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Detection of Genetically Modified Maize by Multiplex PCR Method

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Abstract The GMO (Genetically Modified Organism) labeling system on raw materials has been in Korea since March 2001, and genetically modified organisms (GMOs)-derived foods since July 2001. Therefore, we designed a multiplex PCR method to ascertain the validity of the labeling system and to monitor the status of circulation for genetically modified maize (GM Maize). Five lines of GM Maize (GA21, TC1507, Mon810, NK603, and Bt176) were used, and specific primer pairs were designed to detect each line. Using this method, the different lines of GM Maize were monitored from raw products and processed foods in Korean market. Some of the maize processed foods and raw materials were shown to contain more than one foreign gene. This method was found to be effective for detecting five different GM Maize in a single reaction.

Key words: Genetically modified maize, multiplex PCR

World agriculture has progressed more efficiently and productively to feed the world population. One of the agricultural progresses is genetically modified organisms (GMOs). GMOs have been developed by biotechnology and characterized by insect resistance, herbicide tolerance, improved hypotonicity, and nutritional value in crop. Recently, various GMO species have been circulated on the market, with different events approved in different countries. In Korea, the safety assessments of five different events (GA21, TC1507, Mon810, NK603, and Bt176) among genetically modified maize (GM Maize) were recently approved by Korea Food and Drug Administration (KFDA). Also, some lines of GM Maize are yet to be authorized from KFDA. In response to growing consumer concerns, the GMO labeling system has been enforced in raw material since March 2001, and in GMO-derived foods since July 2001. The labeling system is based on the detection of inserted genes or proteins expressed from inserted genes. Thus, the success of the labeling system is dependent upon the efficiency with which GM-derived material can be detected. Consequently, the development of a practical detection method is required to confirm the validity of labeling system and to monitor the status of circulation for GMO. The method of polymerase chain reaction (PCR) that detects specific genes, and immunological method that use specific proteins, were used for monitoring GMO worldwide [2, 19, 22, 23]. The PCR method has been found to be more sensitive than the protein-based method for the detection of GMO in raw materials and processed foods [21]. Also, with the increasing number of GMOs that are being developed for food applications, the ability to detect various GMOs with high detectability and low experimental time and cost becomes an important feature of any detection methods. Multiplex PCR has adequate sensitivity to simultaneously detect various GMOs in a single reaction, without loss of specificity. The detection method for GMOs by using single or duplex PCR was reported [10, 20], however, the detection method of multiplex PCR for a GMO was not yet developed in Korea.

Therefore, in the present study, the multiplex PCR was designed to efficiently monitor five different lines of GM Maize in a single reaction, and designed primer sets were validated by sequencing of the PCR products.

MATERIALS AND METHODS

Samples

Grains of five references of GM Maize (herbicide tolerant GA21 and NK603 from Monsanto, insect resistant Mon810 from Monsanto, insect and herbicide resistant TC1507 from Mycogen, and Bt176 from Syngenta Seeds) were provided by KFDA. As a negative control, non-GM Maize

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grains were also provided by KFDA. For the monitoring of GM Maize, maize for feeding, imported from the United States and China in 2002, was obtained from a feed manufacturer. Maize for cooking oil, imported from the United States and China in 2002, was obtained from an oil manufacturer. Corn and corn snack were purchased from food markets in Korea, and corn cereal from the United States.

Extraction of Genomic DNA

Genomic DNAs of reference maize and samples were extracted, using the cetyltrimethylammonium bromide (CTAB) method [18]. Milled maize and samples (0.1 g each) were mixed in a 2% (w/v) CTAB buffer, consisting of 100 mM Tris-HCl (pH 8.0), 20 mM EDTA, and 1.4 M NaCl and incubated for 1 h in a water bath at 65°C. Chloroform was added to remove protein, and 2 volumes of CTAB precipitation buffer [0.005% (w/v), 40 mM NaCl] was then added to the supernatant and incubated for 1 h at room temperature. The pellet was dissolved in 1.2 M NaCl, and 5 µl of 10 mg/ml RNase A was added. The mixture was incubated for 30 min at 37°C. An equal volume of chloroform was added. The upper phase was transferred to a new tube, 0.6 volume of isopropanol was added to precipitate DNA pellet. The DNA pellet was washed with 70% ethanol and dried. The DNA pellet was dissolved in 50 µl of distilled water. DNA concentrations were determined by UV-spectrophotometery (220S, Hitachi, Japan) [8]. For multiplex PCR, each genomic DNA of five GM Maize was mixed in equal concentrations.

Oligonucleotide Primers

Five sets of primer pairs were designed for the multiplex PCR to detect and distinguish each GM Maize and synthesized by CoreBioSystem Co. (Korea). The inserted DNAs and amplified regions for GA21, TC1507, Mon810, Bt176, and NK603 are described in Fig. 1. The primers of cry1F 5', cry1F 3', PEPC 1, and CTP 3' were designed on the basis of delta-endotoxin gene derived from *Bacillus thuringiensis* var. *aizawai* [3], promoter from the maize

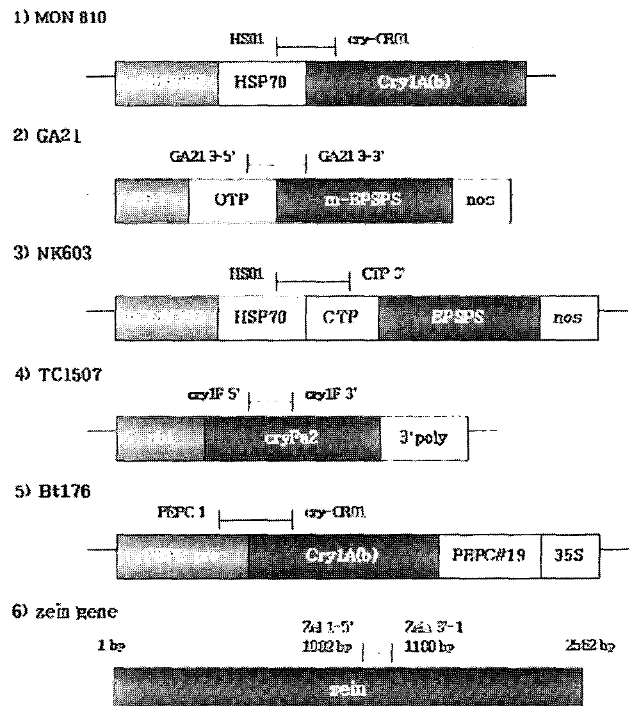

Fig. 1. Schematic diagrams of inserted DNA and amplified regions for the five lines of GM maize.

The primer pairs are represented at the upper side of each line. 1) Mon810: eCMV35S: enhanced cauliflower mosaic virus 35S promoter; HSP70: maize HSP70 intron; Cry1A(b): synthetic cry1A(b) gene derived from *Bacillus thuringiensis* subsp *kurstaki* strain HD-1. 2) GA21: PR I: rice actin I promoter; OTP: intron, optimized transit peptide sequences were derived from ribulose-1,5-bisphosphate carboxylase oxygenase genes isolated from maize and sunflower; m-EPSPS: point-mutated 5-enolpyruvylshikimate-3-phosphate synthase gene (*epsps*) derived from maize; nos: terminator, derived from cauliflower mosaic virus (CMV). 3) NK603: CTP: chloroplast transit peptide leader sequence from *Arabidopsis thaliana* EPSPS gene. 4) TC1507: ubi: promoter and 5' untranslated region from the maize ubiquitin gene including the first exon and intron; cryFa2: cry1F delta-endotoxin from *B. thuringiensis* var. *aizawai*; 3poly: 3 termination/polyadenylation sequences were derived from *A. tumefaciens* open reading frame25. 5) Bt176: PEPC pro: promoter from the maize phosphoenolpyruvate carboxylase gene; PEPC#19: DNA fragment containing the No. 9 intron sequence from maize PEPC; 35S: derived from cauliflower mosaic virus (CMV). 6) zein gene: *Zea mays* 10-kDa zein gene.

Table 1. PCR primers for detection of r-DNAs.

Primer name	Specificity (product size)		Sequence (5'-3')	Reference
HS01	HSP70	Mon810	AGTTTCCTTTTTGTTGCTCTCCT	[17]
cry-CR01	Cry1A(b)	(193 bp)	GATGTTTGGGTTGTTGTCCAT	[17]
GA21 3-5'	OTP	GA21	GAAGCCTCGGCAACGGCA	KFDA
GA21 3-3'	mEPSPS	(133 bp)	ATCCGGTTGGAAAGCGACTT	KFDA
HS01	HSP70	NK603	AGTTTCCTTTTTGTTGCTCTCCT	[17]
CTP 3'	CTP	(328 bp)	ATCGGATAAGCTCGTGGATG	This study
cry1F 5'	cryFa2	TC1507	ACAAGTTCAGTAATTGAAGATTCTC	This study
cry1F 3'	cryFa2	(173 bp)	CGTGAACTAACTAAGTGTCCT	This study
PEPC 1	PEPC pro	Bt176	GGTTACCGCCGATCACATGC	This study
cry-CR01	Cry1A(b)	(248 bp)	GATGTTTGGGTTGTTGTCCAT	[17]
Zel 1-5'	zein	intrinsic gene	CCTCAGTCGCACATATCTACTATACT	[15, 16]
Zein 3'-1	zein	(99b p)	CAAAGAGCTAGGAGAGCGAA	This study

phosphoenolpyruvate carboxylase gene [14], and chloroplast transit peptide leader sequence from *Arabidopsis thaliana* [12], using the GenBank (Accession Nos. AF336114, X15642, and X06613). The primers of HS01 and cry-CR01 were designed by referring to other publication [17]. The primers of GA21 3-5' and GA21 3-3' were obtained from KFDA [13]. The primer pair, Zel 1-5' and Zein 3'-1, was used for detection of the intrinsic zein gene (Accession No. M23537) as a maize internal control [11, 15] and is described in Fig. 1. The sequences of the oligonucleotide primers are shown in Table 1.

Duplex and Multiplex PCR Conditions

Duplex PCR and Multiplex PCR were operated by using a thermal cycler (ASTEC PC808, Japan). The reaction mixture of 25 µl volume contained 2.5 µl of 10× buffer (TaKaRa, Japan), 2.0 µl of 2.5 mM dNTP (TaKaRa, Japan), 1 unit of *Taq* DNA polymerase (TaKaRa, Japan), and concentrations of template DNA and the optimized primer pairs are shown in Table 2. The conditions for Duplex PCR were; pre-incubation at 94°C for 3 min and 40 cycles of denaturation at 94°C for 1 min, annealing at 60°C for 1 min, extension at 72°C for 1 min, and terminal elongation at 72°C for 8 min. Multiplex PCR conditions were the same as duplex PCR conditions, except for annealing at 57°C for 35 s and concentrations of template DNA and primers.

Agarose Gel Electrophoresis

The PCR products were separated by gel electrophoresis and the inserted DNAs were identified from the length of the amplified DNA fragments. After PCR, 10 µl each of products were loaded on a 3.5% agarose gel that contained 0.5 µg/ml ethidium bromide. The φX174 RF DNA/*Hae*III (Gibco-BRL) was used as size standards for the amplified DNA fragments.

DNA Sequencings

The PCR products of five GMM (GA21, TC1507, Mon810, Bt176, and NK603) were extracted from agarose gel using Gel extraction kit (Qiagen, U.S.A.). The pGEM-T easy vector (Promega Co.) was used to clone the amplified DNA fragments and transformed into *Escherichia coli* strain DH5α. The selected *E. coli*, containing the recombinant plasmid, was cultivated and purified. The sequencing of the amplified DNA fragment in pGEM-T easy vector was executed by. GreenGene BioTech Inc. (Korea) with ABIPRISM 3700 DNA analyzer (Perkin Elmer, U.S.A.). The DNA sequences were analyzed with the GenBank [10].

RESULTS AND DISCUSSION

DNA Extraction and PCR Specificity for the Different Lines of GM Maize

All samples were ground into fine powder, and genomic DNAs were extracted by the CTAB method. The purity and quantity of the extracted DNA were evaluated by measuring the 260/280 nm UV absorption and the ratio was between 1.8-2.0. DNAs extracted from each of the five references of GM Maize were identified by a specific primer set. As shown in Fig. 2, duplex PCR with two sets of primers, which consisted of a maize-intrinsic primer

Table 2. Template DNA and Primer concentrations of duplex and multiplex PCRs.

Type	Events	Template DNA (ng)	Specific primer pairs (pM)		Intrinsic primer pairs (pM)	
Duplex	GA21	50	GA21 3-5'	0.5	Zel 1-5'	0.5
			GA21 3-3'	0.5	Zein 3'-1	0.5
	TC1507	50	cry1F 5'	15	Zel 1-5'	4.0
			cry1F 3'	15	Zein 3'-1	4.0
	Mon810	50	HS01	6.0	Zel 1-5'	5.0
			cry-CR01	6.0	Zein 3'-1	5.0
	Bt176	50	PEPC-F2	0.4	Zel 1-5'	0.8
			cry-CR01	0.4	Zein 3'-1	0.8
	NK603	50	HS01	12	Zel 1-5'	15
			CTP 3'	12	Zein 3'-1	15
Multiplex	Mixture	150	GA21 3-5'	20	Zel 1-5'	2.0
			GA21 3-3'	20	Zein 3'-1	2.0
			cry1F 5'	50		
			cry1F 3'	50		
			HS01	9.0		
			cry-CR01	5.4		
			PEPC-F2	0.4		
			CTP 3'	4.0		

Total volume 25 µl.

pair and a GM Maize-specific primer pair, amplified a part of zein gene and each inserted gene, respectively. The intrinsic zein primer set amplified a 99 bp specific DNA fragment. PCR by using each primer set for GA21 (lane 7), TC1507 (lane 8), Mon810 (lane 9), Bt176 (lane 10), and NK603 (lane 11) amplified 133 bp, 173 bp, 193 bp, 248 bp, and 328 bp specific DNA fragment, respectively. With non-GM Maize, no GM Maize-specific primer pair amplified, but a maize-intrinsic primer pair amplified a 99 bp DNA fragment from intrinsic zein gene (lanes 1-5).

Sensitivity of Primer Pairs and Specificity for the Multiplex PCR

In order to optimize multiplex PCR, the optimal condition for primer-to-primer ratio was investigated. In particular, the presence of more than one primer pair in the multiplex PCR brought about not only unexpected PCR products, but also several difficulties, including poor sensitivity and specificity. All the primer pairs in multiplex PCR should have similar amplification efficiencies for their respective target. Therefore, initially equimolar primer concentrations of 0.5 mM each were used in the multiplex PCR. When there was uneven amplification, change of the proportions of various primers in the reaction was required, increasing the amount of primer for weak intensity and decreasing the amount of primer for the strong intensity. As shown in Table 2, this multiplex PCR was optimized by combining each primer at different concentrations. Multiplex PCR with non-GM and GM Maize was performed with six sets of primers in a single tube. The amplified product from a mixture of five reference GM Maize, using multiplex PCR, is shown in lane 12 of Fig. 2. The intrinsic zein primer set amplified a 99 bp specific DNA fragment. The multiplex PCR product was amplified to the size of 133 bp for the introduced genes in GA21, 173 bp in TC1507, 193 bp in Mon810, 248 bp in Bt176, and 328 bp in NK603 (Fig. 2, lane 12). No inserted DNAs from non-GM Maize with six sets of primers were observed, except intrinsic zein gene (Fig. 2, lane 6). The specific bands of each line were clearly distinguished on agarose gel.

Sequencing of PCR Products from GM Maize

On the basis of the sequences obtained from PCR products, the native genes were searched by using the GenBank. These native genes have about 89% or higher homology to the obtained sequences. Sequence comparison between the amplified region and the sequence of native gene is shown in Fig. 3. When the sequences obtained from HSP70, cry1A, cry1Fa2, and PEPC pro were compared with the corresponding native genes, we found that each inserted gene had been modified from the native gene. This modification might increase the expression of the inserted gene in maize [4]. An enhancer element HSP70 was inserted into Mon810 from Monsanto (Fig. 3A) and NK603 from Monsanto (Fig. 3C), respectively, and modified at same sites from the native gene. A target gene cry1A(b) was applied to Mon810 from Monsanto (Fig. 3A) and Bt176 from Syngenta Seeds (Fig. 3E), but some differences in the sources of obtained sequences among the above GM lines were found by sequencing and other publications [4, 16, 17]. The source of synthetic construct insecticidal protein CryIAc1 (cryIAc1) gene (Accession No. AY126450) matches to the sequence obtained from Mon810. The source of synthetic truncated cry1Ac gene (Accession No. U63372) [1] matches to the sequence obtained from Bt176. According to the public information, in order to optimize expression of the Cry1F protein in TC1507, the nucleotide sequence of the cry1Fa2 gene was modified via *in vitro* mutagenesis to contain plant-preferred codons (Fig. 3D). In the case of GA21 and NK603, an intron was inserted as a junction between two elements, OTP and m-epsps in GA21 (Fig. 3B) and also HSP and CTP in

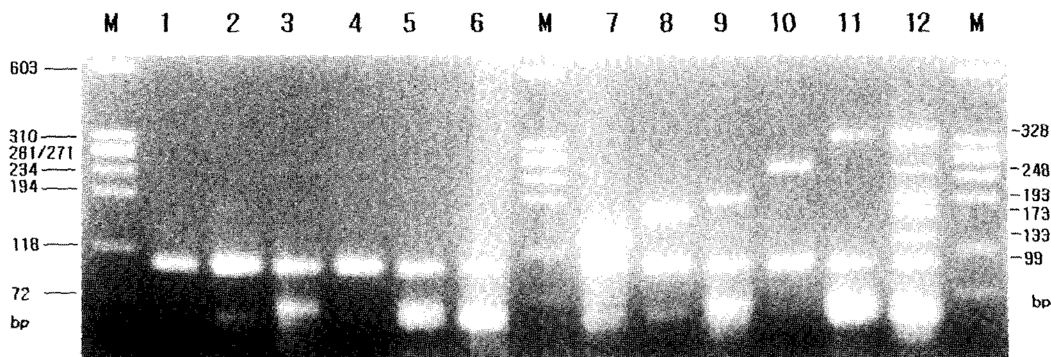

Fig. 2. Agarose gel electrophoresis of Duplex (lanes 1-5 and 7-11) and Multiplex PCR (lanes 6 and 12) products from DNA of non-GM Maize and reference GM Maize.

Lanes 1-6: amplification of maize DNA from non-GM Maize, Lanes 7-12: amplification of maize DNA from GM Maize, Lane M: marker (ΦX174 RF DNA/*Hae*III), Lanes 1, 7: GA21 (133 bp), Lanes 2, 8: TC1507 (173 bp), Lanes 3 and 9: Mon810 (193 bp), Lanes 4 and 10: Bt176 (248 bp), Lanes 5 and 11: NK603 (328 bp), Lanes 6 and 12: maize containing 100% of each of the five lines of GM Maize.

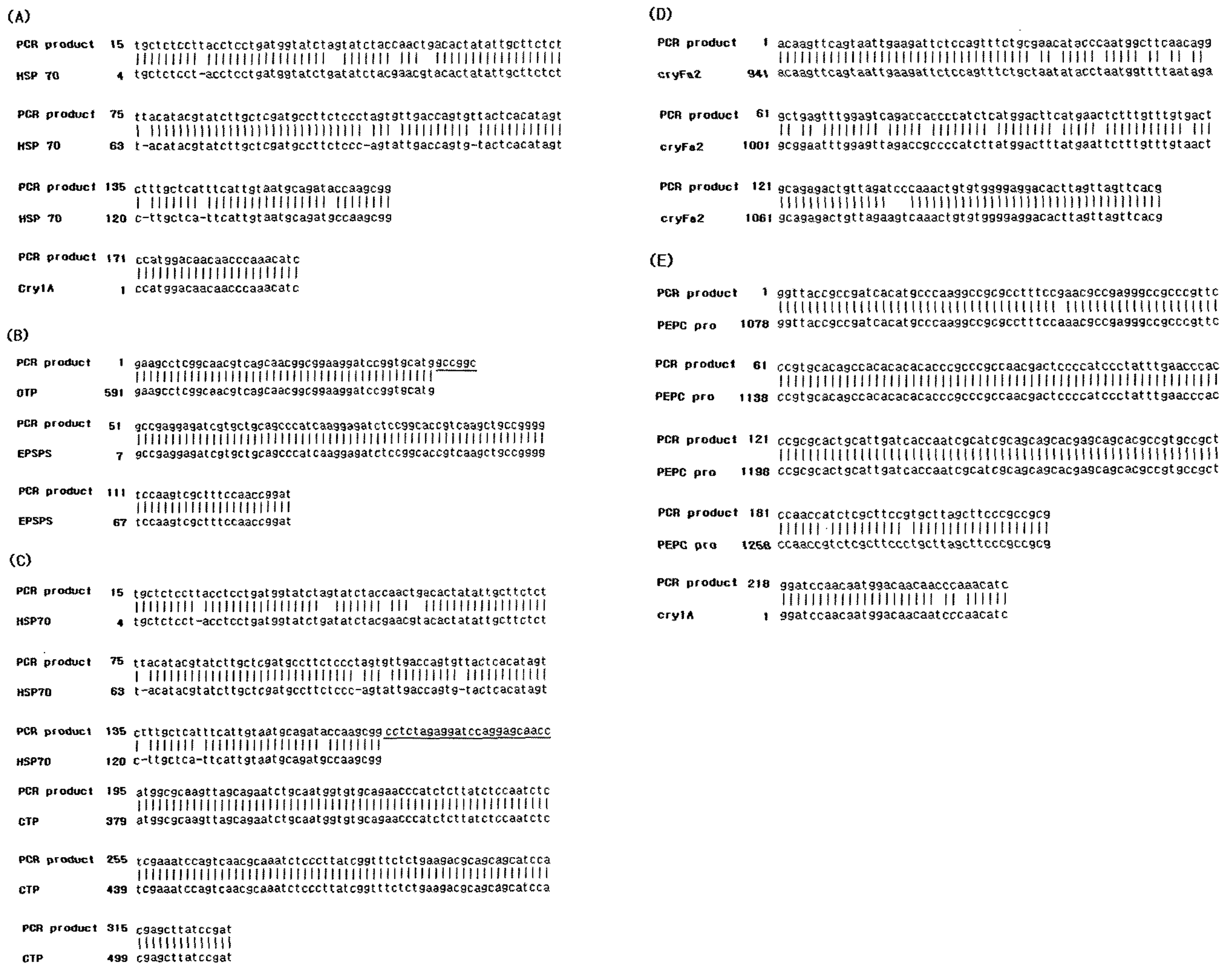

Fig. 3. Sequence comparison between PCR products and the native genes corresponding to each of the five lines of GM Maize.
A: The resulting sequence of the HSP70 and cry1A(b) gene introduced into Mon810. B: The resulting sequence of the OTP and m-EPSPS gene introduced into GA21. C: The resulting sequence of the HSP70 and CTP gene introduced into NK603. D: The resulting sequence of the cryFa2 gene introduced into TC1507. E: The resulting sequence of the PEPC pro and cry1A(b) gene introduced into Bt176. The sequence for comparison referred to HSP70, synthetic construct insecticidal protein cry1A for Mon810, OTP, EPSPS, CTP, cryFa2, PEPC pro and synthetic, truncated cry1A for Bt176 by using the GenBank (Accession No. X03697, AY126450, Y00322, X63374, X06613, AF336114, X15642, and U63372, respectively).

NK603 (Fig. 3C). The underlined sequences represent the intron in Figs. 3B and 3C. According to recent publications [15, 16], an m-epsps gene, which is originally derived from *Z. mays*, is modified at the eight bases from the native gene, but the obtained sequence, using the primer pair GA21 3-5' and GA21 3-3', does not include these modified bases.

Multiplex PCR for the Detection of GM Maize from Samples

We specifically detected the five inserted DNAs and intrinsic zein gene in one reaction by using multiplex PCR. Using this multiplex PCR method, the monitoring of GM Maize from various maize samples in Korean market was performed. Figure 4 shows the same results obtained from different types of maize by both duplex (A to G) and multiplex (H) PCR. Table 3 shows the state of GMM from various maize samples, showing that no PCR amplification related to GM Maize was observed with DNAs extracted from domestic corn (Fig. 4G and Fig. 4H, lane 7) and maize imported from China (Figs. 4A and 4C, and Fig. 4H, lanes 1 and 3). In contrast, raw materials for feeding (Fig. 4B and Fig. 4H, lane 2) and cooking oil imported from the United States (Fig. 4D and Fig. 4H, lane 4) showed PCR products which were resolved as a band of 133 bp in GA21 and 193 bp in Mon810. Corn cereal imported from the United States (Fig. 4E and Fig. 4H, lane 5) and corn snack made from corn imported from the United States (Fig. 4F and Fig. 4H, lane 6) showed a band of 133 bp in GA21. These results explain the presence of safety-approved

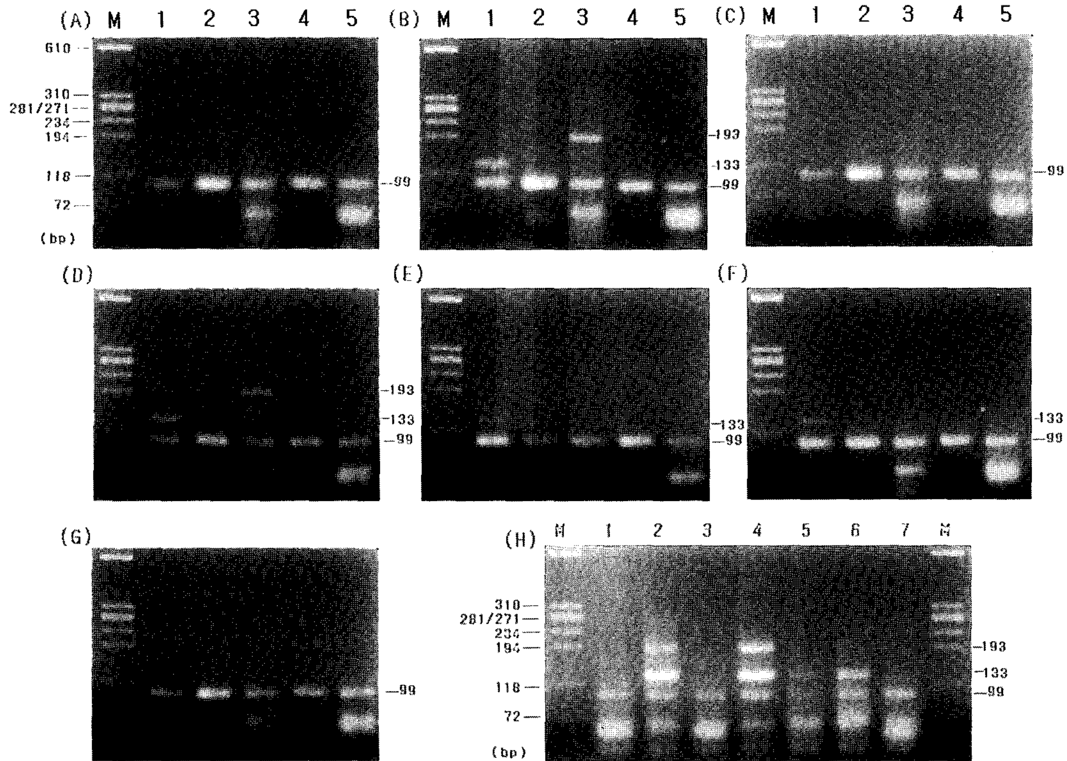

Fig. 4. Agarose gel electrophoresis of duplex (A-G) and multiplex (H) PCR products from different types of maize.
A: Amplification of maize for feeding from China, a feed manufacturer furnished; B: amplification of maize for feeding from U.S.A., a feed manufacturer furnished, C: amplification of maize for cooking oil from China; D: amplification of maize for cooking oil from U.S.A.; E: amplification of corn cereal from U.S.A.; F: amplification of corn snack; G: amplification of domestic corn; Lane M: marker (*φX174 RF DNA/HaeIII*); Lane 1: the primer pairs for detection of GA21; Lane 2: the primer pairs for detection of TC1507; Lane 3: the primer pairs for detection of Mon810; Lane 4: the primer pairs for detection of Bt176; Lane 5: the primer pairs for detection of NK603; H: amplification of different maize and maize derived foods (Lanes 1-7 correspond to samples A-E, respectively) by multiplex PCR.

GM Maize, Mon810, and GA21 in maize imported from the United States, but the amount of GM Maize was not calculated. In order to quantitate the amount of GM maize, real-time PCR should be applied to monitor the GM maize.

Taken together, this detection method, using multiplex PCR, could distinguish the five different lines of GM Maize in a single reaction and reduce experimental time and cost, compared with single or duplex PCR. Thus, this method would be helpful to monitor the reliability of the labeling system of GM Maize in the Korean market.

Table 3. The result of GMM detection on each Maize sample by the PCR method, using the primer pairs to amplify the r-DNA segment.

Samples / Events	Maize for feeding		Maize for cooking oil		Corn cereal	Corn snack	Domestic corn
	A	B	C	D	E	F	G
GA21	-[b]	+[a]	-	+	+	+	-
TC1507	-	-	-	-	-	-	-
Mon810	-	+	-	+	-	-	-
Bt176	-	-	-	-	-	-	-
NK603	-	-	-	-	-	-	-

[a]Detected as GM Maize (+). [b]Detected as non-GM Maize (-).
A-G corresponds to Fig. 4.

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