GENETIC DIVERSITY OF STRAWBERRY MOTTLE VIRUS BASED ON *CP* GENE

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Abstract. The strawberry mottle virus (SMoV) is a huge threat to the strawberry production, which seriously reduces the productivity of strawberries. The systematic study on the distribution, structural variation and genetic diversity of SMoV is useful for the prevention and control of SMoV. In this study, 159 strawberry leaves were randomly collected from 7 major strawberry growing areas in Shanxi Province for RT-PCR detection. The *cp* genes of positive samples were sequenced and analyzed through MEGA5, SDTv 1.2, DnaSP v5.10 and RDP v.4.31. RT-PCR detection showed that 65 samples of the 159 strawberry leaves were positive with a detection rate of 38.46%. The 65 positive samples were isolated, sequenced, and cloned to obtain three SMoV isolates. The phylogenetic tree analysis showed the 25 SMoV isolates were divided into three groups with group 1 containing 14 isolates from China, group 2 containing 10 isolates from Canada, Japan, and the United States, and group 3 containing 1 isolate from Japan. The results of selective pressure analysis and neutrality test in group 1 and group 2 showed that there were significant genetic differences between group 1 and group 2. The negative selection pressure maybe the reason for the genetic diversity of SMoV. Sequence similarity analysis displayed that the nucleotide identity range and the consistency range of amino acids was ranged from 94.68% to 99.53%, while from 98.12% to 99.84% for amino acids. This study demonstrated that SMoV was of high genetic variation, and the negative selection pressure may be the cause of SMoV genetic diversity, which provides theoretical guidance for SMoV in Shanxi.
Keywords: *strawberry, SMoV, coat protein, genetic diversity, virus control*

Introduction

Strawberry (*Fragaria* × *ananassa* Duch.), belonging to the Rosaceae and Fragaria family, is a perennial plant with red fruits. It is known as “Fruit Queen” with extremely high nutritional value (Liston et al., 2014; Qarni et al., 2022), which contains a variety of vitamins, carotene, pectin and rich dietary fiber. Besides, strawberries also have a high economic value, which can be made into a variety of food such as freeze-dried strawberries, strawberry jam and so on (Forbes-Hernández et al., 2017; Kowalska et al., 2018). There are about 24 species worldwide mainly concentrated in Europe, America and Asia (Liston et al., 2014; Staudt, 2009). Among them, there are 13 wild strawberry resources in China mainly distributed in the northwest, southwest, northeast and central regions (Shu et al., 2018). Shanxi, specifically in Jinzhong, Linfen, Taiyuan, Yuncheng, Xinzhou, Shuozhou and Datong, is one of the main strawberry producing regions in China. The cultivar is pineapple strawberry introduced into China in the early 20th century. The cultivated strawberry (*Fragaria* × *ananassa*, 8x) is an economically important crop worldwide (Li et al., 2019).

In recent years, due to the virus infection, the production has been decreasing leading to huge economic losses, which has hindered the development of the strawberry industry. There are more than 30 strawberry viruses in the world. In China, there are strawberry mottle virus (SMoV), strawberry crinkle virus (SCV), strawberry vein banding virus (SVBV), strawberry mild yellow edge virus (SMYEV) and so on (Li et

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al., 2019; Martin and Tzanetakis, 2006; Sharma et al., 2018). The symptoms of strawberry after infected by the virus are small fruits, plant dwarfing, poor production and poor quality (Tzanetakis and Martin, 2013). SMoV was first found in England in 1938 and has been widely distributed on various varieties of strawberries around the world. SMoV classified into the *Secoviridae* family of *Picornavirales* is one of the most common viruses in strawberry plantations in Europe (Feng et al., 2016). The mature SMoV is roughly spherical and 28–30 nm in diameter. It is present in the leaf epidermis, phloem and parenchyma cells, which is mainly transmitted by aphids. *Aphis gossypii Glover* and peach aphid (*Myzus persicae)* are their main dispersal mediators (Cieślińska, 2019). The prevention and control of insect borne virus diseases requires not only controlling the virus itself, but also restraining the spread and prevalence of virus diseases through controlling vectors (Frazier and Sylvester, 1960). However, the non-persistent transmission of SMoV by aphids makes it difficult to control through the application of pesticides (Zhang et al., 2021). Therefore, high-quality crop germplasm resources and varieties with high resistance to virus diseases have become an urgent requirement for the healthy and sustainable development of the strawberry industry.

The genome of SMoV is composed of RNA1 and RNA2 of 7036 and 5619 nt, respectively. The polyprotein of RNA2 displayed some similarity to the large coat protein domain of Satsuma dwarf virus and related viruses (Verchot and Carrington, 1995). The coat protein (CP) encoded by the virus is associated with aphid transmission and is crucial for its long-distance movement (Liu, 2005). Transferring the *cp* gene of the virus into plants to achieve a certain degree of virus resistance is a strategy in plant disease resistance genetic engineering (Schaad et al., 199). In this study, leaf samples were collected from strawberries in 7 regions of Shanxi Province. RT-PCR was performed on *cp* gene, and *cp* gene sequence diversity and population structure analysis were conducted between the obtained isolates and NCBI, aiming to clarify the population structure and genetic diversity of SMoV.

Materials and methods

Field surveys and sample collection

Fresh leaf samples were collected from 7 regions of Shanxi Province including Jinzhong, Linfen, Taiyuan, Yuncheng, Xinzhou, Shuozhou and Datong. 159 samples (68 in 2019 and 91 in 2020) were collected, wrapped in tin foil, stored in liquid nitrogen, transported to the laboratory within 48 h and stored in a -80℃ refrigerator for later use.

Primer design

The *cp* gene sequences of SMoV were obtained from the National Center for Biotechnology Information (NCBI). Based on the conserved domains, Primer 5.0 (PREMIER Biosoft International, Palo Alto, CA, USA) was used to design primer sets to clone the *cp* gene of SMoV. The primer information is as follows: the upstream primer sequence is GGACCTACGGATCTTGGAAG; the downstream primer sequence is ACCCGCGCAACTTGTCGGAG.

Total RNA extraction and RT-PCR amplification and sequencing

Total RNA was extracted from leaf samples using the SuperPure Plantpoly RNA Kit (GeneAnswer, Beijing, China), and cDNA was synthesized using a Prime Script RT

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reagent Kit (TaKaRa, Dalian, China). The PCR reaction was performed in a 25 μL reaction volume comprising 2 μL of cDNA template, 12.5 μL 2×Super Multiplex PCR Mix (Cwbiotech, Jiangsu, China), 2 μL of sense and antisense primers (10 μM each) and 8.5 μL double distilled water. The cycling program was as follows: initial denaturation at 94°C for 3 min, followed by 35 cycles of denaturation at 94°C for 30 s, primer annealing at 51°C for 60 s, primer extension at 72°C for 90 s, and a final extension step at 72°C for 5 min. Amplified PCR products were analyzed by electrophoresis in 1.0% agarose gels.

Purify and recover SMoV positive bands using EasyPure Quick Gel Extraction Kit, and send them to General Biol (Anhui) Co., Ltd. for sequencing. Each positive band should be sequenced at least three times until a consistent sequence is obtained.

Phylogenetic analysis

Blast comparison of *cp* gene sequences was conducted through NCBI, and 22 sequences similar to those in this study were obtained (from the NCBI). The nucleotide sequences were aligned using DNAMAN, and a phylogenetic tree of the *cp* gene of SMoV was constructed by the neighbor-joining method in MEGA 5.0 with a bootstrap value of 1000. The branches with the phylogeny number below 50% were removed.

Genetic diversity analysis

Number of sequences (M), average number of nucleotide differences between sequences (K), haplotype diversity (Hd), average pairwise nucleotide diversity (π), number of segregating sites (S), nonsynonymous mutation frequency (Ka), synonymous mutation frequency (Ks) and ratio of nonsynonymous mutations to synonymous mutations (Ka/Ks) were calculated using DnaSP software. If $Ka/Ks > 1$, there will be positive selection effect. If $Ka/Ks = 1$, there will be neutral selection. If $Ka/Ks < 1$, there will be purification selection effect. The software was further used to perform neutrality tests of Tajima's D, Fu & Li's D and Fu & Li's F to determine the diversity faced by the population. Among them, Tajima's D test is mainly used to measure the ratios of polymorphic sites; Fu & Li's D test is to detect the quantitative difference and the difference in total number of site mutations on the basis of single site mutation; and Fu & Li's F test is to calculate the average value caused by single site mutation and pairwise sequence difference.

Recombination analysis

The *cp* gene of 25 SMoV isolates were subjected to recombination analysis using the recombination detection program RDP v.4.31 software by methods including RDP, GENECONV, BootScan, MaxChi, Chimaera, SiScan and 3Seq. Recombination events with $P < 10^{-6}$ detected by at least five different methods were accepted as reliable results.

Results

RT-PCR detection and amplification products analysis

The primers were designed according to Primier 5.0 software to amplify the whole *cp* gene of SMoV, and the specific band was about 460 bp (*Fig. 1*). The positive control band was clear and bright, which confirmed that the primer for cloning the *cp* gene of SMoV could be used in the follow-up experiments.

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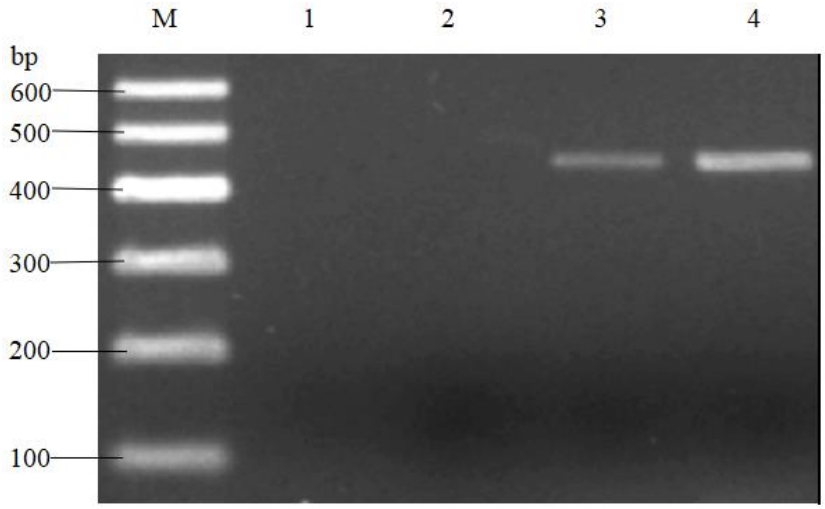

Figure 1. *The tests of SMoV based on the cp gene. M: Marker I;1-4: negative control, healthy samples, virus-infected sample, positive control*

The incidence of SMoV in Shanxi Province

Among 159 strawberry leaves collected from Jinzhong, Linfen, Taiyuan, Yuncheng, Xinzhou, Shuozhou and Datong of Shanxi Province, 65 samples were positive for SMoV by RT-PCR. After sequencing, the sequences of 62 SMoV isolates were found to be 100% consistent with the nucleotides and amino acids of online SMoV through BLAST online analysis. The sequences of 3 SMoV isolates showed differences in nucleotide and amino acid levels compared to the previously reported SMoV isolates, which showed that they were new SMoV isolates. The incidence of SMoV ranged from 22.22% to 58.33% with the highest in Linfen and the lowest in Datong (*Fig. 2*). Total 30 samples among 68 samples were infected by SMoV in 2019, and 35 of 91 were infected in 2020.

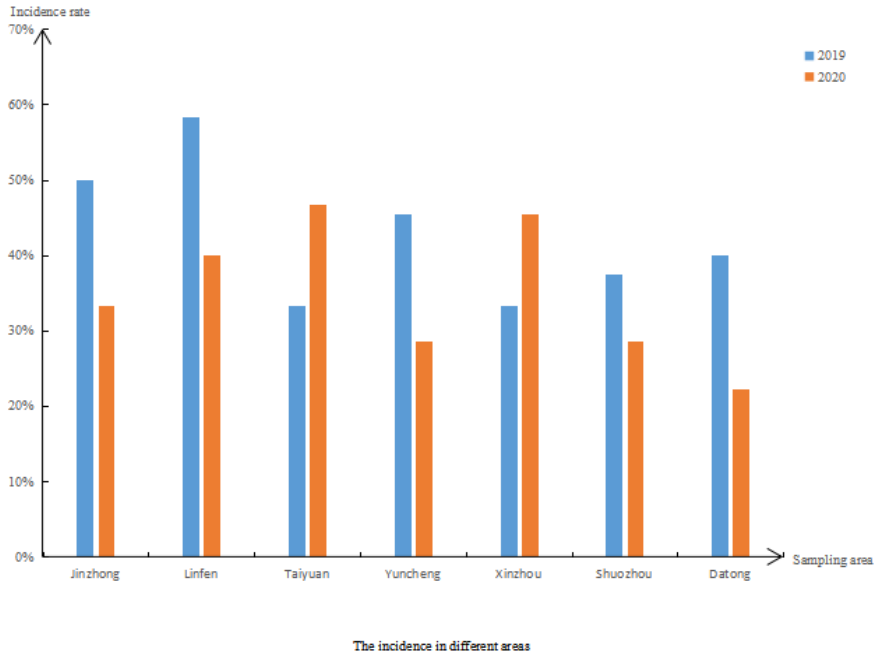

Figure 2. *Incidence of SMoV in different areas of Shanxi Province*

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Phylogenetic tree analysis

According to the comparison results, the *cp* gene of 25 SMoV isolates with high nucleotide consistency (22 from NCBI, 3 obtained by sequencing in this experiment) were analyzed using MEGA 5, which displayed that the 25 SMoV isolates were divided into 3 groups (*Fig. 3*). Group 1 contained 14 isolates from China. Group 2 consisted of 10 isolates including 4 from Canada, 3 from America, 2 from China and 1 from Japan. Group 3 contained 1 isolate from Japan. 2 isolates from Japan and 16 isolates from China were divided into two different groups. These results suggested that the *cp* gene variation of SMoV may be independent of geographic location.

Figure 3. *The Evolutionary Tree of 25 SMoV isolates. The colored branches on the evolutionary tree represent different groupings, with blue representing Group 1, purple representing Group 2, and green representing Group 3; the colored labels represent different countries, red represents China, yellow represents Japan, green represents the United States and purple represents Canada. The red circle represents the isolated new species*

Sequence similarity analysis

The nucleotide identity of the *cp* gene of 25 SMoV isolates ranged from 94.68% to 99.53% (*Fig. 4A*), and the amino acid identity ranged from 98.12% to 99.84% (*Fig. 4B*). The analysis indicates that the *cp* gene are highly conserved in species evolution.

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KU200456.1 had the highest nucleotide identity (99.53%) with MZ328086.1, and LC550285.1 had the lowest nucleotide identity (94.68%) with LC550287.1. Shanxi_1 had the highest amino acid identity (99.84%) with Shanxi_2, and MT070751.1 had the lowest amino acid identity (98.12%) with MT070749.1.

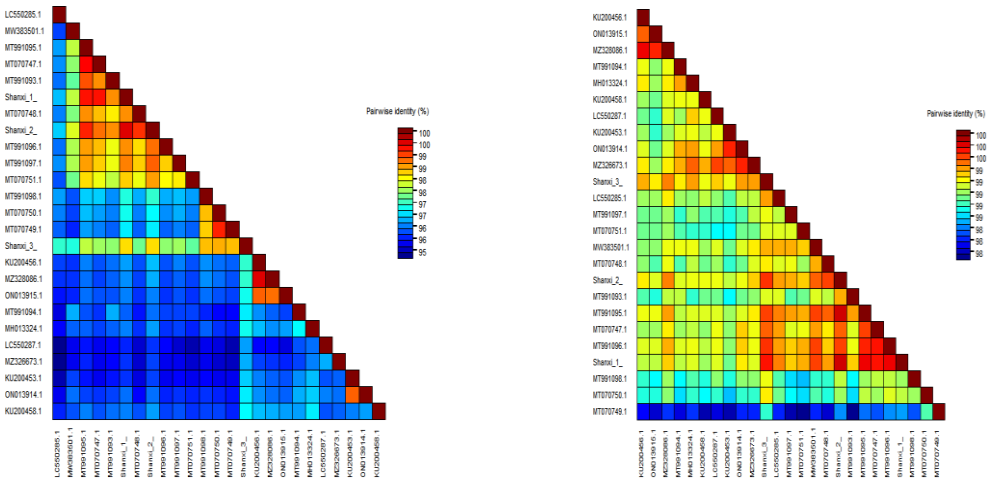

Figure 4. *Two-dimensional distribution of the consistency of nucleotide sequence (A) and amino acid sequence (B) of 25 SMoV cp genes*

Genetic diversity analysis

The *cp* gene sequences of SMoV (obtained through isolation and GenBank website; n = 24) were used to study the genetic diversity by DnaSP software (*Table 1*). The results showed that the number of sequences (M), the average number of nucleotide differences (K), the haplotype diversity (Hd), the number of segregation sites (S), the non-synonymous nucleotide diversity (Ka), the synonymous nucleotide diversity (Ks), and the ratio of nonsynonymous and synonymous nucleotide diversity (ω) of group 1 is significantly higher than group 2, which indicates a higher degree of genetic differentiation. The results showed that the Ka values of various genes were significantly smaller than the Ks values, that is, ω was less than 1 (*Table 1*) indicating that various genes of the SMoV isolates were affected by purification selection. Through the three tests of Tajima's D, Fu & Li's D and Fu & Li's F, the test values of group1 and group2 were all negative (*Table 1*) indicating that the SMoV population was in an expanding stage.

Population genetic differentiation of the two groups showed that the Fst value between groups 1 and 2 was 0.27551, which indicates a large genetic differentiation between the groups of the SMoV isolates (*Table 2*).

Table 1. *Genetic diversity of different SMoV isolates*

Population	M	K	Hd	S	π	Ka	Ks	ω	Fu&Li'sD	Fu&Li'sF	Tajima'sD
Group1	14	144.22	1.000	546	0.02503	0.00357	0.09811	0.0364	-1.09163	-1.16683	-0.81266
Group2	10	219.71	1.000	712	0.03814	0.00316	0.15715	0.0201	-0.65973	-0.77523	-0.76248
Group1/Group2	24	210.77	1.000	1001	0.03659	0.00384	0.15036	0.0255	-1.26354	-1.42138	-1.08707

M: Number of sequences, K: Average number of nucleotide differences between sequences; Hd: Haplotype diversity; S: Number of segregation sites; π: Nucleotide diversity; Ka: Non-synonymous nucleotide diversity; Ks: Synonymous nucleotide diversity; ω: Ka/Ks; ns: non-significant

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Table 2. *Estimates of genetic differentiation among SMoV populations*

Population	Ks*	Kst*	Ks*, Kst* P-value	Z*	Z* P-value	Snn	Snn P-value	Fst
Group 1/Group2	5.05449	0.04228	0.0000***	4.11816	0.0000 ***	0.91667	0.0000***	0.27551

Ks*, Kst*, Z* and Snn are test statistics of genetic differentiation; Fst examines the extent of genetic differentiation between geographical isolates; $0.01 < P < 0.05$; **: $0.001 < P < 0.01$; ***: $P < 0.001$; ns: non-significant

Recombination analysis

To detect whether recombination occurred in the SMoV populations, the nucleotide sequences of 25 SMoV isolates were analyzed using seven methods in the RDP software. The results showed there were no recombination events in the 25 SMoV isolates, which indicates recombination has little impact on the evolution of SMoV.

Discussion

SMoV is a huge threat to the strawberry production. The identification of virus types in the main strawberry cultivation areas of China in the 1980s and 1990s showed that most of the strawberries in the cultivation areas were infected with SMoV (Freeman and Mellor, 1962). In the infections, yield losses up to 30% have been reported (Wang et al., 1991). In this experiment, 65 samples collected in 7 regions of Shanxi Province were tested positive by RT-PCR, and the incidence rate was 38.46%. SMoV is almost distributed in all strawberry planting areas such as Linfen, Taiyuan, Jinzhong and so on. The prevention and control of SMoV has become one of the main problems that the strawberry industry needs to solve urgently.

High genetic variation was present in SMoV. The nucleotide identity of the *cp* gene of 25 SMoV isolates ranged from 94.68% to 99.53%, and the amino acid identity ranged from 98.12% to 99.84%. There are several possible reasons for the high genetic differentiation of SMoV. Firstly, the strong negative selection results in the variation of *cp* gene. Secondly, the use of antiviral chemical pesticides, such as thiacloprid, imidacloprid and acetamiprid, has accelerated the emergence of resistant virus strains, driving the evolution of the virus. Moreover, the combinations use of drugs make aphid control increasingly difficult. Thirdly, the RNA viruses themselves lack a correction function for RNA polymerase. Finally, the virus is highly variable and replicates quickly. High diversity of SMoV brings both an opportunity and a great challenge to future anti-virus breeding.

Comprehensive agricultural, chemical, biological and other control technologies are effective strategies to control SMoV. In order to improve the production and increase the economic benefits of strawberries, some measures to effectively control the SMoV can be taken. Choosing strawberry seedlings without virus or with high disease resistance is the most direct and effective measure to prevent virus infection (The Science News-Letter, 1955). At present, the virus-free technology of strawberry seedlings is mature, and there are many effective virus-free technologies, such as anther culture, shoot-tip meristematic tissue culture, heat treatment and so on (Quiroz et al., 2017; Zou et al., 2022). At the same time, it is crucial to establish and optimize a rapid virus detection system. The control of aphids can also suppress the spread of the virus

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very well by spraying some agents to reduce aphids. Strengthen the farmland management, keep good farmland sanitation, the appropriate application of fertilizer, the reasonable use of organic fertilizer, and remove the plants infected by the virus were also proved to be effective measures for the control of virus (Huang et al., 2011; Quiroz et al., 2017). For genetic engineering, the genes of virus, such as *cp* gene, were introduced into the strawberry genome to obtain the antiviral strawberry, which was widely used in the control of virus (Finstad et al., 1995; Fitch et al., 1992). The comprehensive application of agricultural, physical, chemical and molecular biological methods may be important to prevent and control SMoV in the future.

Conclusion

SMoV is widely distributed in Shanxi and a limiting factor for strawberry production. In this study, 159 strawberry leaves were collected from Jinzhong, Linfen, Taiyuan, Yuncheng, Xinzhou, Shuozhou and Datong of Shanxi Province, of which 65 samples were positive for SMoV. The incidence of SMoV was ranged from 22.22% to 58.33%. The highest incidence was found in Linfen and the lowest in Datong. The prevention and control of SMoV has become one of the urgent needs to control of SMoV.

The negative selection pressure may be the reason for the high genetic variation of SMoV. 25 SMoV isolates were analyzed using MEGA 5, which displayed that the 25 SMoV isolates were high genetic diversity. Since the Ka values of various genes were significantly smaller than the Ks values, negative selection pressure may be usual among SMoV isolates. In-depth understanding of the genetic diversity of SMoV and the reason is of great significance for the effective control of SMoV. The application of agricultural, physical, chemical and molecular biological methods may be necessary to control SMoV in the future.

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Conflict of interests. The authors declare that there is no conflict of interests.

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