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# Biological and molecular characterisation of the two Polish *Wheat streak mosaic virus* isolates and their transmission by wheat curl mites

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**Abstract:** *Wheat streak mosaic virus* (WSMV) is a serious and widespread pathogen in the wheat-producing areas in the USA while, in Europe, it has been considered a minor threat to cereal crops. In the past, WSMV was detected in wheat, triticale and maize plants in Poland by DAS-ELISA. Here, we present the biological and molecular characterisation of WSMV-Sze and WSMV-Sosn isolates collected from western and southern Poland and report their transmissibility by the widespread wheat curl mite (WCM) lineage MT-8. The performed bioassays revealed that the analysed WSMV isolates infect wheat, barley, triticale, rye, oat and maize, but they differ in the symptoms induced on the infected plants. Moreover, they infect *Bromus hordeaceus* Linnaeus, which is increasingly recognised as a virus reservoir. The full-length genome sequence of both isolates was obtained and compared with the others described to date. The phylogenetic analysis revealed that the Polish isolates are clustered with the earlier described type B isolates of WSMV from Europe and Iran. The recombination analysis revealed the presence of recombinant variants in WSMV population and indicated that the WSMV-Sosn might originated from the intra-species recombination of the WSMV-Sze and WSMV-Cz isolates.

**Keywords:** phylogenetic relationships; recombination; virus diversity; MT-8; WSMV; WCM

*Wheat streak mosaic virus* (WSMV) is a type member of the *Tritimovirus* genus within the *Potyviridae* family (Adams et al. 2012). The virus is transmitted exclusively by the wheat curl mite species complex (WCM) *Aceria tosichella* Keifer (Prostigmata: Eriophyidae), a vagrant eriophyid mite of ca. 250 µm in body size (Slykhuis 1955; Navia et al. 2013) and at a low rate by seeds (Coutts et al. 2014). It affects many hosts within the *Gramineae* family, including wheat (*Triticum aestivum* Linnaeus) and other economically important cereals as well as several wild grasses (Singh et al. 2018). The virus induces chlorotic streaks, leaf mosaic, yellowing and stunting, and can lead to severe wheat yield losses reaching up to 87% (Hunger et al. 1992). WSMV has been found in most of the wheat growing regions of the world (Navia et al. 2013). Due to the dependence of the spread and intensity of the virus disease in crops on its vector occurrence and efficiency, it is worthwhile to supplement WSMV isolate reports with data on the WCM presence and transmission effectivity tests. Up to now, two lineages designated as MT-1 and MT-8 have been reported in cereal crops globally, equivalent of the North American and Australian "type 2" or "WCM2" and "type 1"or "WCM1", respectively (Carew et al. 2009; Hein et al. 2012; Skoracka

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et al. 2013, 2014). In North America, both lineages, while in Australia only "WCM 2" (MT-1), have been shown to transmit WSMV to wheat (Schiffer et al. 2009). So far, no studies were published for the European WCM populations and the local WSMV isolates. Both lineages are confirmed to be present in Poland, whereas MT-8 is more prevalent than MT-1 (Skoracka et al. 2017).

Here, we report the biological and molecular characterisation of two naturally occurring WSMV-Sze and WSMV-Sosn isolates in Poland and their transmissibility by the widespread WCM lineage MT-8.

## MATERIAL AND METHODS

WSMV-Sze was isolated from a triticale plant naturally co-infected with *Brome mosaic virus* (BMV), collected from Szelejewo (western Poland) in 2013 (Trzmiel et al. 2015). WSMV-Sosn was isolated from a wheat cv. Bogatka plant with leaf mosaic symptoms found near Sośnicowice (southern Poland) in 2016. The WSMV isolates were transmitted mechanically to a set of 4–10 test plants, including cereals and wild grasses (Table 1). An amount of 500 mg of tissue was ground in 2 mL of a 0.05 M phosphate buffer (pH 7.0) and inoculated onto the Carborundum-dusted leaves of all the test plants. The plants were inoculated at the two-leaf stage and maintained in a greenhouse under standard conditions (16 h of light and 8 h of darkness at 23 °C). The WSMV infections were confirmed by a double antibody sandwich enzyme-linked immunosorbent assay (DAS-ELISA) (Clark & Adams 1977) using a commercial kit (Loewe Biochemica GmbH, Germany) about 14 days after inoculation. Molecular analyses were performed with the symptomatic wheat cv. Muszelka and barley cv. Conchita plants inoculated with WSMV-Sze and WSMV-Sosn, respectively. The total RNA was extracted from the infected plant tissue using a NucleoSpin RNA Plant Kit (Macherey-Nagel, Germany), according to the manufacturer's instructions. The complete genome sequence of the Polish WSMV isolates were assembled from several overlapping genome fragments. For the purpose of this study, a set of original primers (Table 2) was designed using the Primer3 program (http://frodo.wi.mit.edu/) (Rosen & Skaletski 2000) based on the full-length nucleotide sequence of WSMV-Cz (AF454454). The RNA of WSMV-Sze was amplified using a OneStep RT-PCR Kit (Qiagen, Germany). The reactions

Table 1. Detection of WSMV-Sze and WSMV-Sosn in the test plants after artificial inoculation by DAS-ELISA

| Test plants | WSMV-Sze | | WSMV-Sosn | |
|---|---|---|---|---|
| | no. of infected plants/ no. of inoculated plants | symptoms | no. of infected plants/ no. of inoculated plants | symptoms* |
| *Avena sativa* L. cv. Arden | 8/10 | as | 21/21 | as |
| *Bromus hordeaceus* L. | 1/4 | dw | 6/7 | dw |
| *Echinochloa crus-galli* (L.) P. Beauv. | 4/10 | sm | 2/5 | sm |
| *Hordeum vulgare* L. cv. Conchita | 8/21 | sm | 18/43 | sm, n |
| *Secale cereale* L. cv. Dankowskie Amber | 1/30 | sm | 7/23 | sm |
| *Triticum aestivum* L. cv. Muszelka | 32/35 | dw, sm | 2/12 | sm |
| x *Triticosecale* Wittm. ex A. Camus cv. Maestozo | 5/25 | sm | 30/36 | sm |
| *Zea mays* subsp. *mays* cv. Waza | 3/10 | sm | 4/4 | sm |
| *Zea mays* L. cv. Polonez | 7/8 | sm, n | 8/8 | sm, n |

as – asymptomatic infection; dw – dwarfism; sm – systemic mosaic; n – necrosis

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Table 2. Primers used in this study

| Primer | Sequence 5'– 3' | Position[1] |
|---|---|---|
| WSMVfull-F | AATTAAACCAACCCAAATCG | 1–20 |
| WSMV-HC-F | TGTGCACATGGACGCATTCAG | 1 124–1 144 |
| WSMV5-R | CCTTTCTTGGTAATGCTTCC | 1 258–1 277 |
| WSMV-Hc-R | CATATCCACTAACCATTCAGC | 2 417–24 317 |
| WSMV1-F | GCGTAAGATCAACAAAGATGC | 2 398–2 418 |
| WSMV1-R | AGTCCAAATGCCAACAAGGC | 4499–4 519 |
| WSMVlukaI-F | CGGAGCCTATTTATTAAAG | 3 091–3 109 |
| WSMVlukaI-R | GTGTTTTACAACTGGCTTAAGAGC | 3 890–3 913 |
| WSMV2-F | GCGCCAACTGATCAACAAATC | 4 363–4 383 |
| WSMV2-R | GAAGTGATACGGCGCCAAGAG | 6 095–6 115 |
| WSMVlukaII-F | GCGTGTGCCAGCAGTTAACC | 4 909–4 928 |
| WSMVlukaII-R | GTGAAGGCTTTCTGCAGATCC | 5 743–5 763 |
| WSMV3-F | CACTTGATCATATCCATCAG | 6 015–6 034 |
| WSMV3-R | GTACTTCCACTGCTCGGTGCAC | 8 244–8 265 |
| WSMVlukaIII-F | CAGTTCTGACACATGAAGCAT | 6 867–6 887 |
| WSMVlukaIII-R | CAGCCTCATCGTCCTCTGCC | 7 504–7 523 |
| WSMV-CPeur-F | ACCTAACTGGGAACCGAACWG | 8 118–8 137 |
| WSMV-CP-R | GTATTCCGCGTAGCCTGTTC | 9 094–9 113 |
| WSMV-F2* | CGAGTGATGATCGAGGAGAG | 8 930–8 949 |
| WSMV-R2* | GAAACTGTGCGTGTTCTCCC | 9 192–9 211 |
| WSMVfull-R | GTAGCGTCGCCCTAACCCAC | 9 365–9 384 |
| WSMV-5' RACE | CAC CTC AAT ACT GCA ATC C | 688–670 |
| WSMV-3' RACE | CACCTTGCTTCCTGGATGGG | 8 731–8 750 |

*The primers used for virus detection in the wheat curl mite; [1]the primer's position according WSMV-Cz AF454454

were performed in a final volume of 10 µL according to the manufacturer's instructions. No amplicons were observed for WSMV-Sosn; therefore, the reverse transcription (RT) and polymerase chain reaction (PCR) reactions were conducted separately, but with the same primers. The first-strand cDNA was synthesised using a SuperScript® Reverse Transcriptase (Thermo Fisher Scientific, USA) with random hexamer primers following the manufacturer's recommendations. The PCR test was carried out using 1 µL of the RT mixture with 5 µL of a 2 × Dream Taq Master mix (Thermo Fisher Scientific, USA), 0.4 µL of a Primer Mix (10 µmol/µL each) and sterile Milli-Q water for a final volume of 10 µL. Amplification was performed as follows: 94 °C for 2 min, 40 cycles of 94 °C for 30 sec, 50 °C or 55 °C for 30 sec, 72 °C for 30 sec to 180 sec (depending on the product size) and a final cycle of 72 °C for 7 minutes. The nucleotide sequences of each WSMV isolate were established according to Trzmiel et al. (2016). Subsequently, they were analysed using the Standard Nucleotide BLAST online tool (http://blast.ncbi.nlm.nih.gov/Blast.cgi) and edited using the BioEdit software (version 7.2.5) (Hall 1999). Finally, the 5' and 3' terminal sequences were received by the 5' and 3' RACE System for the Rapid Amplification of the cDNA Ends (Thermo Fisher Scientific, USA). The complete sequences of both Polish isolates were submitted to the National Center for Biotechnology Information (NCBI) GenBank database. Prior to the phylogenetic analysis, the occurrence of potential recombination events within the WSMV isolates was analysed using the recombination detection program (RDP; version 4), GENECONV, Chimaera, MaxChi, BootScan, SiScan and 3Seq methods implemented in the RDP program (Martin et al. 2015) with the default settings. The recombination events were considered as significant if four or more of these methods had a $P < 0.05$ in addition to the phylogenetic evidence of recombination. The phylogenetic analyses were performed for the two Polish isolates and eighteen previously characterised isolates with a known full-length genome

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sequence. Oat necrotic mottle virus- Type-NE, a distinct *Tritimovirus* species, is most closely related to WSMV (Stenger & French 2004). So, it was used as an outgroup. Multiple alignments were performed using the ClustalW software (Thompson et al. 1994). The phylogenetic relationships of both the coat protein (CP) gene and the complete genome nucleotide (nt) sequences were analysed using the Maximum Likelihood algorithm (ML) implemented in MEGA (version 6.1) (Tamura et al. 2013). The best nt substitution models were chosen as Kimura 2-parameter (K2+G) and General Time Reversible (GTR+G+I), and the bootstrap values were calculated using 1 000 random replicates. The phylogenetic trees were visualised using TreeExplorer implemented in the MEGA program.

The mite's ability to transmit the virus isolates was tested by transferring the mites from an infected source plants to test plants. The WCM sources for the study were two non-viruliferous laboratory stock colonies of the MT-8 lineage, maintained on isolated wheat plants in a rearing room with temperatures of 21–24 °C and verified to be free of WSMV by periodical DAS-ELISA tests of the host plants. The stock colonies originated from wild populations from Poland: colony no. 1 [GenBank acc. no. KC422635 (Skoracka et al. 2014)] was used to transmit WSMV-Sze, and colony no. 2 [GenBank acc. no. MG194318 and MG076844 (Skoracka et al. 2018)] was used for the WSMV-Sosn transmission.

The viruliferous mite populations were obtained by moving ca. 25 non-viruliferous mites from the stock colony to the source plants, which were mechanically inoculated with WSMV at four weeks prior to the transfer and tested positive. The mites were left to feed and reproduce on the source plants for ten to fourteen days. The mite's ability to transmit the WSMV-Sze and WSMV-Sosn isolates was tested by transferring single mites (adult females) by hand from an infected source plant to twenty, two-week-old test plants of the wheat cv. Muszelka. The mites were observed for a few minutes on the test plant until they had successfully established on the new host. All the source and test plants were kept in mesh cages (mesh diameter: approx. 99 µm) at 21–26 °C. Finally, after ca. 35 days, the plants were checked for the mite population presence (which indicated the successful transfer) and tested for WSMV by DAS-ELISA. The prevalence of infected plants per assay was calculated as a percentage of the WSMV-positive test plants compared to the number of test plants, with 95% binomial confidence intervals.

The presence of WSMV in the WCM vector was verified by RT-PCR. The total RNAs isolation from the mites was performed using a NucleoSpin RNA Plant Kit (Macherey-Nagel, Germany) following the manufacturer's instructions with minor modifications. For the isolation, four WCM samples consisting of at least fifty individuals were collected from the transmission assays and stored at –80 °C. For each sample, thawed on ice, 100 µL of a lysis buffer supplemented with 2-mercaptoethanol was added, mixed vigorously (~500 rpm), incubated at 95 °C for 2 min, immediately chilled on ice and centrifuged at 14 000 rpm for 5 min. The supernatant was transferred to a filtration column and then centrifuged at 12 000 rpm for 1 minute. The obtained filtrate was mixed with the same volume (~100 µL) of a binding solution (ethanol 70%) and loaded by centrifugation (12 000 rpm for 1 min). The next steps of isolation: desalting, digesting the DNA and washing the silica membrane, were carried out following the manufacturer's instructions. The RNA was eluted in 20 µL of RNase-free water and stored at –20 °C.

For the detection of WSMV in the WCM, the first-strand cDNA was synthesised using a SuperScript® Reverse Transcriptase (Thermo Fisher Scientific, USA) with a WSMV-R2 gene-specific reverse primer following the manufacturer's recommendations. The reaction mixture contained a maximum amount of, i.e., 11 µL of the total RNA. The PCR reaction using 2 × Dream Taq Master mix (Thermo Fisher Scientific, Waltham, USA) and WSMV-F2/WSMV-R2 primer pairs (Table 2) was carried out as described above.

## RESULTS AND DISCUSSION

The results obtained in the present study revealed that the Polish WSMV isolates infect many plant species of the *Gramineae* family (Table 1). Both isolates were efficiently transmitted to all economically important cereals: WSMV-Sze and WSMV-Sosn induced systemic severe leaf mosaics on wheat, barley and triticale plants; only mild leaf mosaics on rye and asymptomatic infections of the tested oat plants. Furthermore, the mechanical inoculation of fodder maize resulted in lesions followed by systemic necrosis and plant death, whereas only slight leaf mosaic was observed on sweet maize. In addition, both WSMV isolates caused systemic leaf mosaic on barnyard grass and soft brome. Some

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attention should be paid especially to the first one, as the observation of the infections in this study and previous sampling indicated that it is an alternative host for WCM in Poland (Trzmiel & Szydło 2012) and a potential reservoir of the virus which may facilitate the virus introduction into cereal crops.

Due to the close relationships in the virus-vector complex, a risk assessment on the part of the WSMV requires a holistic approach to the topic. The virus has been observed in many European countries (Navia et al. 2013), most recently in Lithuania (Urbanavičienė et al. 2015), Germany (Schubert et al. 2015) and the Ukraine (Mishchenko et al. 2018). The study by Gadiou et al. (2009) showed that the dispersal of the virus may be efficient within the continent.

This study provided the first data for European WSMV isolates' transmission by local populations of WCM (MT-8). The presence of the virus in the mite samples was confirmed by molecular methods. The specific RT-PCR product (284 bp) was detected in three out of four tested samples (data not shown). The obtained nucleotide sequence consisted of the nucleotide sequence of the corresponding region (fragment CP gene) of the Polish WSMV isolates (99% similarity). For both isolates (WSMV-Sze and WSMV-Sosn), the single mites were able to transmit the viruses, with prevalence values of 10% (95% CI: 1.2–31.7%) for WSMV-Sze, and 40% (95% CI: 19.1–64%) for WSMV-Sosn. Wosula et al. (2016) suggested that the differences in the WSMV transmission efficiency by WCM could be caused by the virus genetic variability, especially in the helper-component proteinase (HC-Pro) region. For that reason, the determination of the genetic variation of the studied virus isolates is essential. In addition, a detailed analysis of the HC-Pro and CP, which, according to Tatineni and Hein (2018), were identified as vector transmission determinants of WSMV, was conducted. The analysis showed only two amino acid mutations in the 5' end of the Hc-Pro: tyrosine/histidine (71 residue) and serine/alanine (281 residues). However, a zinc-finger-like motif ($His_{13}$-X2-$Cys_{16}$-X29-$Cys_{46}$-X2-$Cys_{49}$) in the Hc-Pro as well as aspartic acid residues at amino acid positions 289 and 326 in the CP region, which are required for the efficient mite transmission by WCMs, were unchanged.

The existing nucleotide sequences of European isolates of WSMV mostly cover the 3' fragment of the polyprotein with the coding sequence of the CP region. Up to now, only four complete genome sequences from Europe have been determined and deposited in the GenBank database. The analysis of a complete nucleotide sequence (9381 nt) for WSMV-Sze (MH939145) and WSMV-Sosn (MH959146) indicated that the Polish isolates are closely related to each other and share a 98.2 and 99.1% nucleotide and amino acid sequence identity, respectively [Table S1 and S2 in electronic supplementary material (ESM)]. The obtained nucleotide and deduced amino acid sequences of the studied isolates were compared with the corresponding sequences of four other European isolates and one Iranian isolate belonging to type B of the WSMV isolates. The obtained results revealed the highest nucleotide sequence similarity of WSMV-Sze to -Marmagne and WSMV-Sosn to -Czech (98.4 and 98.6%, respectively). In general, the group of type B isolates of WSMV showed a 91.6 to 98.6% nucleotide identity to the Polish isolates (Table S1 and S2 in ESM). The genome sequence analysis showed that both the studied isolates represent the European-type WSMV. Like the other members of this group, they have a typical triplet deletion (GAG) at the nucleotide positions 8 412 to 8 414, resulting in a lack of the glycine amino acid at position 2 761 in the CP region of the polyprotein (Gadiou et al. 2009). Moreover, the results revealed that all the predicted polyprotein proteinase cleavage sites were located at the same positions in the studied, as well as the other known, WSMV isolates. Both the Polish isolates have the same conserved *Cla*I restriction site in the coding sequence of the CP gene (Schubert et al. 2015), as WSMV-Hoym and WSMV-Cz, they contain the same HGLRWY/CEP motif in the putative protein P1/helper-component proteinase protease cleavage site (Choi et al. 2002; Schubert et al. 2015) and the same conserved motif QYCVYE/S in the deduced large nuclear inclusion (NIb)/CP junction cleaved by a small nuclear inclusion protein (NIa) proteinase (Rabenstein et al. 2002). A pairwise comparison of both Polish WSMV isolates' cistrons revealed that some part of the proteins of the replication complex, including 6K1, NIa-Pro, NIb as well as the CP were the most conserved (100% homology of he amino acid sequences and the highest identity of the nucleotide sequences, 99.3, 99.4, 99.7 and 97.8%, respectively). However, in general, the comparative analysis of each region of the polyprotein of the type B group of WSMV isolates revealed their genetic variability and the presence of numerous point mutations (Table 3). Taken together, the obtained data showed that the isolates are more divergent than was previously demonstrated in the CP-based nucleotide sequence studies provided by Gadiou et al. (2009).

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Next, the recombination analysis revealed that the Polish isolate WSMV-Sosn potentially underwent a recombination event with WSMV-Czech (AF454454) (major parent) or/and WSMV-Sze (minor parent). Moreover, the other recombinant variants were predicted within the WSMV population which are illustrated in Supplemental Figure 1 and S1 (Figure S1 in ESM).

The identified sequence variability was supported by the phylogenetic analysis, which confirmed that both Polish isolates are grouped together in cluster B. In contrast to the CP-based phylogeny, the analysis based on the whole genome sequence revealed the presence of only three clusters (A, B and D) without C, which were reported by Stenger and French (2009). Our results are consistent with the data presented by Schubert et al. (2015). The extent of the genetic diversity of the WSMV has been evaluated between various isolates from different origins. Variability based on the whole genome divided the WSMV isolates into three major clades, namely clade A, clade B and clade D. Clade A represents isolates from Mexico, known as El-Batán. Clade B contains isolates from Europe, Russia and Turkey, whereas clade D includes isolates from North and South America, Australia, Canada and Turkey. It has been shown that the European isolates (cluster B, European/Asian) only recombined with isolates from within this cluster, whereas clusters A and D (mainly American/Asian) also contain sequences of the European isolates. The genetic diversity of the WSMV populations and the occurrence of recombination events within the virus population should be taken into account in future cultivar resistance evaluations. Moreover, the analysis performed in this study strongly supports the idea that recombination has shaped the WSMV population structure. Recombination clearly plays a significant role in the evolution of RNA viruses by generating genetic variations, reducing the mutational load and by producing new viruses. The occurrence of recombination events within the WSMV isolates with recombination host spots, which are localised in dif-

Table 3. A summary of non-synonymous (NS) and synonymous (S) point mutations in the coding regions sequences between WSMV-Sze and WSMV-Sosn and other known type B isolates of *Wheat streak mosaic virus*

| Genome region | **WSMV-Sze** | | | | | |
|---|---|---|---|---|---|---|
| | -Sosn | -Austria | -Czech | -Hoym | -Marmagne | -Saadat-Shahr |
| | S/NS | S/NS | S/NS | S/NS | S/NS | S/NS |
| P1 | 27/9 | 23/8 | 26/8 | 23/11 | 24/9 | 86/11 |
| HC-Pro | 32/2 | 38/3 | 25/2 | 27/2 | 17/4 | 139/15 |
| P3 | 13/2 | 10/2 | 10/0 | 9/1 | 3/0 | 39/2 |
| 6K1 | 1/0 | 1/1 | 2/0 | 2/0 | 1/0 | 2/0 |
| CI | 40/6 | 46/10 | 40/5 | 38/6 | 37/12 | 247/9 |
| 6K2 | 2/2 | 4/2 | 2/2 | 4/3 | 2/2 | 4/2 |
| NIa-Vpg | 12/2 | 9/2 | 8/0 | 9/2 | 9/1 | 57/2 |
| NIa-Pro | 4/0 | 9/2 | 8/0 | 9/2 | 9/1 | 57/2 |
| NIb | 3/0 | 35/6 | 28/6 | 26/5 | 24/4 | 96/7 |
| CP | 22/0 | 27/5 | 26/3 | 29/5 | 17/4 | 86/5 |
| | **WSMV-Sosn** | | | | | |
| | -Sze | -Austria | -Czech | -Hoym | -Marmagne | -Saadat-Shahr |
| P1 | 27/9 | 17/3 | 15/1 | 15/4 | 24/4 | 78/9 |
| HC-Pro | 32/2 | 28/1 | 14/0 | 17/0 | 31/6 | 130/3 |
| P3 | 13/2 | 11/5 | 9/3 | 7/4 | 12/3 | 40/5 |
| 6K1 | 1/0 | 2/1 | 3/0 | 3/0 | 2/0 | 3/0 |
| CI | 40/6 | 40/8 | 30/3 | 28/4 | 53/10 | 244/7 |
| 6K2 | 2/2 | 2/1 | 0/0 | 2/1 | 0/0 | 2/0 |
| NIa-Vpg | 12/2 | 31/2 | 6/1 | 7/1 | 12/2 | 13/0 |
| NIa-Pro | 4/0 | 9/2 | 8/0 | 9/2 | 11/1 | 59/2 |
| NIb | 3/0 | 38/6 | 29/6 | 27/5 | 28/4 | 102/7 |
| CP | 22/0 | 19/1 | 8/3 | 11/3 | 15/2 | 79/3 |

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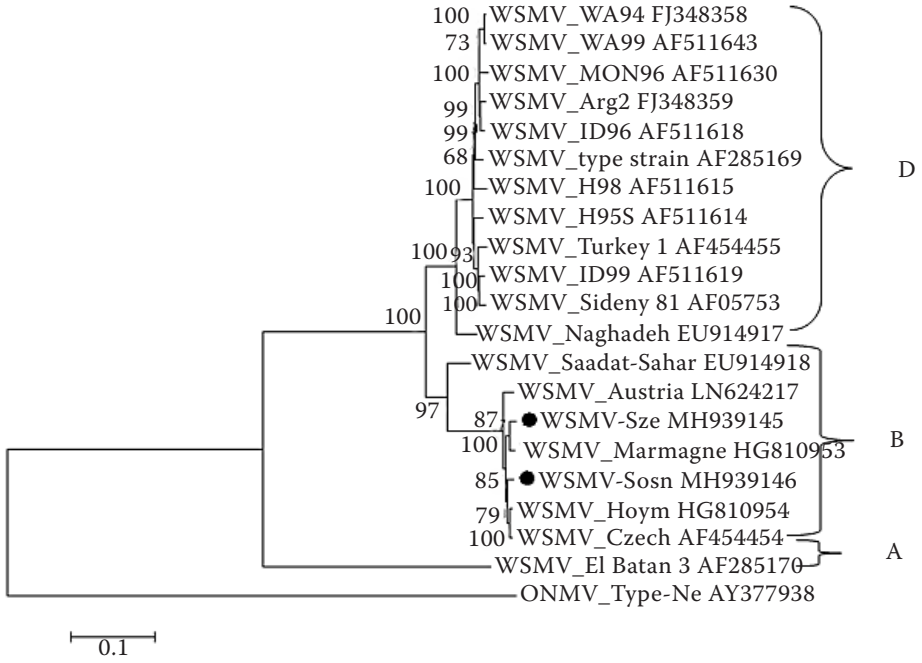


Figure 1. Maximum likelihood tree based on the complete genome nucleotide sequences of the *Wheat streak mosaic virus* isolates

The nucleotide sequence of the *Oat necrotic mottle virus* was used as an outgroup; the numbers of each major node indicate bootstrap values out of 1 000 replicates (provided only when > 50%); the scale bar represents the number of substitutions per base, and corresponds to the nucleotide diversity among the taxa; the analysed isolates are indicated by black dots

ferent genome regions, suggests that new variants may be created thereby displaying different genetic and biological properties.

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