Molecular Genetic Analysis of *Cucumber mosaic virus* Populations Infecting Pepper Suggests Unique Patterns of Evolution in Korea

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ABSTRACT

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Studying genetic structure and diversity of viruses is important to understand the evolutionary mechanisms that generate and maintain variations in viral populations. *Cucumber mosaic virus* (CMV) is endemic in most pepper fields in Korea. Currently, no effective methods for control of CMV are available due to many environmental and biological factors such as the extensive evolutionary capacity of CMV. Thus, analyzing the genetic structure of CMV populations may facilitate the development of strategies for the control of CMV. In this study, 252

pepper (*Capsicum annuum*) samples showing virus symptoms were collected by field surveys performed throughout Korea in 2007. Reverse-transcription polymerase chain reaction analyses revealed that, in total, 165 collected samples were infected with CMV. Forty-five CMV isolates were randomly selected within each regional subpopulation and analyzed by full-genome sequencing. Analyses of genetic diversity showed that the 2b gene of CMV is under weaker purifying selection than the other genes. Based on the phylogenetic analysis of RNA1, the CMV isolates from pepper were divided into three clusters in subgroup I. Our full-genome sequence-based molecular analyses of the CMV Korean population suggest that the subpopulations of CMV have been geographically localized in pepper fields in Korea.

Pepper (*Capsicum annuum* L.) is an economically important vegetable crop worldwide. Pepper is broadly used as a spice and flavor ingredient in Korea. Although consumption of pepper is increasing, viral diseases often cause epidemics in pepper in Korea. Viral diseases usually induce severe symptoms on pepper fruit, such as stunting, necrosis, malformation, and mosaic, resulting in a significant loss of the yield and commercial value (1,4,18).

At least 10 families of plant viruses (i.e., *Partitiviridae*, *Bromoviridae*, *Comoviridae*, *Tombusviridae*, *Luteoviridae*, *Bunyaviridae*, *Potyviridae*, *Geminiviridae*, *Rhabdoviridae*, and *Virgaviridae*) are known to infect pepper worldwide (3,12). In Korea, many viruses have been reported to infect pepper (3,4,18,21). The list includes *Cucumber mosaic virus* (CMV), *Broad bean wilt virus 2* (BBWV2), *Pepper mottle virus* (PepMoV), *Pepper mild mottle virus* (PMMoV), *Pepper severe mosaic virus*, *Bell pepper mottle virus* (BPMV), *Pepper vein mottle virus*, *Pepper vein chlorosis virus*, and *Potato virus X* (21). Viral disease caused by CMV has occurred most frequently and caused the most severe damage to pepper plants in Korea (3,4,18).

CMV, the type member of the genus *Cucumovirus*, is an isometric virus with a tripartite genome of positive-sense, single-stranded RNA (28). CMV is distributed worldwide, has the widest host range, and is transmitted by >60 aphid species in a non-persistent manner (13,28). CMV can be divided into two major subgroups, I and II, based on the sequence similarity and serological relationships, and subgroup I can be further divided into

subgroups IA and IB (13,28,31). Several studies have analyzed the genetic structure of CMV populations in Spain, California, and Italy based on RNase protection assays (RPAs) and analyses of partial genomic sequences (5,8,9,24,25). A recent review has described population genetics of CMV on a global scale and noted the importance of analyzing sequences of all three genome segments of CMV to better understand population genetics of CMV (13).

Studying genetic structure and diversity of viruses is important to understand their molecular evolutionary histories in relation to virulence, dispersion, and emergence of epidemics. CMV is endemic in most pepper fields in Korea (3,4,18). Currently, the only effective control of CMV is considered to be through genetic resistance. The durability of resistance against plant viruses is highly related to evolutionary potential of viruses (11). Thus, analyzing the molecular genetic structure of CMV populations infecting pepper may facilitate the development of strategies for the control of CMV. In the present study, we determined full genome sequences of 45 CMV isolates from naturally infected pepper plants in Korea. We examined the genetic structure and diversity of this CMV population by comparison with previously reported sequences of 13 CMV strains and *Peanut stunt virus* (strain ER) based on analyses of complete genome sequences.

MATERIALS AND METHODS

Virus isolates and reverse-transcription polymerase chain reaction. In total, 252 pepper (*C. annuum*) samples showing typical virus symptoms were collected in commercial pepper fields in Korea during the summer of 2007. To identify viruses from the pepper tissues, total RNAs were extracted from the collected samples and subjected to reverse-transcription polymerase chain

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reaction (RT-PCR) analyses using specific primers for CMV (5'-AAGAARCTTGTTCGCGCAT-3' and 5'-TGGTCTCCTTT TRAGGCCCCCA-3'), BBWV2 (5'-AAACAAACAGCTTTTCG TTCCG-3' and 5'-GCCATCTCATTGGCATGGA-3'), or PepMoV (5'-AATGGCAGTCCCCAAA-3' and 5'-TCTCTCATGCC AACTACGA-3'). PCR reaction was performed using GoTaq DNA polymerase (Promega Corp., Madison, WI) under the following conditions: initial denaturation at 94°C for 2 min; and 35 cycles consisting of 20 s at 94°C, 30 s at 50°C, and 60 s at 72°C. The resulting PCR products were subjected to sequencing to confirm whether the target viruses were specifically amplified.

Cloning and sequencing. Forty-five CMV isolates were randomly selected for each geographic region to examine the population genetics of CMV infecting pepper, and their complete genome sequences were determined (Fig. 1). Total RNAs extracted from the CMV-infected tissues were subjected to RT-PCR to amplify full-length genomic RNA1, RNA2, and RNA3 cDNAs using specific primer pairs (5'-GTTTATTTACAAGAGCGTACG GTT-3' and 5'-TGGTCTCCTTTAGGAGGCCC-3' for RNA1 and RNA2, and 5'-GTAATCTTACCACTGTGTGTGT-3' and 5'-TGGTCTCCTTTGAGAGACCC-3' for RNA3). The 5' and 3' end sequences of CMV RNAs were determined by a 5' and 3' rapid amplification of cDNA ends (RACE) protocol (Boehringer Mannheim, Mannheim, Germany) using specific primer pairs Xec-primer (5'-AAAGAATTCCCCCCCCCCCC-3') and 5'-TCCTT TATCGCCGTGGGAGGCTAC-3' for the 5' end of RNA1, Xec-primer and 5'-TCCTCGGGAGTGTGCGACACC-3' for the 5' end of RNA3, Xec and 5'-CCTACTGGTACCTTGAAAGCCAT-3' for the 5' end of RNA3, Anchor-primer (5'-GACCAGC CGTATCGATGTCGACTTTTTTTTTTTTTT-3') and 5'-TTGCTCGTGCTTAGCGG-3' for the 3' end of RNA1, Anchor-primer and 5'-ACAAAGTCCCAGCGAGAG-3' for the 3' end of RNA2, and Anchor-primer and 5'-TTGATTCTACCGTGTGGG TGACAGT-3' for the 3' end of RNA3. PCR reaction was performed as described above using appropriate primer pairs. The resulting PCR fragments were cloned in pGEM-T easy vector (Promega Corp.) and were sequenced by the dideoxynucleotide termination method using various specific primers (the compo-

sition of the primers used in DNA sequencing is available upon request). Subsequently, more than three clones for each fragment were completely sequenced. To verify sequence discords among the clones and identify the dominant sequence in the viral population, direct sequencing of RT-PCR products of entire genomes was performed. The nucleotide sequence data have been submitted to GenBank database with accession numbers listed in Table 1.

Sequence analyses. Multiple alignments for the CMV sequences were obtained using the ClustalX2 program and manually edited using MacClade 4.0 (22,26). Nucleotide and deduced amino acid sequence similarities were analyzed using AlignX implemented in the Vector NTI Suite (Invitrogen, Carlsbad, CA). Pairwise genetic distances and pairwise synonymous (*dS*) and nonsynonymous (*dN*) substitutions were analyzed by Kimura's two-parameter method (19) and the Pamilo-Bianchi-Li method (23,29), respectively, using the MEGA5 program (32). The phylogenetic relationships of the CMV sequences were analyzed by the maximum likelihood (ML) method (Tamura-Nei model) implemented in the MEGA5.1 program using *Peanut stunt virus* (PSV) strain ER as an outgroup. Bootstrap values were calculated using 1,000 random replications. The calculated trees were displayed using the MEGA5.1 program. Spatial analyses of molecular variance (SAMOVAs) were performed using the SAMOVA 1.0 program, as described previously (7,14). For SAMOVAs, 100 initial conditions were used as recommended (7) and the population indexes F_{SC} , F_{ST} , and F_{CT} , quantifying variations within populations, between populations, and between groups, respectively, were calculated. Positive selection at individual codons was statistically examined by the fixed-effects likelihood (FEL) and mixed-effects model of evolution (MEME) methods available from the DATAMONKEY server (<http://www.datamonkey.org>).

RESULTS

The appearance of viral infection in pepper in Korea. Field surveys were performed throughout Korea in 2007. We sampled pepper tissues showing typical virus symptoms such as mosaic,

Fig. 1. Geographic locations of the collected pepper tissue samples and *Cucumber mosaic virus* (CMV) isolates in Korea. Numbers indicate the count of pepper tissue samples collected from the indicated areas. CMV isolates given in parentheses are collected from the indicated areas.

mottling, vein clearing, malformation, and local chlorosis. In total, 252 pepper tissue samples were collected from 22 areas in Korea (Fig. 1). To identify viruses from the collected pepper tissues, we performed RT-PCR using specific primers for CMV, BBWV2, or PepMoV because these three viruses are prevalent in pepper plants in Korea (3,4,18). Out of 252 collected samples, 233 samples (92.5%) were infected with at least one of the three viruses and 55, 20, and 1 samples were singly infected with BBWV2, CMV, and PepMoV, respectively. Infection with BBWV2 was detected in 205 samples (81.3%), while 165 samples (65.5%) were infected with CMV. Mixed infection of CMV and BBWV2 was detected in 138 samples (54.8%) and this double infection, which causes severe damage on pepper by synergistic symptom expression, was most prevalent in pepper fields in Korea. Infection with PepMoV was detected in 92 samples (36.5%) and 12 and 7 samples were doubly infected with PepMoV + BBWV2 and PepMoV + CMV, respectively. In addition, 72 pepper samples (28.6%) were found to be triply infected with CMV, BBWV2, and PepMoV. Interestingly, approximately 99% of PepMoV-infected samples were mixed-infections with CMV or BBWV2, suggesting that the dynamics of infection and transmission of PepMoV in pepper fields might be highly correlated with those of CMV and BBWV2.

Molecular characterization of the CMV population. CMV is endemic in Korea and has caused severe damage persistently in most pepper fields (3,4,18). Thus, to examine the population of CMV infecting pepper, 45 CMV isolates were randomly selected for each geographic region (Fig. 1) and their complete genome sequences were determined. The sequences are available in the GenBank database with the accession codes listed in Table 1.

The molecular variability of the population of CMV from pepper was examined by comparing either complete nucleotide sequences or deduced amino acid sequences of the 45 CMV isolates. Widespread nucleotide variations were detected throughout the genome and more significant variations were observed in the 5' and 3' untranslated regions (UTRs) of RNA2 and the 5' UTR and central intercistronic region of RNA3 (Fig. 2). Comparison of diversity of the nucleotide sequences revealed that the genomic regions of 1a, 2a, and 2b exhibited relatively high variability compared with those of the movement protein (MP) and coat protein (CP) coding regions (Fig. 2). However, when analyzing the pairwise genetic differences at *dN* and *dS* nucleotide positions, the *dN/dS* ratio of the 2b coding region was significantly higher than those of the other genomic regions (Table 2). Interestingly, the genomic region of 1a exhibited the lowest *dN/dS* ratio despite exhibiting the highest nucleotide diversity

TABLE 1. Pepper *Cucumber mosaic virus* isolates used in this work and GenBank accession numbers

Province	Area	Isolate	Accession number		
			RNA1	RNA2	RNA3
Gyeonggi	Pocheon (PC)	RP10	KC527784	KC527694	KC527739
		RP11	KC527785	KC527695	KC527740
		RP12	KC527786	KC527696	KC527741
		RP13	KC527787	KC527697	KC527742
	Suwon (SW)	RP4	KC527778	KC527688	KC527733
		RP5	KC527779	KC527689	KC527734
		RP6	KC527780	KC527690	KC527735
		RP29	KC527802	KC527712	KC527757
	Yeoju (YJ)	RP7	KC527781	KC527691	KC527736
		RP3	KC527777	KC527687	KC527732
Chungnam	Cheongyang (CY)	RP14	KC527788	KC527698	KC527743
		RP15	KC527789	KC527699	KC527744
		RP16	KC527790	KC527700	KC527745
		RP17	KC527791	KC527701	KC527746
	Seosan (SS)	RP42	KC527812	KC527722	KC527767
		RP44	KC527813	KC527723	KC527768
		RP45	KC527814	KC527724	KC527769
		RP38	KC527808	KC527718	KC527763
	Boeun (BE)	RP39	KC527809	KC527719	KC527764
		RP40	KC527810	KC527720	KC527765
Chungbuk	Chungju (CJ)	RP41	KC527811	KC527721	KC527766
		RP34	KC527806	KC527716	KC527761
		RP35	KC527807	KC527717	KC527762
		RP24	KC527797	KC527707	KC527752
	Danyang (DY)	RP18	KC527792	KC527702	KC527747
		RP19	KC527793	KC527703	KC527748
	Buan (BA)	RP8	KC527782	KC527692	KC527737
		RP9	KC527783	KC527693	KC527738
	Muju (MJ)	RP1	KC527775	KC527685	KC527730
		RP2	KC527776	KC527686	KC527731
Jeonbuk	Naju (NJ)	RP20	KC527794	KC527704	KC527749
		RP22	KC527795	KC527705	KC527750
		RP23	KC527796	KC527706	KC527751
		RP48	KC527817	KC527727	KC527772
	Yanggu (YG)	RP49	KC527818	KC527728	KC527773
		RP50	KC527819	KC527729	KC527774
		RP25	KC527798	KC527708	KC527753
		RP26	KC527799	KC527709	KC527754
	Pyeongchang (PCh)	RP27	KC527800	KC527710	KC527755
		RP28	KC527801	KC527711	KC527756
Jeonnam	Hadong (HD)	RP46	KC527815	KC527725	KC527770
		RP47	KC527816	KC527726	KC527771
		RP30	KC527803	KC527713	KC527758
		RP31	KC527804	KC527714	KC527759
	Gyeongbuk	RP33	KC527805	KC527715	KC527760
	Gyeongnam				
	Gyeongbuk				

(Table 2). In all, the results suggested that 2b of CMV is under weaker purifying selection than the other genes in the CMV population.

Genetic structure of the CMV population. We performed phylogenetic analyses to determine the relationships among CMV isolates from pepper by including the reported sequences of 13 CMV strains and isolates (Fig. 3). We also included an isolate of PSV (strain ER) as an outgroup in the phylogenetic analyses. The phylogenetic trees were reconstructed by the ML method based on the alignments of the complete nucleotide sequences of RNA1, RNA2, and RNA3 (Fig. 3). Phylogenetic analyses revealed that all the CMV isolates from pepper belonged to subgroup I. When the phylogenetic analyses were performed using RNA1 sequences, the CMV isolates from pepper were further split into three clusters (Fig. 3). On the other hand, in the phylogenetic trees reconstructed with RNA2 or RNA3 sequences, the CMV isolates from pepper were largely subdivided into two clusters (Fig. 3). This clustering of the CMV population from pepper was further supported by the genetic diversity analyses. The genetic diversities within and between subpopulations, which were designated based on the phylogenetic trees, were estimated by Kimura's two-parameter method (19). The analyses showed that the genetic diversities within subpopulations were somewhat low compared with those between subpopulations (Table 3).

Based on the phylogenetic analyses, we examined whether the genetic relationship is correlated with geographic location of the CMV population. Interestingly, the phylogenetic analyses performed with RNA1 sequences highly supported geographic clustering of the CMV isolates from pepper (Fig. 4). Although the CMV isolates collected from the middle inland areas of Korea fell into cluster A, the CMV isolates collected from the south and far-north areas belonged to clusters B and C, respectively (Figs. 3 and 4). Furthermore, RNA1-based phylogenetic analyses revealed the CMV in mixed infections belonged to different subpopulations at the boundary regions (Fig. 4). To further assess the geographic

structure of the CMV population in Korea, spatial analyses of molecular variance were performed using the SAMOVA program (7). The number of groups (K value) ranged between two and five. The best partitioning of the spatial genetic diversity of the CMV population was obtained when the geographic subpopulations were divided into four groups ($K = 4$; $F_{SC} = 0.282$, $P < 10^{-5}$; $F_{ST} = 0.804$, $P < 10^{-5}$; $F_{CT} = 0.726$, $P < 10^{-5}$) (Table 4). The barriers between the groups of subpopulations defined by the SAMOVA analysis ($K = 4$) were represented in Figure 4. The SAMOVA result was highly correlated with the population clustering detected in the RNA1-based phylogenetic tree (Figs. 3 and 4). In all, our results suggest that the CMV subpopulations may be geographically localized in pepper fields in Korea.

Genetic reassortment is an important evolutionary process in the diversification of CMV populations (8,13,24,25,31). A previous study examined a large number of CMV isolates by RPAs and found that genetic exchanges have occurred by segment reassortment in natural CMV populations (8). Careful examination of our phylogenetic analyses, including the phylogenetic tree of the concatenated sequences of entire genomes (i.e., RNA1 + RNA2 + RNA3) of CMV strains and isolates, also strongly indicates that reassortment has occurred in the CMV population from pepper (Fig. 3). We identified at least nine possible reassortants: for RP1 and RP2, their RNAs3 belong to cluster A in the RNA3-based tree while the other two genomic RNAs belong to cluster B in the RNA1- or RNA2-based trees; For RP10, RP11, and RP13, their RNAs1 belong to cluster C in the RNA1-based tree while the other two genomic RNAs belong to cluster A in the RNA2- or RNA3-based tree; for RP15 and RP27, their RNAs1 belong to cluster B in the RNA1-based tree while the

TABLE 2. Nucleotide diversity for different genomic regions of the *Cucumber mosaic virus* population isolated from pepper in Korea

Region	Nucleotide diversity ^a			
	<i>d</i>	<i>dN</i>	<i>dS</i>	<i>dN/dS</i>
1a	0.060 ± 0.003	0.012 ± 0.002	0.198 ± 0.010	0.061
2a	0.049 ± 0.003	0.017 ± 0.002	0.131 ± 0.009	0.130
2b	0.048 ± 0.007	0.031 ± 0.007	0.089 ± 0.021	0.348
MP	0.029 ± 0.003	0.009 ± 0.002	0.078 ± 0.011	0.115
CP	0.032 ± 0.004	0.008 ± 0.003	0.090 ± 0.013	0.089

^a Values are means ± standard error of the mean; *d* = nucleotide diversity estimated by the Kimura's two-parameter method; *dN* and *dS* = nucleotide diversity at nonsynonymous and at synonymous position, respectively, estimated by the Pamilo-Bianchi-Li method.

TABLE 3. Genetic diversity of population of *Cucumber mosaic virus* RNA1, RNA2, and RNA3 isolated from pepper in Korea

Population ^b	Nucleotide diversity between subpopulations ^a		
	Cluster A	Cluster B	Cluster C
RNA1			
Cluster A	0.019 ± 0.0015	...	...
Cluster B	0.096 ± 0.006	0.018 ± 0.0013	...
Cluster C	0.094 ± 0.005	0.077 ± 0.004	0.043 ± 0.0025
RNA2			
Cluster A	0.028 ± 0.0048	...	...
Cluster B	0.098 ± 0.014	0.030 ± 0.0055	...
RNA3			
Cluster A	0.010 ± 0.0013	...	...
Cluster B	0.075 ± 0.008	0.020 ± 0.0021	...

^a Pairwise genetic diversity was analyzed by the Kimura's two-parameter method using the MEGA5 program. Numeric values indicate nucleotide diversity ± standard error.

^b Genome and subpopulation. Subpopulations were designated based on the phylogenetic trees shown in Figure 3.

Fig. 2. Nucleotide and encoded amino acid sequence similarities in the *Cucumber mosaic virus* (CMV) population. Full-length sequences of RNA1, RNA2, and RNA3 of 45 CMV isolates from pepper were aligned by ClustalX2 and analyzed using AlignX by setting the window size to 5 to estimate sequence similarities. The similarity index '+0.8' on the y-axis means that sequences are perfectly conserved. **A**, Genome map of CMV RNAs is shown to scale. **B**, Similarity profile of CMV genomic nucleotide sequences. **C**, Similarity profile of CMV-encoded amino acid sequences.

other two genomic RNAs belong to cluster A in the RNA2- or RNA3-based tree; and for RP26 and RP45, their RNA3s belong to cluster B in the RNA3-based tree while the other two genomic RNAs belong to cluster A in the RNA1- or RNA2-based tree (Fig. 3).

Effects of selection in the CMV population from pepper. To examine the role of natural selection in the whole CMV population (including the Korean pepper isolates and the other global isolates and strains) at the molecular level, the rate of

nonsynonymous substitutions per nonsynonymous site (dN) and the rate of synonymous substitutions per synonymous site (dS) were analyzed. Selection at individual codons was statistically examined by two different methods, FEL and MEME (20,27). The FEL method fits codon models to each site independently and performs a likelihood ratio test to evaluate the difference between dN and dS (i.e., $dN > dS$ indicates positive selection, $dN < dS$ indicates negative selection, $dN \approx dS$ indicates neutral evolution). The MEME method fits a codon model with two

Fig. 3. Phylogenetic analyses for the complete genome sequences of RNA1, RNA2, RNA3, and RNA1 + RNA2 + RNA3 of the *Cucurbit mosaic virus* (CMV) population. An isolate of *Peanut stunt virus* (PSV) was included as an out-group. Phylogenetic trees were reconstructed by the maximum-likelihood method applying the Tamura-Nei model method for nucleotide sequence analyses. Numbers on the branches indicate bootstrap percentages based on 1,000 replications (only values $>70\%$ are shown). Possible reassortants are indicated with asterisks. GenBank accession numbers of RNA1: Fny (D00356), Leg (D16403), Y (D12537), Mf (AJ276479), IA (AB042292), Ix (U20220), Tf (Y16924), Nt9 (D28778), CTL (EF213023), LS (AF416899), Q (X02733), Trk7 (AJ007933), LY (AF198101), and ER-PSV (U15728). GenBank accession numbers of RNA2: Fny (D00355), Leg (D16406), Y (D12538), Mf (AJ276480), IA (AB042293), Ix (U20218), Tf (Y16925), Nt9 (D28779), CTL (EF213024), LS (AF416900), Q (X00985), Trk7 (AJ007934), LY (AF198102), and ER-PSV (U15729). GenBank accession numbers of RNA3: Fny (D10538), Leg (D16405), Y (D12499), Mf (AJ276481), IA (AB042294), Ix (U20219), Tf (Y16926), Nt9 (D28780), CTL (EF213025), LS (AF127976), Q (M21464), Trk7 (L15336), LY (AF198103), and ER-PSV (U15730).

TABLE 4. Spatial analysis of molecular variance for the *Cucurbit mosaic virus* population in Korea

Source of variation ^a	df	Sum of squares	Variance components	Percentage of variance (%)	P value
$K = 2^b$					
Among groups	1	617.485	34.292	57.44	<0.001
Among populations within groups	16	692.910	12.379	20.73	<0.001
Within populations	27	352.917	13.034	21.83	<0.001
$K = 3^c$					
Among groups	2	853.642	47.119	69.86	<0.001
Among populations within groups	15	456.752	7.291	10.81	<0.001
Within populations	27	351.917	13.034	19.33	<0.001
$K = 4^d$					
Among groups	3	949.992	48.216	72.65	<0.001
Among populations within groups	14	360.402	5.118	7.71	<0.001
Within populations	27	351.917	13.034	19.64	<0.001
$K = 5^e$					
Among groups	4	973.326	47.293	72.27	<0.001
Among populations within groups	13	337.069	5.116	7.82	<0.001
Within populations	27	351.917	13.034	19.92	<0.001

^a Geographic location of each population is indicated in Figure 4. K = the number of groups.
^b PC, HD, NJ, YG, US versus YJ, SW, SS, BE, CY, MJ, BA, GC, PCh, CJ, DY, YJ, CG.
^c HD, NJ, US versus PC versus YJ, SW, SS, BE, CY, MJ, BA, GC, PCh, CJ, DY, YJ, CG, YG.
^d HD, NJ, US versus PC versus YG versus YJ, SW, SS, BE, CY, MJ, BA, GC, PCh, CJ, DY, YJ, CG.
^e HD, US versus NJ versus PC versus YG versus YJ, SW, SS, BE, CY, MJ, BA, GC, PCh, CJ, DY, YJ, CG.

categories of lineage-specific dN substitution rates to each site, β^- (one that is $\leq dS$) and β^+ (one that is unrestricted), and calculates the proportion of branches estimated to be part of the dN substitution rate class (p^+) (27,33). We report only concordant results detected by the two methods with a stringent significance level ($P < 0.05$). Our approach identified four positively selected sites in the 1a-coding gene, three positively selected sites in the 2a-coding gene, three positively selected sites in the MP-coding gene, and one positively selected sites in the CP-coding gene (Table 5). However, no positive selection was found in the 2b-coding gene in the whole CMV population.

DISCUSSION

Understanding genetic structures of virus populations and their evolutionary mechanisms is an important aspect of managing viral diseases and the risk of emerging new viruses. There have been several studies attempting to examine population genetics of CMV based on serological analyses, RPA, and analyses of partial genomic sequences (5,8,24,25). However, accumulating evidence suggests that genetic exchanges by recombination and reassortment might have played an important role in CMV evolution (13,25,31). This implies that more complete analyses with full

Fig. 4. Geographic distribution of the *Cucurbit mosaic virus* (CMV) isolates in Korea. CMV isolates from pepper were clustered into three subpopulations (clusters A, B, and C) based on the phylogenetic analysis of RNA1 (Fig. 3). Whereas the CMV isolates in cluster A were prevalent throughout the middle inland areas, the CMV isolates in clusters B and C were dominant in the south and far-north areas of Korea, respectively. Barriers between the groups of subpopulations defined by spatial analyses of molecular variance ($K = 4$) are indicated with solid black lines and dotted lines.

TABLE 5. Positively selected sites in the whole *Cucurbit mosaic virus* population, including the Korean pepper isolates and the other global isolates and strains

Gene ^c	Site	FEL ^a		MEME ^b			
		Normalized $dN - dS$	P value	β^-	β^+	p^+	P value
1a	542	2.98	0.024	0.011	18.36	0.83	0.005
	550	1.52	0.025	0	1.29	1	0.034
	551	1.50	0.027	0	1.37	1	0.039
	566	1.37	0.031	0	1.36	1	0.040
2a	134	1.95	0.031	0	6.30	1	0.006
	139	2.53	0.009	0	1.84	1	0.015
	268	2.67	0.027	0.183	14.53	1	0.022
MP	259	3.51	0.014	0	9.25	0.35	0.002
	269	2.54	0.033	0	1.49	1	0.045
	271	2.89	0.020	0	1.67	1	0.031
CP	138	4.06	0.006	0	6.55	0.40	0.004

^a Fixed-effects likelihood; dN = nonsynonymous and dS = synonymous.
^b Mixed-effects model of evolution. Values of β^- and β^+ mean dN substitution rates to each site, one that is $\leq dS$ and one that is unrestricted, respectively. Values of p^+ indicate the proportion of branches estimated to be part of the dN substitution rate class.
^c MP = movement protein and CP = coat protein.

genome sequences of CMV populations are required for understanding the genetic structure of CMV populations and their evolutionary histories. In the present study, we investigated the molecular genetic structure of CMV populations at a geographic scale based on analyses of full genome sequences of a large number of CMV isolates from pepper.

Mixed infections with two or more viruses are known to be common in virus-infected crop fields (3,12,18). Our field surveys also revealed that 62.3% of the collected pepper tissues were mixed infected with at least two viruses (i.e., CMV + BBWV2, CMV + PepMoV, BBWV2 + PepMoV, or CMV + BBWV2 + PepMoV). In many cases, mixed viral infections cause synergistic symptom enhancement, resulting in a great loss of yield and commercial value of crops. Synergistic symptom responses by coinfection of CMV with other viruses, including *Zucchini yellow mosaic virus*, *Tobacco necrosis virus*, and *Abutilon mosaic virus*, have been reported (34–36). Sometimes the enhancement of the synergistic symptom expression is related to an increase in titer of the infecting viruses (30). We also observed synergistic symptom responses on the mixed-infected pepper samples, especially with PepMoV, which is a *Potyvirus* sp. (data not shown).

In this study, we determined the complete genome sequences of 45 CMV isolates from pepper and analyzed the genetic diversity and phylogenetic structure of this population. Analyses of the pairwise nucleotide and deduced amino acid similarities among the CMV isolates revealed that nucleotide variations were distributed throughout the entire genome but the 5' and 3' UTRs of RNA2 and the 5' UTR and central intergenic region of RNA3 were significantly more variable than the protein-coding regions (Fig. 2). This suggests that the protein-coding regions are under stronger evolutionary constraint than the noncoding regions. Many of viral proteins are compact and play essential roles in the virus life cycle. Thus, genetic flexibility of the viral protein-coding regions is likely to be very limited. In addition, our analyses revealed that the 2b gene of CMV is under weaker purifying selection than the other genes in the CMV population, indicating the 2b protein may be structurally or functionally more flexible. It has been considered that different evolutionary dynamics for different genomic regions are closely associated with functional or structural constraints on conserved regions (10). Overall, plant viruses tend to have greater evolutionary constraints on the proteins involved in replication (i.e., the 1a and 2a proteins) and less conservation of MPs and capsid proteins.

Phylogenetic analyses of the 45 CMV isolates from pepper provide a framework for classifying the CMV subpopulations according to geographic origins. This CMV collection can be largely divided into three subpopulations based on the phylogenetic analyses of RNA1 sequences (Fig. 3). This phylogenetic classification is highly correlated with geographic clustering of the CMV subpopulations according to latitude (Fig. 4). The geographic clustering of the CMV subpopulations in Korea was further supported by SAMOVA analyzed by the SAMOVA program (Fig. 4; Table 4). Interestingly, a recent study performed by Ben Tamarzizt et al. (2) showed geographic localization of CMV subpopulations in Tunisia. It was suggested that there was a high genetic differentiation between two local CMV subpopulations in Tunisia and this differentiation was associated with the localities of sampling (2). Although more detailed examinations are required to clarify the relationship between phylogenetic and geographic clustering, it is possible that temperature affects the geographic colonization of CMV in pepper fields in Korea in relation to pepper cultivars cultivated in each local area and ranges of alternate hosts and insect vectors.

Analyses of the whole CMV population detected a total of 11 possible positively selected sites in the 1a-, 2a-, MP-, and CP-coding genes (Table 5). Several studies have shown that the CMV proteins such as 1a and 2a function as an elicitor for plant defense responses (6,15–17). In particular, a recent study showed that a

CMV isolate isolated from a pepper cultivar harboring the *Cmr1* gene, which confers resistance to CMV, contained mutations in the 1a protein (15), indicating that CMV 1a protein may be a target for resistance responses in pepper. In this regard, the positive selections found in the CMV population (Table 5) might be the result of adaptation of CMV to resistant plants or novel hosts.

In the present study, we have examined the genetic structure and diversity of the CMV population from pepper based on analyses of full genome sequences. Our results showed that each genomic region is under different evolutionary constraints in the population of CMV from pepper. Consistent with previous studies (8,13,24,25,31), our complete genome sequence-based analyses also suggested that reassortment might have occurred in the CMV Korean population. Conclusively, our study suggests that analyses of viral populations on a restricted geographic scale can provide other detailed aspects of evolution that cannot be determined from studies on a worldwide-scale.

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