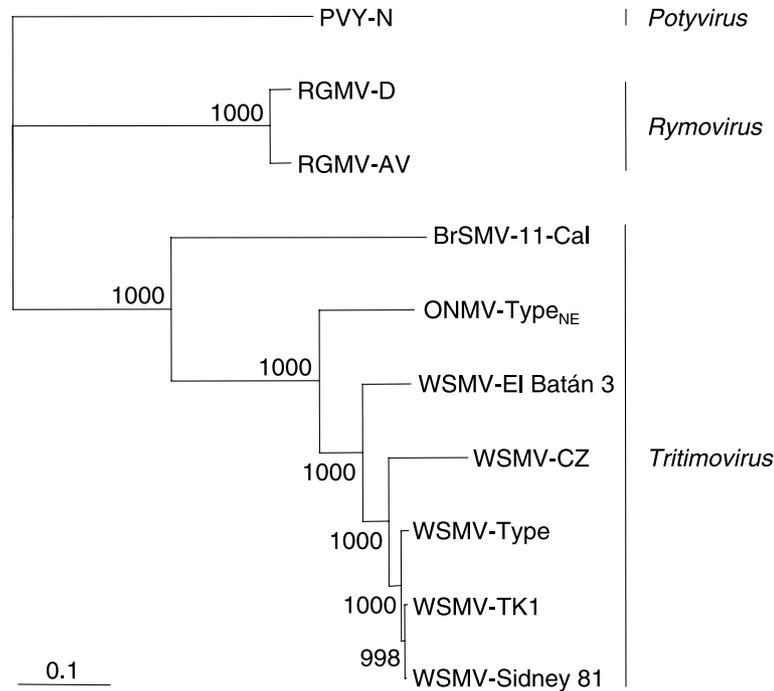
**Table 2.** Percent sequence identity<sup>a</sup> of ONMV-Type<sub>NE</sub> compared to other tritimoviruses (*Wheat streak mosaic virus* [WSMV] and *Brome streak mosaic virus* [BrSMV]), a rymovirus (*Ryegrass mosaic virus* [RGMV]), and a potyvirus (*Potato virus Y* [PVY])

Strain	WSMV					BrSMV 11-Cal	RGMV AV	PVY N
	Sidney 81	Type	TK1	CZ	El Batán 3			
Complete genome	72.9 (80.5)	72.9 (80.4)	73.0 (80.5)	72.8 (80.5)	72.4 (80.5)	52.4 (47.0)	39.7 (25.9)	39.2 (23.3)
5'-UTR	72.3	70.8	70.8	70.0	70.8	50.4	46.0	61.0
P1	68.7 (70.2)	68.9 (70.2)	68.5 (69.6)	67.5 (70.2)	68.9 (71.6)	39.0 (22.0)	35.0 (20.4)	31.5 (14.9)
HC-Pro	66.9 (70.1)	67.3 (69.8)	67.2 (69.5)	65.3 (69.5)	65.8 (68.0)	51.5 (42.8)	35.3 (17.0)	37.0 (17.0)
P3	79.5 (86.7)	79.3 (86.7)	79.0 (86.7)	78.9 (86.3)	79.6 (86.0)	49.9 (40.1)	32.8 (16.7)	30.1 (12.1)
6K1	73.2 (88.2)	71.9 (86.3)	71.2 (88.2)	72.5 (88.2)	73.2 (88.2)	57.5 (52.9)	36.6 (25.5)	39.3 (18.0)
CI	76.1 (89.0)	76.1 (89.3)	76.3 (89.1)	77.2 (89.6)	74.7 (89.1)	56.4 (52.7)	39.9 (26.6)	39.9 (25.2)
6K2	68.6 (70.6)	68.0 (70.6)	69.3 (70.6)	68.6 (70.6)	66.7 (72.5)	56.2 (51.0)	38.6 (21.6)	37.9 (15.7)
NIa	74.3 (79.6)	74.0 (79.3)	74.2 (79.6)	73.9 (79.3)	71.7 (80.5)	55.7 (51.1)	41.9 (27.2)	39.2 (24.5)
NIb	72.8 (84.2)	72.9 (84.0)	73.3 (84.2)	74.1 (84.0)	73.7 (83.6)	60.1 (61.8)	47.1 (40.9)	48.6 (38.3)
CP	71.8 (78.3)	71.4 (77.7)	72.1 (78.6)	70.9 (78.0)	73.7 (78.2)	52.2 (43.4)	39.5 (23.9)	39.7 (22.5)
3'-UTR	78.2	78.9	78.2	78.2	74.2	44.5	38.0	37.0

<sup>a</sup>Percent nucleotide sequence identity followed in parentheses by percent amino acid sequence identity, if applicable

complete polyprotein sequences) clearly places ONMV within a clade composed of tritimoviruses, and further illustrates the distant relationship of ONMV with species of the genera *Rymovirus* and *Potyvirus* (Fig. 2).

Pairwise comparison of terminal untranslated regions (5'-UTR and 3'-UTR) or individual cistrons (demarcated by proteinase cleavage sites listed in Table 1) of ONMV-Type<sub>NE</sub> and other tritimoviruses are indicated in Table 2. As with the complete genome, the WSMV strain used in pairwise comparison of individual elements had minimal effect on sequence identity relative to ONMV-Type<sub>NE</sub>. Among individual cistrons, ONMV-Type<sub>NE</sub> and WSMV shared the greatest sequence identity in the P3 cistron (~79% nt, ~86% aa), and the least amount of sequence identity in the HC-Pro cistron (~67% nt, ~69% aa). Among ONMV Type<sub>NE</sub> and BrSMV-11-Cal, the highest level of sequence conservation was for the NIb cistron (60.1% nt, 61.8% aa identity), with the P1 cistron being the most divergent (39.0% nt, 22.0% aa identity). Among ONMV and RGMV or PVY, NIb



**Fig. 2.** Neighbor-joining analysis based on an alignment of complete polyprotein amino acid sequences of representative taxa of the family *Potyviridae*. The phylogram presented is based on 1000 bootstraps, with bootstrap support for each node indicated. Branch lengths are proportional to genetic distance (indicated by scale below). PVY-N was designated as an outgroup to root the phylogram. Generic affiliation of taxa are indicated at right

also was the most conserved cistron, albeit at identity values considerably less than that within the genus *Tritimovirus* (Table 2).

As alignment of the ONMV-Type<sub>NE</sub> and WSMV polyprotein sequences did not require introduction of gaps other than in the amino-terminal region of the CP cistron mentioned above, divergence between the two viruses may be unambiguously quantified in several other ways. Between the two viruses there were 919 fixed differences at synonymous codon sites and 583 fixed differences at nonsynonymous sites. In contrast, there were 2148 synonymous and 310 nonsynonymous variable sites among the five WSMV strains. The difference in the ratios of fixed and polymorphic sites again support the idea that ONMV-Type<sub>NE</sub> is a distinct species. Further, the excess of fixed amino acid replacements between the two species suggests that there is differential adaptive selection [8] acting on the polyproteins of the two viruses, whereas the pattern of substitutions within WSMV may be best explained by genetic drift [1, 16]. Additional evidence for divergence can be seen in the nonsynonymous site differences between the two viruses. Codons leading to amino acid sequence differences between ONMV-Type<sub>NE</sub> and the five WSMV strains tended to undergo multiple substitutions, with an average of 284 codons differing at two positions and 94 differing at all three

codon positions. Multiple substitutions also occurred within 101 synonymous codons. Lastly, the average ratio of transversion versus transition nt substitutions between ONMV-Type<sub>NE</sub> and WSMV was 1.1 while among the five WSMV strains it was 2.1. This parallels the trend seen previously using 3'-terminal nt sequences [5, 10, 16].

Collectively, the substitution pattern data are consistent with a hypothesis that divergence is due to accumulation of recurrent mutations at most variable sites, and that at least one of the branches leading from ONMV-Type<sub>NE</sub> and WSMV to their most recent common ancestor is longer than that indicated by percent nt distance alone. Furthermore, a most parsimonious explanation for the inability of ONMV to infect wheat is that this phenotype likely represents a derived character state in which, during the divergence of ONMV, this lineage lost the ability to infect wheat. As BrSMV and WSMV both infect wheat and share a most recent common ancestor basal to that of ONMV and WSMV (Fig. 2), infection of wheat, therefore, is most likely an ancestral character state of the clade represented by the three known species of tritimoviruses.

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