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Analysis of Genetic Diversity of *Phytophthora infestans* in Korea by Using Molecular Markers

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Abstract A total of 367 isolates of *Phytophthora infestans* was collected from the leaf samples of late blight disease from five provinces in Korea over the three growing seasons of 2002–2004. Of the 367 isolates, 337 isolates were of the A1 mating type, and 30 isolates were of A2 mating type, showing that the majority was A1 mating type. Profiles of *Gpi* and *Pep* defined four allozyme genotypes among the total of 367 isolates. All four allozyme genotypes could be distinguished on the basis of *Gpi* profiles alone, whereas all isolates were homozygous at the *Pep* locus (100/100). The mitochondrial DNA haplotype of all isolates were the IIa haplotype. Amplification of the genomic DNAs extracted from eight isolates of each mating type by polymerase chain reaction with the selected primer (OPC-5 primer) produced a total of 20 DNA bands, of which 11 bands were polymorphic. According to the RAPD analysis using the OPC-5 primer, 106 isolates including two standard isolates were separated into 8 groups at the similarity level of 92.5%. The RAPD groups were not correlated with the allozyme genotypes and the isolated locations. All of the eight RAPD groups were identified in Gangwon-do, suggesting that Gangwon-do is the center of origin of the *P. infestans* in Korea. A 600-bp DNA band generated with the OPC-5 primer was specific to A1 mating type isolates, but not detected with A2 mating type, showing that the specific PCR primer can distinguish different mating types in *P. infestans*.

Key words: *Phytophthora infestans*, allozyme loci (*Gpi* and *Pep*), mt DNA haplotype, RAPD

Late blight, caused by the heterothallic oomycete *Phytophthora infestans*, is most commonly associated with the Irish Potato Famine of the 1840s and is one of the most devastating potato diseases in many parts of the world. The *P. infestans* of the late blight of potatoes generally consists of two mating types, which are called A1 and A2 [3, 12]. The genetic mechanism for differentiating the A1 and A2 types in *Phytophthora* has been the subject of much speculation, particularly since oomycetes are diploid, and therefore, mating type alleles potentially operate in a heterozygous condition. Mating type was shown to be determined by a single locus, which displayed a pattern of non-Mendelian inheritance [25].

P. infestans is a diploid oomycete that probably evolved in the highlands of central Mexico [10]; Mexican populations have a greater genetic diversity for allozymes [17, 37] and DNA fingerprints [17]. In contrast, populations from other parts of the world were almost totally monomorphic for the allozyme and restriction fragment length polymorphism (RFLP) loci. Over the last 20 years, new genotypes migrating from Mexico have established and replaced the old monomorphic genotype around the world [8, 11, 12, 17, 18, 36]. For example, in Europe before the 1980s, the US-1 clonal lineage was the predominant lineage [11, 15]; however, other *P. infestans* genotypes have more recently replaced US-1.

Mitochondrial DNA (mtDNA) polymorphisms of *P. infestans* have been reported to be useful for monitoring pathogen populations, because they are easily detected and uniparentally (and probably maternally) inherited [38]. Previously, hybridization of digested total DNA with labeled cloned mtDNA [7, 13, 33] and digestion of isolated mtDNA [10, 28] have been used to study these polymorphisms. Primers to amplify the known polymorphic sequences of the *P. infestans* genome by polymerase chain reaction (PCR) can be designed using the recent sequence data of the mitochondrial genome [5, 34].

The randomly amplified polymorphic DNA (RAPD) technique has been used as an auxiliary tool for the genetic analysis, classification, or identification of plant pathogenic

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fungi, GMO plant, and human pathogens such as *Fusarium*, *Rhizoctonia*, *Colletotrichum* [2, 27, 32], maize [21], and *Helicobacter pylori* [30]. Punja *et al.* [35] described novel genotypes of *P. infestans* in British Columbia and New Brunswick during the 1990s by using RAPD analysis, and postulated that sexual reproduction was responsible for the variation.

The first objective of this study was to detect the genotypic diversity of *P. infestans* in Korea by using RAPD, mtDNA, and allozyme patterns. The second objective was to look for a DNA marker linked to the determinants of mating type in *P. infestans* with RAPD analysis.

Materials and Methods

Source of Isolates

From 2002 to 2004, potato and tomato leaflets with symptoms of late blight were sampled from commercial fields and research stations in five provinces (including Jeju-do) of Korea (Fig. 1). Fungi isolation was made by placing diseased leaf fragments on potato tuber slices in Petri dishes and then incubating them at 22°C. After 5–7 days, fungi grew on the surface of the tuber slices. Each isolate was derived by transferring hyphal tips on a selective medium of V-8 juice agar (200 ml of V-8 juice, 4.5 g of calcium carbonate, 20 g of agar, 800 ml of distilled water) containing 500 ml/l of ampicillin, 200 ml/l of vancomycin, 50 ml/l of rifampicin, 100 ml/l of pimaricin, 35 ml/l of PCNB, and 10 ml/l of benomyl. Purified isolates were maintained at 22°C on V-8 juice rye agar medium.

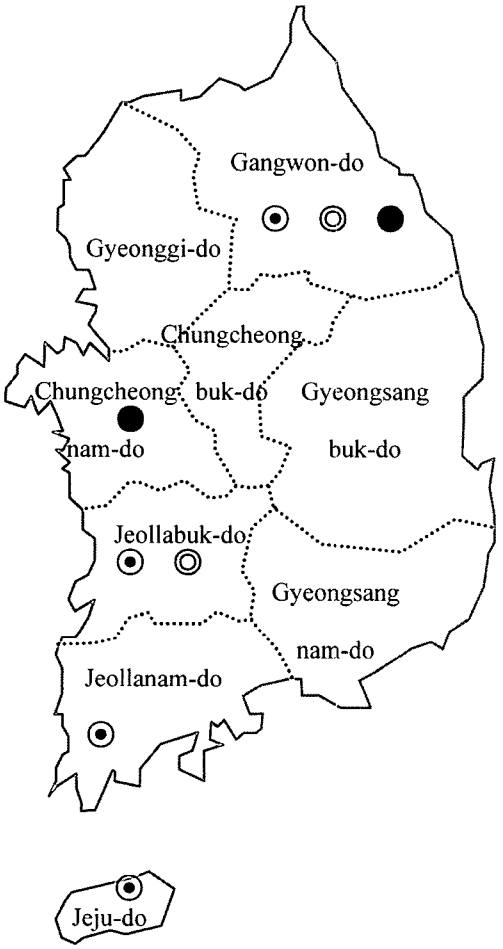

Fig. 1. Map of South Korea, showing the sites where the isolates of *Phytophthora infestans* were collected and the distribution of mating types.
⊙: A1, potato; ◎: A2, potato; ○: A2, tomato.

Mating-Type Tests

To determine the mating type of an isolate, a mycelial agar disk (7 mm in diameter) was cut from the colony edge of *P. infestans* grown on V-8 juice agar for 7–10 days. The agar disk of unknown mating type was placed at the center of the medium, and both standard A1 and A2 mating type isolates of *P. infestans* were then placed 3 cm apart from the center on opposite sides. The mating type was designated by observing oospores in the contact zone between each standard and unknown isolates.

Allozyme Genotype Analysis

All isolates collected from 2002 to 2004 were analyzed for genotype at the two allozyme loci of glucose-6-phosphate isomerase (*Gpi*) and peptidase (*Pep*). Mycelia for allozyme analyses were grown in V-8 juice rye agar and rye broth medium. Allozyme genotypes were determined with cellulose acetate electrophoresis (CAE) [19]. Mycelia grown in solid medium were ground in liquid nitrogen with a pestle, and liquid culture mycelia were freeze-dried. Tissue samples were then placed in 1.5-ml microcentrifuge tubes, and 100 µl of cold sterile water was added. Samples were centrifuged at 13,000 rpm for 2 min and kept on ice until loading. Small aliquots of each sample were then transferred from the supernatant into wells on the sample well plate and were loaded on the gels.

Gel electrophoresis of samples was carried out on cellulose acetate as described by Goodwin *et al.* [19]. Staining for *Gpi* and *Pep* allozymes was accomplished using agar overlays [19].

Genomic DNA Extraction

All isolates of *P. infestans* collected from 2002 to 2004 were grown in rye broth medium and were freeze-dried. The total genomic DNA of each isolate was extracted by a modification of the method described by Goodwin *et al.* [16].

Mitochondrial DNA Haplotype and RAPD Analyses

Mtochondrial DNA haplotype was determined using the PCR-based method of Griffith and Shaw [20]. To investigate mitochondrial DNA haplotype, we used four primer pairs

Table 1. Oligonucleotide primers used to investigate mitochondrial DNA haplotypes of *Phytophthora infestans* isolates in Korea.

Primer	Sequence	Length (base)
F1	5'-GCAATGGGTAAATCGGCTCAA-3'	21
R1	5'-AAACCATAAGGACCACACAT-3'	20
F2	5'-TTCCCTTTGTCCTCTACCGAT-3'	21
R2	5'-TTACGGCGGTTTAGCACATACA-3'	22
F3	5'-ATGGTAGAGCGTGGGAATCAT-3'	21
R3	5'-AATACCGCCTTTGGGTCCATT-3'	21
F4	5'-TGGTCATCCAGAGGTTTATGTT-3'	22
R4	5'-CCGATACCGATACCAGCACCAA-3'	22

(Table 1) identified by Carter *et al.* [4]. PCRs with an Authorized Thermal Cycler (Eppendorf AG, Hamburg, Germany) were optimized to maximize the yield of the desired PCR products and reduce levels of nonspecific products. Amplification was as follows for all primer combinations (final concentrations): deoxynucleoside triphosphates, 200 μM each; oligonucleotide primer, 0.34 mM each; 1× reaction buffer (50 mM KCl, 10 mM Tris-HCl [pH 8.3], 2.75 mM $MgCl_2$); Taq DNA polymerase, 0.2 μl (1 U). Twenty ng of total DNA was mixed with 23 μl of a master mix of the other PCR reactants in 0.5-ml microcentrifuge tubes (final volume, 25 μl). The PCR conditions were as follows: 1 cycle of 94°C for 90 s and 40 cycles of 94°C for 40 s, 55°C for 60 s, and 72°C for 90 s, and a final cycle at 72°C for 120 s.

Five μl of the amplified DNA was digested with the restriction enzymes of CfoI, MspI, and EcoRI in 20-μl volume at 37°C for 4 h. The digested DNA samples were then mixed with 4 μl of 6× gel-loading buffer (0.25% bromophenol blue, 40% sucrose), and 15 μl was loaded into a slot on a 2% agarose gel in 1× Tris-borate-EDTA (TBE) buffer (containing 0.5 μg/ml of ethidium bromide). The gel was run at 60 V for 5–6 hours. Restriction patterns were visualized with a UV transilluminator (Ultra-Violet Products Ltd., Cambridge, U.K.) at 302 nm, and the images were recorded by a gel documentation system.

To estimate the genetic diversity among *P. infestans* isolates collected from 2002 to 2004 and to look for a DNA marker linked to the determinants of mating type loci, RAPD fragments were generated for all isolates. RAPD primers were selected by screening genomic DNA from 8 isolates of each mating type (A1 and A2). Sixty primers [QIAGEN Operon oligonucleotide (kits C, D, and E)] were screened to obtain primers that exhibited polymorphisms and yielded consistent, easily-scorable banding patterns. DNA amplification was performed in an Eppendorf Thermal Cycler with one cycle of 94°C for 5 min, 36°C for 2 min, and 72°C for 2 min; 35 cycles of 94°C for 1 min, 36°C for 1 min, and 72°C for 2 min; and a final cycle at 72°C for 10 min. Reactions were carried out in 25-μl volumes containing 1× DNA polymerase buffer (10 mM Tris-HCl, 40 mM KCl, 1.5 mM $MgCl_2$, pH 9.0), 0.2 mM each of dNTP, 0.4 μM primer, 1 U *Taq* DNA polymerase, and 20 ng of DNA. Amplification products were separated in 1.2% agarose gels (containing 0.5 μg/ml of ethidium bromide) at a constant 60 V in 1× TBE buffer for 5–6 hours at room temperature. DNA fragments were visualized under 302 nm UV light and photographed.

RAPD Data Analysis

The RAPD bands were manually scored and analyzed as binary data, with 1 representing the presence and 0 representing the absence of a band at a particular location in each lane. Bands were assumed to be independent, and those of identical size were assumed to have identical sequences. Data were analyzed with the computer software NTSYS-PC. Similarity matrices were obtained based on the simple matching coefficients, using the SIMQUAL program in the software package. Cluster analysis of matrix values was performed by the unweighted pair-group method with arithmetic averages (UPGMA) using the SHAN algorithm of NTSY-PC. Dendrograms showing the relationships among the RAPD pattern were generated from these matrices using the TREE program from NTSYS-PC.

RESULTS

Mating Type and Allozyme Genotype

Among the total of 367 isolates of *P. infestans* collected in five provinces of Korea from 2002 to 2004, 337 isolates were A1 mating type, and 30 were A2 mating type, showing that the majority was A1 mating type (Table 2). A2 mating type appeared in the isolates collected in the potato fields of Pyeongchanggun of Gangwon-do in 2002, of Bosunggun and Muhangun of Jeollabuk-do in 2003, and the tomato fields of Buyergun of Chungcheongnam-do in 2002 and of Gangneung of Gangwon-do in 2003 (Fig. 1). All isolates derived from tomato plants were A2 mating type.

Profiles of *Gpi* and *Pep* and mating types defined 4 allozyme genotypes among the total of 367 isolates (Table 2). In 2002, 2 allozyme genotypes were detected; most isolates belonged to JP-1 type (*Gpi*: 100/100; *Pep*: 100/100). In 2003, 4 allozyme genotypes were detected, of which 111 isolates were JP-1 type, 9 and 1 were US-17 and US-14 types (*Gpi*: 100/122; *Pep*: 100/100), respectively, and 2 were KR-1 type (*Gpi*: 100/130; *Pep*: 100/100). In 2004, 3 allozyme genotypes were found, of which 111, 78, and 1 isolates were JP-1 type, US-17 type, and KR-1 type, respectively. The genotypes of JP-1 type, US-14, type US-17 type, and KR-1 type used in this study were named from the different allozyme and mtDNA haplotype patterns.

Table 2. Differentiation of mating type, allozyme pattern, and mtDNA haplotype of *Phytophthora infestans* isolates collected in Korea from 2002 to 2004.

Mating type	Allozyme pattern		mtDNA haplotype	Host	Number of isolates	Genotype[a]
	Gpi	*Pep*				
2002						
A1	100/100	100/100	IIa	Potato	38	JP-1
A2	100/100	100/100	IIa	Potato	11	JP-1
A2	100/100	100/100	IIa	Tomato	4	JP-1
A2	100/122	100/100	IIa	Potato	1	US-14
2003						
A1	100/100	100/100	IIa	Potato	98	JP-1
A2	100/100	100/100	IIa	Potato	8	JP-1
A2	100/100	100/100	IIa	Tomato	5	JP-1
A1	100/122	100/100	IIa	Potato	9	US-17
A2	100/122	100/100	IIa	Potato	1	US-14
A1	100/130	100/100	IIa	Potato	2	KR-1
2004						
A1	100/100	100/100	IIa	Potato	111	JP-1
A1	100/122	100/100	IIa	Potato	78	US-17
A1	100/130	100/100	IIa	Potato	1	KR-1

[a]JP population of *P. infestans* is standard isolates found in Japan. US population is standard isolates found in the United States. KR-1 is a new isolate not detected throughout the world.

Changes in the genotypes of the *P. infestans* isolates collected from 2002 to 2004 showed that US-17 type increased gradually, whereas JP-1 type decreased (Fig. 2). All isolates were homozygous at the *Pep* locus (100/100) with the exception of two standard isolates (KA-2 Korean isolate and DN 3085 Japanese isolate) belonging to US-1 (data not shown).

Mitochondrial DNA Haplotype and RAPD Analyses

A total of 367 isolates of *P. infestans* collected in 4 provinces and JeJu-shi of Korea from 2002 to 2004 were analyzed for mitochondrial DNA haplotype using restriction enzyme digestion patterns of PCR products (Fig. 3). The mitochondrial DNA haplotype of all isolates were the IIa haplotype (Table 2), and the 2 standard isolates were Ib haplotype.

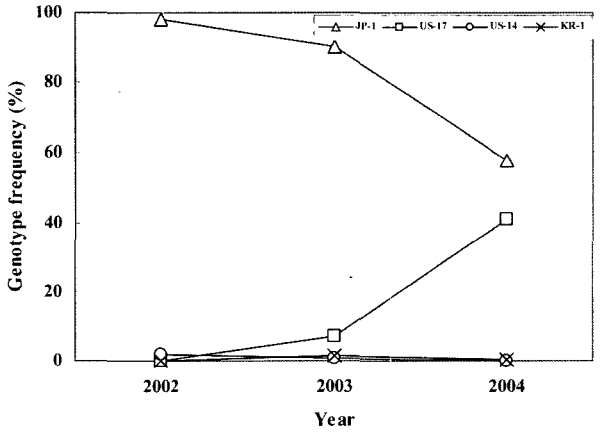

Fig. 2. Genotypic changes based on allozyme loci and mitochondrial DNA haplotype in Korean isolates of *Phytophthora infestans* collected from 2002 to 2004 (JP-1: A1, Gpi, 100/100, Pep, 100/100, IIa; US-17: A1, Gpi, 100/122, Pep, 100/100, IIa; US-14: A2, Gpi, 100/122, Pep, 100/100, IIa; KR-1: A1, Gpi, 100/130, Pep, 100/100).

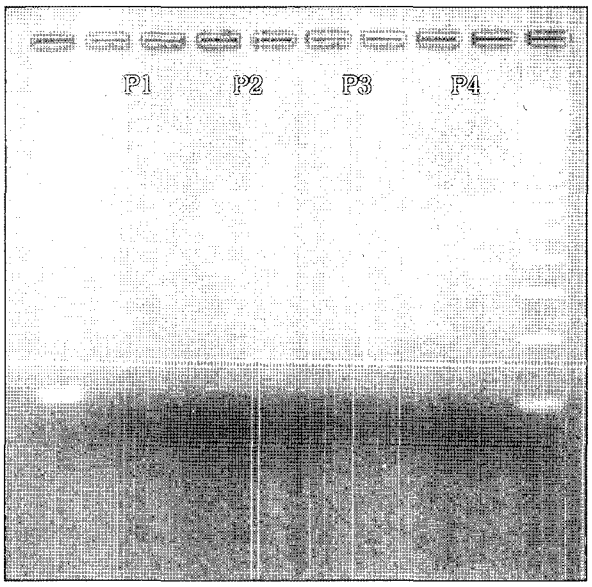

Fig. 3. Restriction enzyme digestion patterns of PCR products amplified from the DNA of *Phytophthora infestans* with the primer pairs of F1-R1 (P1, cut with CfoI), F2-R2 (P2, cut with MspI), F3-R3 (P3, cut with EcoRI), and F4-R4 (P4, cut with EcoRI). Amplifications were conducted with DNA from isolates representing each of the two mitochondrial DNA haplotypes (Ib and IIa). Among the mtDNA haplotypes produced with each primer pair, left is Ib haplotype, and right is IIa haplotype.

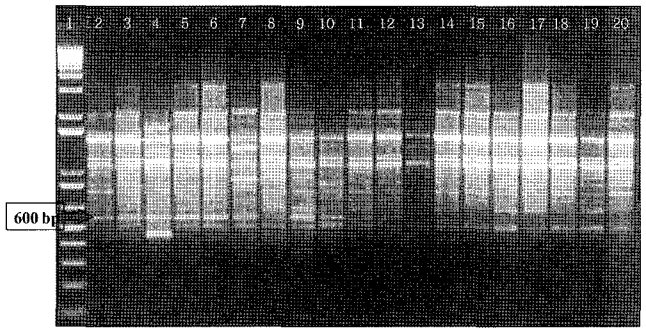

Fig. 4. RAPD profiles generated with the primer OPC-5 for selected *Phytopthora infestans* isolates.
Lane 1: molecular size marker (1kb ladder marker). Lanes 2-12: RG 1 to 11 (A1 mating type). Lanes 13-20: RG 12 to 19 (A2 mating type).

To estimate the genetic diversity among the *P. infestans* isolates collected from 2002 to 2004, genomic DNA of the 104 isolates and the 2 standard isolates (KA-2 and DN-3085) were analyzed using RAPD. Based on the diversity of RAPD profiles and specific band markers linked with specific mating type, the OPC-5 primer was selected among the 60 QIAGEN Operon primers tested. A total of 20 DNA bands were produced with the OPC-5 primer, of which 11 bands were polymorphic (Fig. 4). Values of similarity coefficients of each groups found with the OPC-5 primer ranged from 0.650 to 1.000 for all *P. infestans* isolates. The result was similar to the similarity coefficients of the RAPD group for *P. infestans*, ranging from 0.600 to 1.000, described by Abu-El Samen *et al.* [1]. Results from the RAPD analyses showed that hierarchical clustering separates the isolates into 2 major RAPD groups (RG1 and RG3) and 6 minor groups at the similarity level of 92.5% (Fig. 5 and Table 3). The RG 8 group included only one isolate. The 2 major RAPD groups and RG8 were composed of A1 mating type, whereas RG5, RG6, and RG7 were composed of A2 mating type. RG2 and RG4 constituted A1 and A2 mating types. If 100% similarity level is considered to separate *P. infestans* isolated in Korea into distinct groups, then 19 groups were identified among the total isolates analyzed with RAPD profiles (Fig. 5). When RAPD groups were separated by 100% similarity value, RG 1 to 11 were composed of A1 mating type, and RG 12 to 19 were composed of A2 mating type (Fig. 4). As revealed by the above investigation, the OPC-5 primer can distinguish RAPD groups by distinct mating types. In addition, an about 600-bp DNA band only specific for A1 mating type was detected by RAPD analysis (Fig. 4).

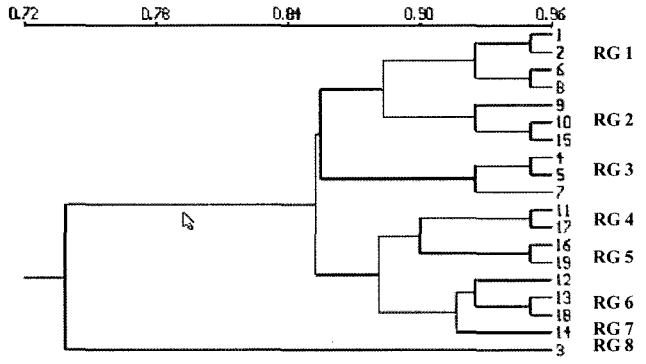

Fig. 5. Dendrograms of *Phytophthora infestans* isolates collected in Korea based on RAPD markers. Genetic distances among these isolates were revealed by cluster analysis with the unweighted pair group method with arithmetic averages.

Table 3. RAPD group, mating type, and allozyme genotypes of *Phytophthora infestans* isolates collected in Korea from 2002 to 2004.

RAPD group	Number of isolates	Mating type		Allozyme genotypes[a]
		A1	A2	
RG 1	43	43	0	JP-1, US-17, US-1[b]
RG 2	10	7	3	JP-1
RG 3	26	26	0	JP-1, US-17, KR-1
RG 4	7	2	5	JP-1, US-14
RG 5	4	0	4	JP-1, US-14
RG 6	12	0	12	JP-1
RG 7	3	0	3	JP-1
RG 8	1	1	0	JP-1
Total	106	79	27	5

[a]JP population of *P. infestans* are standard isolates found in Japan. US population are standard isolates found in the United States. KR-1 is a new isolate not detected throughout the world.
[b]US-1 is a standard isolate that belongs to the old origin population.

The RAPD groups of RG2, RG6, RG7, and RG8 were composed of only one allozyme genotype (JP-1 type), whereas other groups were composed of 2 or more allozyme genotypes (Table 3). RAPD groups and allozyme genotypes varied depending on distinct location, of which the isolates collected from Gangwon-do were differentiated

Table 4. Distribution of RAPD groups and allozyme genotypes of *Phytophthora infestans* collected in Korea from 2002 to 2004.

RAPD group	Provinces[a]				
	GW	JLB	JLN	CCN	JJ
RG 1	○	○	○		
RG 2	○	○			○
RG 3	○	○			○
RG 4	○	○			○
RG 5	○	○			
RG 6	○	○		○	
RG 7	○			○	
RG 8	○				
No. of RAPD groups	8	6	1	2	3
No. of allozyme genotypes	4	2	2	1	1

[a]GW: Gangwon-do. JLB: Jeollabuk-do. JLN: Jeollanam-do. CCN: Chuncheongnam-do. JJ: Jeju-shi.

into all of the 8 RAPD groups and 4 allozyme genotypes (Table 4).

Discussion

The mating type of *P. infestnas* has been studied in many countries including Korea. The change of mating type probably comprises three steps. The first step was that, prior to the 1980s, only the A1 mating type occurred in worldwide populations of *P. infestans* outside of Mexico [14, 22]. The second step was that A2 mating type strains occurred in most parts of the world [26]. The third step was that A1 mating type again displaced A2 mating type as reported in many other countries [6]. Change of the mating types of *P. infestans* in Korea was also identical with the above investigations [39]. In the present study, A1 isolates were always collected from potato plants, whereas all the isolates collected from tomato plants were only A2 mating type (Table 2), indicating a strong host specificity of the lineages of *P. infestans* in Korea.

Current methods usually used to analyze the allozyme of *P. infestans* are SDS-PAGE, starch-gel electrophoresis, and Cellulose acetate electrophoresis (CAE), of which the SDS-PAGE method was used on the enzyme analysis of *Aspergillus flavus* [24] and *Pseudomonas fragi* [23]. However, because the CAE method can rapidly detected *Gpi* and *Pep* isozymes on *P. infestans,* we used the CAE method in this study. Until now, 14 allozyme genotypes of *P. Infestans* have been detected by the *Gpi* locus [9]. The allozyme genotype of *P. infestans* in Korea was first reported by Koh *et al.* [29] in 1994. At that time, 2 allozyme genotypes (US-1 and JP-1) were investigated in Korea, and the majority of *P. infestans* isolates were JP-1. However, profiles of *Gpi* and *Pep* defined 3 allozyme genotypes among the total of 367 isolates collected from 2002 to 2004, and most isolates were JP-1 and US-17 types (Table 2). Furthermore, the previously reported US-1 population was not detected in this study, but rather US-17 type isolates increased gradually (Fig. 2). These findings demonstrate that the old population of JP-1 and new population of US-17 type have substituted the original US-1 population in Korea.

Until recently, 4 mitochondrial DNA haplotypes (Ia, Ib, IIa, IIb) of *P. infestans* have been identified [20]. Koh *et al.* [29] first reported that two mtDNA haplotypes (Ib, IIa) exist in Korea. However, mtDNA haplotype analysis in this study detected only one mitochondrial haplotype, IIa, whereas that of US-1 isolates (Ib) of the old population was not detected (Table 2). This uniqueness in mtDNA haplotype is most likely due to the maternal inheritance nature of mt DNA [38].

Mahuku *et al.* [31] indicated that RAPD markers provide useful information about the genome, because they detect length polymorphisms arising from base sequence changes, insertions, deletions, substitutions, or inversions, either at or between the priming sites. In the present study, RAPD analysis separated the collected *P. infestans* isolates into 8 groups at the similarity level of 92.5% (Fig. 5), and all of the 8 RAPD groups were distributed in Gangwon-do (Table 4). Gangwon-do is the largest potato-producing area in Korea, yielding about 35% of total potato according to the year 2003 statistics of the Ministry of Agriculture and Forestry in Korea. Furthermore, many potato breeding research institutes, including Natinal Institute of Highland Agriculture, are located in Gangwon-do, where a diverse potato cultivar gene source may be reserved. We suggest that *P. infestans* has diversely been differentiated by host. Therefore, Gangwon-do is believed to be the center of origin of the *P. infestans* in Korea. The result was similar to that described by Goodwin *et al.* [18] that the center of origin would most likely be the area of highest genetic diversity. Different RAPD groups were composed of different allozyme genotypes (Table 4). Thus, RAPD groups were not correlated with allozyme genotypes and isolated locations, in accordance with the finding described by Mahuku *et al.* [31]. When the RAPD groups were separated by different mating type isolates at the similarity level of 100%, however, the RG 1 to 11 groups were composed of A1 mating type, and the RG 12 to 19 groups were composed of A2 mating type. The 600-bp DNA band produced with the OPC-5 primer was used to determine different mating types (Fig. 5). Judelson *et al.* [25] also reported an RAPD marker distinguishing mating types; however, they used primers different from that used in the present study. Therefore, the 600-bp DNA band can be utilized to determine *P. infestans* mating types. It is also expected that DNA cloning and sequencing of the 600-bp band could provide additional information for mating type determination.

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