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I.-R. Choi
University of Nebraska - Lincoln

J.S. Hall
University of Nebraska - Lincoln

M. Henry
Centro Internacional De Mejoramiento de Maiz y Trigo, Mexico City, Mexico

Lingyu Zhang
University of Nebraska - Lincoln

G.L. Hein
University of Nebraska Panhandle Research and Extension Center, Scottsbluff, Nebraska, U.S.A.

See next page for additional authors

Authors
I.-R. Choi, J.S. Hall, M. Henry, Lingyu Zhang, G.L. Hein, Roy C. French, and D.C. Stenger

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Contributions of genetic drift and negative selection on the evolution of three strains of wheat streak mosaic tritimovirus

Brief Report

I.-R. Choi[1], **J. S. Hall**[1] **M. Henry**[2], **L. Zhang**[1,*], **G. L. Hein**[3], **R. French**[1], and **D. C. Stenger**[1]

[1]USDA-ARS and Department of Plant Pathology, University of Nebraska, Lincoln, Nebraska, U.S.A.
[2]Centro Internacional De Mejoramiento de Maiz y Trigo, Mexico City, Mexico
[3]Department of Entomology, University of Nebraska Panhandle Research and Extension Center, Scottsbluff, Nebraska, U.S.A.

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Summary. Genome sequences of three *Wheat streak mosaic virus* (WSMV) strains were compared. The Type and Sidney 81 strains of WSMV from the American Great Plains were closely related, with sequence identities of 97.6% (nucleotide) and 98.7% (amino acid). In contrast, the El Batán 3 strain from central Mexico was divergent, and shared only 79.2–79.3% (nucleotide) and 90.3–90.5% (amino acid) sequence identity with Type and Sidney 81. All three WSMV strains were serologically related, however the El Batán 3 capsid protein (CP) had 15 fewer amino acid residues. Phylogenetic analysis of the CP cistron indicated that Type, Sidney 81, and nine other American isolates of WSMV were closely related and distinct from the El Batán 3 sequence. Nucleotide substitutions among the WSMV strains were not randomly distributed across the genome with more variation within P1, HC-Pro, and CP, and less within P3. One 400-nucleotide region of the genome, corresponding to the 3′-end of P3, was strikingly deficient in silent substitutions. Nonetheless, the ratio of synonymous to non-synonymous substitutions throughout the genome was essentially the same for all three WSMV strains. Collectively, our data indicate that both genetic drift and negative selection have contributed to the evolution of WSMV strains.

*

*Pioneer Hi-Bred International, Johnston, IA 50131, U.S.A.

Wheat streak mosaic virus (WSMV) is the type species of the recently established genus *Tritimovirus* of the family *Potyviridae* [30]. The genus *Tritimovirus* includes WSMV and *Brome streak mosaic virus* (BrSMV) [12, 13]. Both tritimoviruses are transmitted by eriophyid mites to monocotyledonous hosts and are phylogenetically distinct from eriophyid mite-transmitted viruses in the genus *Rymovirus* [14, 27, 30]. WSMV has a genome organization similar to that of other monopartite members of the family *Potyviridae*, encoding a polyprotein that is subsequently cleaved by viral-encoded proteinases into 8–10 mature proteins capable of complex protein-protein interactions [6].

WSMV occurs throughout the major wheat growing areas of the United States and Canada, and in epidemic years may contribute to substantial losses [2]. The complete nucleotide sequence (GenBank accession AF057533) of the Sidney 81 strain of WSMV from Nebraska has been determined [30]. The Type strain of WSMV from Kansas [20] may be distinguished from Sidney 81 by the inability of Type to infect the inbred maize line SDP2 [5]. The El Batán 3 strain recently has been isolated from the Central Highlands of Mexico [28]. All three strains are efficiently transmitted from wheat to wheat by the eriophyid mite *Aceria tosichella* (Keifer) [2, 5, 28]. In this communication, we report the complete nucleotide sequences of the WSMV Type (GenBank accession AF285169), and El Batán 3 (GenBank accession AF285170) strains. We further examine the extent and distribution of sequence divergence among the three strains to infer major forces driving WSMV strain evolution.

Type and Sidney 81 are well characterized strains of WSMV. El Batán 3 was initially diagnosed as a WSMV strain based on symptom expression, eriophyid mite transmission, and serology [28]. Confirmation of El Batán 3 as a strain of WSMV was accomplished by reverse transcription-polymerase chain reaction (RT-PCR) of the CP cistron amplified from a total RNA sample using primers described previously [9, 21]. The resulting PCR product was slightly smaller in size than those obtained for Sidney 81 or Type (data not shown). Western blotting of total soluble protein extracted [3] from wheat infected with each WSMV strain revealed that the CP of all three strains reacted to antibodies raised to Type CP and that the El Batán 3 CP appeared to be of smaller size relative to the CP of Type and Sidney 81 (Fig. 1).

Virions of Type and El Batán 3 were purified from wheat (*Triticum aestivum* L.) cv. 'Centurk' as described [18], with modifications indicated [30]. Viral RNA of each strain was purified and used as templates for oligo dT primed reverse transcription [30]. Second strand synthesis was accomplished with the Klenow fragment of DNA polymerase I and *Not* I/*Eco*R I adapters were ligated to the dsDNA products.

High molecular weight (>9 kbp) dsDNA products of El Batán 3 were gel purified and ligated to *Not* I digested pACYC-177N1 (pACYC-177 from New England Biolabs with the unique *Sma* I site modified to a *Not* I site) and transformed into *E. coli* strain JM109. pWSMV-M10-11 contained a ~ 9.3 kbp cDNA insert of the El Batán 3 genome (nts 23-9339), lacking only 22 nucleotides of the 5′-end. The complete nucleotide sequence of pWSMV-M10-11 was deter-

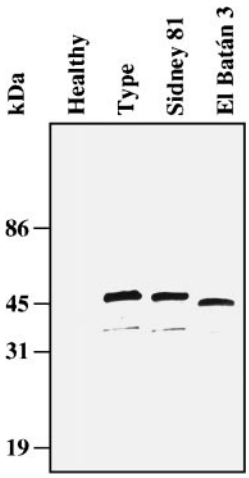

Fig. 1. Serological relatedness of the coat protein (CP) of three *Wheat streak mosaic virus* (WSMV) strains. Presented is a western blot of total soluble protein samples (20 μg) extracted from healthy wheat or wheat infected with WSMV strains Type, Sidney 81, or El Batán 3. The blot was probed with antibodies raised to the CP of WSMV-Type. The mobility and size (kDa) of prestained protein size markers are indicated at left. Note that the CP of El Batán 3 has increased mobility relative to the other two strains. Minor bands of less than full length represent degradation products of the WSMV CP commonly observed during infection [3]

mined for both strands by a primer walking strategy using two primers annealing to regions of pACYC-177N1 flanking the insert and 40 custom oligonucleotide primers to obtain internal sequence.

Type dsDNA products contained a prominent product of ∼2 kbp in size, and only traces of larger products. These were size selected (2–4 kbp) on an agarose gel, ligated to *Not* I digested pGEM5zf+, and transformed into *E. coli* JM109. The 3′-end sequence of the Type genome was determined from four cDNA clones produced by this procedure. To obtain additional Type cDNA clones, RT-PCR using the Expand High Fidelity PCR System (Boehringer-Mannheim, Indianapolis, IN) was performed on a total RNA sample from infected wheat. First strand cDNA was prepared by reverse transcription with both oligo dT and random hexamer primers included in the reaction to ensure complete coverage of the Type genome. PCR was performed using primers based on the Sidney 81 sequence to yield three overlapping products (nts 1-3172, 2339-6691, and 5414-9384) that were ligated into pGEM-T (Promega) and transformed into *E. coli* strain JM 109. Two clones from each PCR product were sequenced completely on both strands by primer walking. Sequence discrepancies between pairs of PCR-derived clones were resolved by obtaining sequence of a third clone for each region in question.

5′-end sequences of both El Batán 3 and Type were determined from three clones each produced using the 5′/3′ RACE kit (Promega, Madison, WI) as described [30]. All sequencing was performed by the Iowa State University DNA Sequencing Facility, Ames, IA. Complete nucleotide sequences were compiled using the Sequencher 3.1 program (Gene Codes, Ann Arbor, MI). To eliminate potential errors resulting from mismatches between Type cDNA and Sidney 81-derived primers, these regions were trimmed prior to compilation of the Type sequence. Open reading frames (ORFs) were identified using the DNA Inspector *IIe* program (Textco, West Lebanon, NH).

The complete nucleotide sequences of Type and El Batán 3 were 9384 and 9339 nts, respectively, exclusive of the variable-length polyadenylated 3′-end. Conceptual translation revealed each encoded a single polyprotein ORF typical

Table 1. Sequence identity and substitution rates among three *Wheat streak mosaic virus* strains

Strain	Sidney 81		Type	
	% Identity	ks/ka	% Identity	ks/ka
Sidney 81	100 (100)	–	–	–
Type	97.6 (98.7)	0.0886/0.0058	100 (100)	–
El Batán 3	79.2 (90.5)	0.7357/0.0561	79.3 (90.3)	0.7364/0.0591

First number indicates percent nucleotide identity; numbers in parentheses indicate percent amino acid identity. Synonymous (ks) and nonsynonymous (ka) rates are also given for each pairwise comparison

of monopartite members of the family *Potyviridae*. Complete nucleotide and polyprotein sequences of Sidney 81, Type, and El Batán 3 were aligned using Clustal X [33] and compared in Table 1. The Sidney 81 and Type genomes were closely related at both the nucleotide and protein level, whereas the El Batán 3 sequence diverged from both Type and Sidney 81. The position (between nts 8272 and 8273) of a 45 nt gap in the El Batán 3 sequence, relative to Sidney 81 and Type, was determined based on the single gap in aligned polyprotein sequences and the Clustal X nucleotide sequence alignment output was manually adjusted accordingly. This 45 nt gap encodes 15 amino acid residues within the CP cistron, and accounts for the smaller sizes of the El Batán 3 CP PCR product and CP described above. Sequencing of the El Batán 3 CP PCR product (data not shown) revealed the same 45 nt gap as in pWSMV-M10-11. Collectively, the occurrence of identical sequence gaps in two independent clones and the apparent smaller size of the El Batán 3 CP (Fig. 1) strongly indicate that the 45 nt gap in the El Batán 3 sequence is not an artifact of cloning. The 45 nt gap occurs within a serine-glycine rich region, positioned 28 amino acid residues downstream of the NIb-CP junction cleaved by NIa proteinase [30] to produce mature CP. This gap could reflect a deletion unique to El Batán 3, or an insertion in the common ancestor of Type and Sidney 81 after separation from the El Batán 3 lineage.

CP nucleotide sequences of Type, Sidney 81, and El Batán 3 were aligned with CP sequences of nine additional American isolates of WSMV [4, 22] and BrSMV [13]. Because the WSMV CP sequences of Chenault et al. [4] do not include the 5′-terminal sequences of the CP cistron, sequences were trimmed to the region (corresponding to Sidney 81 nts 8369–9238) available for all taxa. A maximum likelihood distance tree was generated using the Puzzle 4.02 program [31] and the HYK model of substitution with other parameters set at default values. The unrooted tree was visualized using the TreeView program [24]. The resulting phylogenetic analysis (Fig. 2) indicated that the CP cistrons of Type and Sidney 81 were closely related to all other American isolates, and confirmed that Type and Sidney 81 are representative of genotypes common in the United States. The branch bearing El Batán 3 was longer (reflecting genetic distance) and

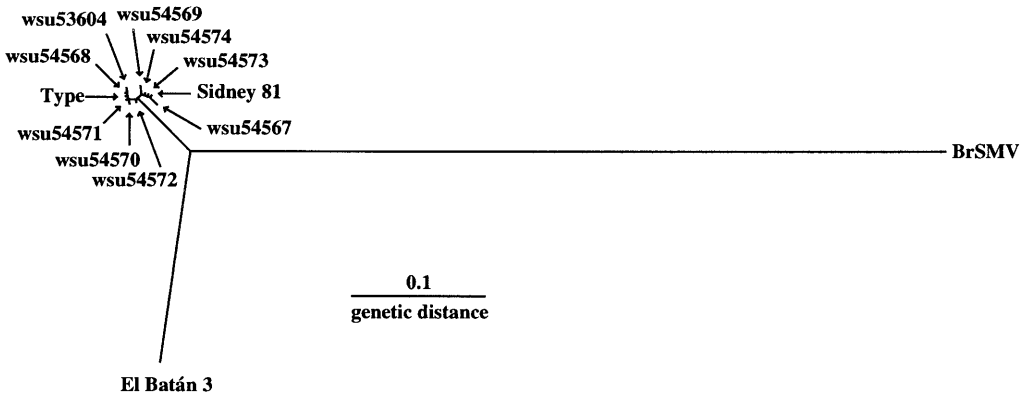

Fig. 2. Phylogenetic relationships among strains and isolates of *Wheat streak mosaic virus* (WSMV). Presented is a maximum likelihood distance tree, constructed using the HYK model of the Puzzle program, depicting the relationship of coat protein (CP) cistron nucleotide sequences for WSMV strains Type, Sidney 81, and El Batán 3 with CP sequences determined [4, 22] for nine other American isolates of WSMV. The CP cistron of the distinct tritimovirus *Brome streak mosaic virus* (BrSMV) was included for comparison. Branch lengths are scaled to reflect genetic distance. Support values calculated for all nodes were $\geq 67\%$ (not shown)

separate from branches emanating from the node exclusively shared by all American WSMV taxa. Nonetheless, El Batán 3 is more closely related to American WSMV genotypes than to the European tritimovirus species BrSMV (Fig. 2).

Prior to this study, all WSMV isolates examined at the molecular level shared high sequence identity [4, 22]. Analysis of genetic variation among field isolates of WSMV from Nebraska [21] and the Texas Panhandle (R. French, unpublished) indicated that WSMV genotypes occurring in the Great Plains comprise a single population. The identification of El Batán 3 as a divergent strain of WSMV occurring in a geographically isolated area suggests a second WSMV population in North America. WSMV also is reported to occur widely in the Old World [7, 8, 16, 19, 23, 25, 29, 34]. Unfortunately, there are no sequences available for any Old World isolate of WSMV, thus relationships among New and Old World WSMV populations remain undefined.

The distribution of amino acid substitutions in the polyprotein sequences of the three WSMV strains was examined using the Sequence Similarity Presenter program [10] with a window size of 4 residues and a window shift of 1 residue. The Sidney 81 polyprotein sequence served as the reference sequence for all two-way comparisons. The polyprotein sequence of BrSMV [13] was included in the analysis to contrast intra- and inter-species differences. Synonymous (ks) and non synonymous (ka) nucleotide substitution rates were calculated using the Sites computer program [15] and their distribution along the aligned WSMV genomes were analyzed by a sliding window approach implemented in the program DnaSP 3.0 [26]. The same program was used for Chi square tests of 2×2 contingency tables of ks/ka and transitions/transversions ratios.

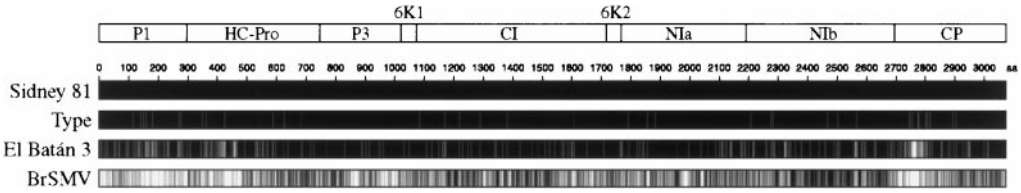

Fig. 3. Distribution of amino acid substitutions in the *Wheat streak mosaic virus* (WSMV) polyprotein. Presented is an alignment of polyprotein sequences of three WSMV strains (Sidney 81, Type, and El Batán 3) and the distinct tritimovirus *Brome streak mosaic virus* (BrSMV). The organization of the polyprotein is presented above. The analysis was conducted using the Sequence Similarity Presenter program [10] using a window size of 4 and a window shift of 1 amino acid residues. The amino-terminal portion of the BrSMV P1 protein sequence that is not present in WSMV was excluded from the analysis. Each polyprotein was compared to Sidney 81. Shading denotes the degree of similarity within each window; ranging between black (4/4 amino acid residue matches per window) and white (0/4 amino acid residue matches per window)

Amino acid substitutions among Sidney 81, Type, and El Batán 3 were distributed along the entire polyprotein with the P1, HC-Pro, and CP cistrons being most variable (Fig. 3). Similar patterns of intraspecies variation have been observed among strains of the potyviruses *Potato virus A* (PVA) [17] and *Yam mosaic virus* [1]. There were many more synonymous (ks) and nonsynonymous (ka) nucleotide substitutions between El Batán 3 and the other two isolates than between the two American isolates (Fig. 3 and Table 1). The ks/ka ratios were 13.1 between El Batán 3 and Sidney 81, 13.0 between El Batán 3 and Type, and 15.3 between Type and Sidney 81. These values were not significantly different from each other by a Chi square test ($P = 0.36$). In contrast, the ratio of transitions (ts) to transversions (tv) between Type and Sidney 81 (ts/tv = 4.68) was significantly different ($P < 10^{-3}$) from those between El Batán 3 and Sidney 81 or between El Batán 3 and Type (ts/tv = 1.56 for both). The 5′-noncoding region of El Batán 3 was only 69% identical to the U.S. isolates while the 3′-noncoding region of El Batán 3 shared 89% (Type) to 91% (Sidney 81) identity. Both noncoding regions of the American isolates differed from each other by less than 2%.

Pairwise synonymous differences among the three WSMV genomes were examined as a function of nucleotide position by a sliding window procedure (window size 50, step size 10). Thus, potential variation in apparent substitution rates can be viewed along the viral genome. The frequency of silent substitutions was constant over most of the polyprotein open reading frame, with one exception (Fig. 4A). A 400 nt segment of the genome (Sidney 81 nt positions 2745–3150) corresponding to the 3′-end of the P3 cistron had a striking and statistically significant lower synonymous substitution rate than the rest of the genome. This same region of the WSMV genome also contained few nonsynonymous substitutions (Fig. 3). A similar analysis conducted with five sequences of the potyvirus *Potato virus A* (PVA) revealed that this distantly related virus also lacked intraspecies variation in the 3′-end of P3 (Fig. 4B), although sequence conservation between

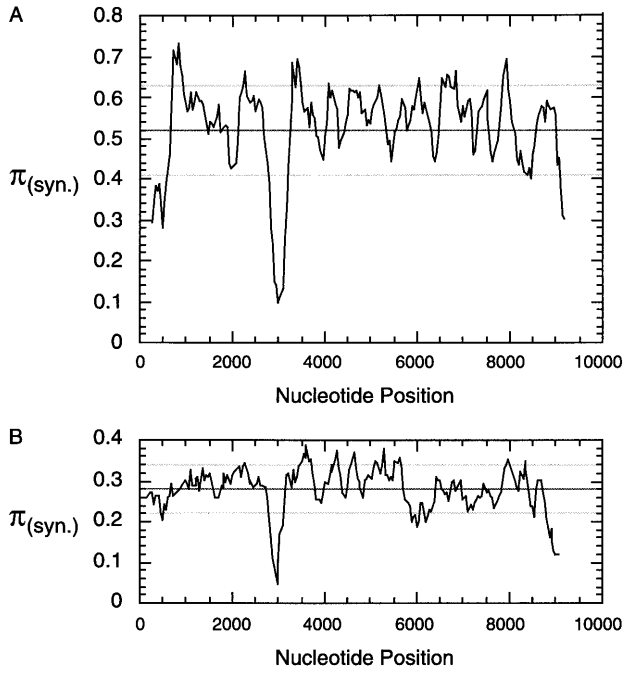

Fig. 4. Distribution of synonymous nucleotide substitutions in the polyprotein open reading frame of **A** *Wheat streak mosaic virus* and **B** *Potato virus A*. Presented are plots of average pairwise identities [$\pi_{(syn.)}$] as a function of nucleotide position calculated using the DnaSP 3.0 program [26]. Black horizontal line indicates the mean; gray horizontal lines denote one standard deviation above and below the mean. Note the lack of intraspecies variation in the region of the genome (nts 2745–3150) corresponding to the 3′-end of the P3 cistron

these two divergent species is low. The same result was obtained when three sequences of *Potato virus Y* were examined (data not shown). Collectively, this indicates strong selection at the intraspecies level for nucleotide sequence conservation within the 3′-end of the P3 cistron. Reduced relative substitution rates could be due to several reasons, such as overlapping ORFs or RNA regulatory elements with highly conserved nucleotide sequence requirements. None of the virus sequences examined had ORFs in common in this region other than the polyprotein, and the genome expression strategy of the family *Potyviridae* make it unlikely that overlapping ORFs are utilized by these viruses. This strongly implies the presence of a species-specific *cis*-acting RNA regulatory element of as yet unknown function.

American isolates of WSMV show little genetic differentiation, whereas the El Batán 3 strain from Mexico has clearly diverged from the American isolates. The simplest explanation is extended genetic isolation of the two North American populations. It has been suggested that the genomes of some plant RNA viruses might be relatively rigid, where even silent changes are subject to negative selection [11]. The considerable divergence of Type and Sidney 81 from El Batán 3 implies that the WSMV genome is actually quite plastic and able to tolerate many nucleotide substitutions. The predominance of silent substitutions is

consistent with a period of neutral mutation and genetic drift after the American and Mexican populations separated. Amino acid substitutions have occurred at the same relative rate as synonymous changes (i.e., ks/ka ratios are equivalent for all two-way comparisons), also suggesting a demographic history dominated by stochastic variation rather than positive selection.

Genetic distances among the WSMV strains likely reflect time elapsed since separation from a common ancestor. Tajima [32] proposed that sites in which exactly two nucleotides are present among three gene sequences allow a simple means for testing the molecular evolutionary clock hypothesis. There are 78 such sites for the Sidney 81 sequence and 91 sites for Type, both relative to El Batán 3 and each other. The relevant chi-square test [32] is $\chi^2 = (91–78)^2/(91+78) = 1.0$ ($P = 0.317$ for one degree of freedom); thus the molecular evolutionary clock null hypothesis cannot be rejected by our data.

We propose that much of the divergence among strains of WSMV may be explained by drift in which genetic isolation facilitates accumulation and fixation of neutral mutations. Time of isolation then accounts for the amount of divergence between strains. However, selection also is an important factor. Clearly, much of the WSMV genome is constrained by negative selection such that certain regions of the genome retain high sequence identity. The GDD polymerase motif within NIb represents a well known example of sequence conservation likely constrained by negative selection. The P3 mutation cold spot may well represent another example where negative selection acts to conserve a sequence element at the intraspecies level. Drift and selection are not mutually exclusive forces and may occur concurrently. Thus, it is the combined effects of both forces acting simultaneously on different regions of the genome that ultimately determine the extent of divergence within a viral species.

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Authors' address: Dr. D. C. Stenger, USDA-ARS, 344 Keim Hall, University of Nebraska, Lincoln, NE 68583, U.S.A.

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