IDENTIFICATION OF AIRBORNE FUNGI FROM INDOOR AIR OF CHICKEN HOUSES BY RAPD-PCR

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ABSTRACT

The objectives of this study were to examine the airborne fungal concentrations, size and compositions as well as the dominant genera in chicken houses. Aerosol fungi were collected from chicken house indoor with Andersen-6 stage air sampler and cultured in RBC media. Species were identified by morphological characteristics and RAPD. The concentrations of aerosol fungi in chicken houses ranged from 1.8–3.0×10^3 CFU/m^3 air. 89 *Fusarium* isolates collected from the chicken houses were characterized by RAPD. Out of 30 primers, five primers reproducibly generated polymorphic patterns for fungal species of a test panel, with 74 characteristic fragments. These primers were then used in RAPD assay of 91 isolates. RAPD profiles were clustered with UPGMA algorithm. This analysis helped clarify *Fusarium* isolates that were difficult to produce conidia for morphological identification.

Keywords: chicken house; airborne fungi; RAPD; genetic distance; *Fusarium*

INTRODUCTION

Fusarium species are common dominant mould in animal farm environment [1 2 3 4]. Traditionally, these species are identified by their morphological and physiological characteristics especially at vegetative stage. These methods are simple and convenient. However, a taxonomic system based on morphology is often factitious. It does not necessarily reflect evolutionary history. Morphology of the genus *Fusarium* is highly variable and extremely complex. In addition, it is difficult or sometimes impossible to obtain the reproductive stages. Even well-trained mycologist may not be able to diagnose one thirds of *Fusarium* isolates to species level [5]. Thus, Identification of *Fusarium* species has been considered the most difficult among fungi [6]. Application of random amplified polymorphic DNA (RAPD) technology in identification of *Fusarium* may be fast and accurate.

In recent years, many studies have focused on fungal aerosol concentrations in hospital, residential and public environment [7, 8, 9, 10]. A literature search indicates that fungal aerosol in animal husbandry environment has not yet been considered as occupational hazard. Here we report the concentration and identification of airborne *Fusarium* species in poultry house with RAPD approach. This study is necessary for evaluation of occupational hazardous exposure for workers in animal husbandry environment.

1. MATERIALS AND METHODS

1.1 Sample Collection

Air samples were collected between November, 2003 and December, 2005 from three chicken farms (Tai'an Manzhuang Chicken Farm, Zaozhuang Mengzhuang Township Chicken Farm and Liuling Tiekuang Chicken Farm) in Shandong Province. Collection was performed using an Anderson 6-stage air sampler [11, 24, 25] at a speed of 28.3 L/min and 50 cm above ground. Each air sample was collected in 2–4 min depending on the environment. For each farm, samples were taken three times alternated by one week. In each time three to five samples were collected.

1.2 Fungal Culture and Identification

Fungi were cultured with RBC media [12] in alternatively lighted incubator for 3–7 d at 25°C. Colonies were counted according to their morphology under Olympus stereoscope. Pure cultures were established and preserved. Fungal isolates were tested in SNA (saccharose nirenberg agar), PDA (potato dextrose agar) and PSA (potato sucrose agar) plates. *Fusarium* species were identified according to their colony characteristics and reproductive structure in reference to the systems of Booth (1971) [13], Gerlach & Nirenberg (1982) [14], Nelson (1983) [15] and Joffe (1986) [16].

1.3 DNA Extraction

The fungal isolates were cultured at 25°C for 6–7 d in 0.5 mL liquid medium (glucose yeast medium) in 1.5 mL Eppendorf tube. Three cultures were made for each isolate. *Aspergillus niger* and *Trichoderma viride* were also cultured as controls. Genomic DNA was isolated as described [17] and DNA concentration was determined by electrophoresis in 1% agarose gel and imaged by GDS 8000 (UVP, US).

1.4 RAPD-PCR

1.4.1 Materials 100 bp DNA ladder, 10×PCR buffer, 10×ExTaq Buffer, dNTP (25 mmol/dm^3 each), ExTaq DNA Polymerase (5U/µL) were purchased from Takara Biotechnology (Dalian) Co., Ltd. Random primers (10bp in size) were purchased from Shanghai Sangon. PCR amplification was performed with PE2400 or PE2700 thermal cycler (ABI, USA).

1.4.2 RAPD Reaction Mixture The volume of each reaction was 25µl. The reaction mixture contains: (1) random primer (table 1) 0.2 µmol/dm^3; (2) dNTP 0.2 µmol/dm^3; (3) PCR buffer 2µL (10 mmol/dm^3 Tris-HCl, pH9.0, 50 mmol/dm^3 KCl, 1.5 mmol/dm^3 $MgCl_2$); (4) genomic DNA 1µL (5 ng DNA); (5) *Taq* DNA polymerase 0.2µL (1U); and (6) highly purified water 18.8 µL.

1.4.3 Thermal Cycle ① Pre-denaturating at 94°C for 200 sec. ② 40 cycles of denaturation at 94°C for 1 min, annealing at 36°C for 1 min, and extension at 72°C for 2 min. ③ Final extension at 72°C for 5 min. Reaction products were preserved at 5°C.

1.4.4 RAPD Product Visualization RAPD products were mixed with loading dye (phenol blue-EDTA-glycerol). The product mixtures were separated by electrophoresis in 2% agarose gel for 1–2 h at 10 V/cm. Gel images were recorded and analyzed with UVP system GDS 8000.

1.4.5 Screening for RAPD Primers Primers were suspended in TE buffer in a working concentration of 20 μM. They were tested against a panel of 6 *Fusarium* species with the condition described in section 1.4.1 through 1.4.4. The panel contains isolate No. 61 (*F. ventricosum*), 15 (*F. morniliforme*), 57 (*F. oxysporum*), 60 (*F. graminearum*), 59 (*F. poae*) and 39 (*F. equiseti*). Five primers that were able to produce 4–10 bands for each of the panel species were selected for the analysis of 91 isolates. Reactions were duplicated for each isolate.

1.4.6 Data Analysis The presence of a band in an isolate was represented by "1", while the absence was represented by "0". Distance between two samples were calculated by equation of D=1–2Nxy/Nx+Ny, where Nxy is the sum of bands in the two samples; Nx is the number of bands in sample x and Ny is the number of bands in sample y. Distance matrix was produced using unweighted pair-group method with arithmetic mean (UPGMA) algorithm and a dendrogram was generated by EPCLUST software.

2. RESULTS

2.1.1 Fungal Concentration Airborne fungal concentrations in closed chicken house were between 1.8~3.0×10^3 CFU/m^3. The chicken density was 5.9~10.2 chicken/m^2. Temperature variation was within 3°C. Humidity was 47% ~ 73%.

2.1.2 Airborne *Fusarium* 150 isolates of airborne *Fusarium* were finally obtained from the three farms. Of which 89 strains were picked randomly for RAPD assay. They belong to the following species: *F. moniliforme, F. moniliforme* var. *intermedium*, *F. morniliforme* var. *subglutinans*, *F. oxysporum*, *F. equiseti*, *F. solani*, *F. semitectum*, *F. graminearum*, *F. ventricosum*, *F. poae*, *F. avenaceum*, *F. nivale*.

2.2 Primer Screening

30 primers were screened using DNA samples extracted from 6 fungal strains. Five primers (*e. g.* P15, P17, P19, P24 and P29) were able to produce highly polymorphic bands. The number of bands produced varied from 5 to 15. They were selected for the analysis of the 89 isolates and 2 control strains.

2.3 RAPD Patterns

The five selected primers were used to amplify DNA from 89 isolates and 2 control strains. Reactions were performed under the same condition and with the same thermal cycler. PCR products were separated by agarose gel electrophoresis and analyzed with high resolution gel documentation equipment. PCR product sizes were obtained by comparing with the molecular weight standard. The product patterns were similar among isolates belonging to the same species while the patterns exhibit greater variation among species. Each species displayed a unique characteristic profile (Fig 1).

2.4 Pattern Matrix Generation

A matrix was generated according to the presence or absence of RAPD-PCR fragments in basic pairs (bp). The bands within the following molecular size (bp) are marked as presence: 1950–

2250(r1), 1750–1950(r2), 1550–1750(r3), 1350–1550(r4), 1150–1350(r5), 950–1150(r6), 850–950(r7), 750–850(r8), 650–750(r9), 550–650(r10), 450–550(r11), 350–450(r12), 250–350(r13), 150–250(r14), 100(r15). The presence of a band within the above range was assigned a value ‘1’, while the absence of a band was assigned a value ‘0’. 91 isolates were tested with the five primers. 73 bands were obtained. A band pattern matrix was generated (data not shown here).

2.5 Genetic Distance

Isolates were clustered using UPGMA to calculate genetic distance. A dendrogram was generated from the distance matrix (Fig. 2). The distances among isolates are small. When threshold distance was set at 0.62, the 91 isolates were divided into 11 groups.

3. DISCUSSION

3.1 Plentiful diversity of aerosol fungi was found in the three tested chicken farms. The most frequent aerosol fungi are *Aspergillus,* some of which are opportunistic pathogens. For example, *A. fumigatus* and *A. terreus* were recorded to infect human and animals. Animal tests have shown that some *Aspergillus* species (e. g. *A. flavus*, *A. parasiticus*, *A. versicolor*) may produce aflatoxins that induce tumor or reduce white blood cells. The other frequent fungi in the farms include *Penicillium, Alternaria, Acremonium* and *Fusarium.* Some *Penicillium* species may clinically infect human beings who have been affected by leukaemia or lymphoma, or infect brain or lung and produce ochratoxins. Some *Alternaria* species may clinically cause skin infection such as hypersensitivity, pneumonitis or asthma. Some species of *Alternaria* may produce mycotoxins that induce oesophageal cancer. *Acremonium* may cause chromomycosis or phaeohyphomycosis. They commonly infect brain or skin. It is surprisingly found that *Fusarium* is a very common genus in these tested chicken houses. *Fusarium* species are very common in agricultural environment and some well-known for producing various mycotoxins. A few *Fusarium* species may induce skin or cornea ulcers, even in rare case being associated with cancer [12, 18–22]. The various fungal species may show certain virulence to the animals. It is necessary to further the study of their pathogenic ability to chickens.

3.2 When threshold is set at 0.62, the tested *Fusarium* isolates can be differentiated into species that can be identified by morphological method. However, the five RAPD primers were unable to distinguish among *F. moniliforme F. moniliforme* var. *subglutinans* and *F. moniliforme* var. *intermedium* because of their close relationship. 75% isolates of the same species were clustered into the same group. Nearly 25% isolates were not clustered to groups in which they were supposed to be. This explains the variability of *Fusarium* species from molecular biology point of view. Some isolates (*e.g.* 27 and 28) are extremely close. They might belong to the same species of different isolates. The difference in collecting sites may result in different genetic distance of the same *Fusarium* species, such as Dalian isolate and Tai’an isolate of *F. moniliforme.* Its genetic distance between airborne isolates and isolates from wheat grains is relatively far-away, suggesting that the complicated environmental factors may cause variation of *Fusarium* species. This result is consistent with the instability of *F. moniliforme* [23].

F. graminearum did not produce conidia even cultured on SNA plate, which makes identification by morphology difficult. An isolate (No. 43) which is difficult identified did not form conidia on either PDA or PSA. Its purple pigment-producing colony indicates that it is

Fusarium. By clustering analysis of RAPD patterns, it can be confidently assigned to *F. graminearum.* Using RAPD marker, some difficult isolates can be identified. It is concluded that RAPD assay could provide as an effective tool for the identification of suspected fungal isolates.

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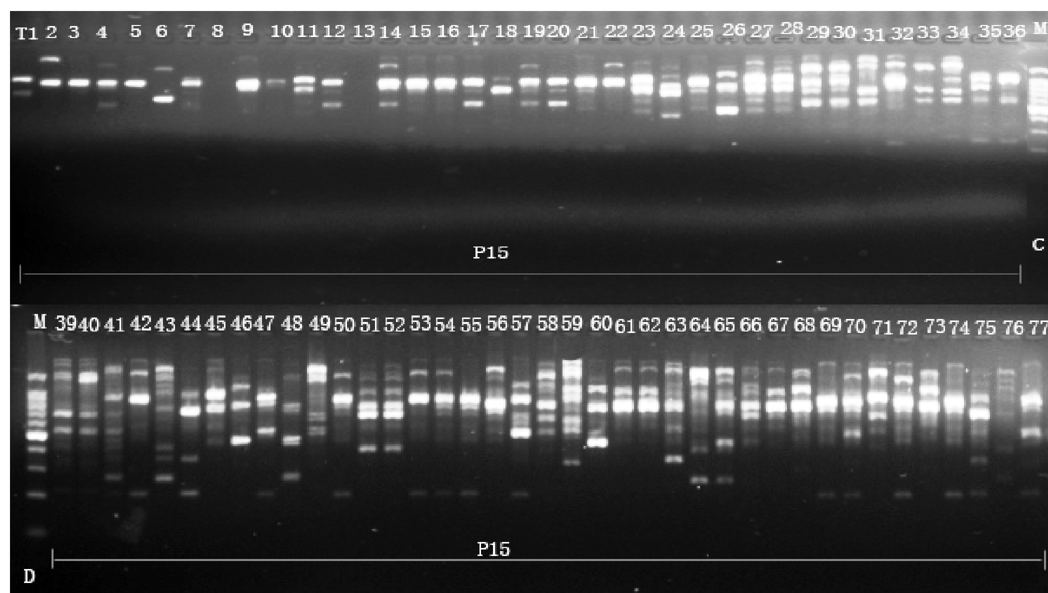
REFERENCES

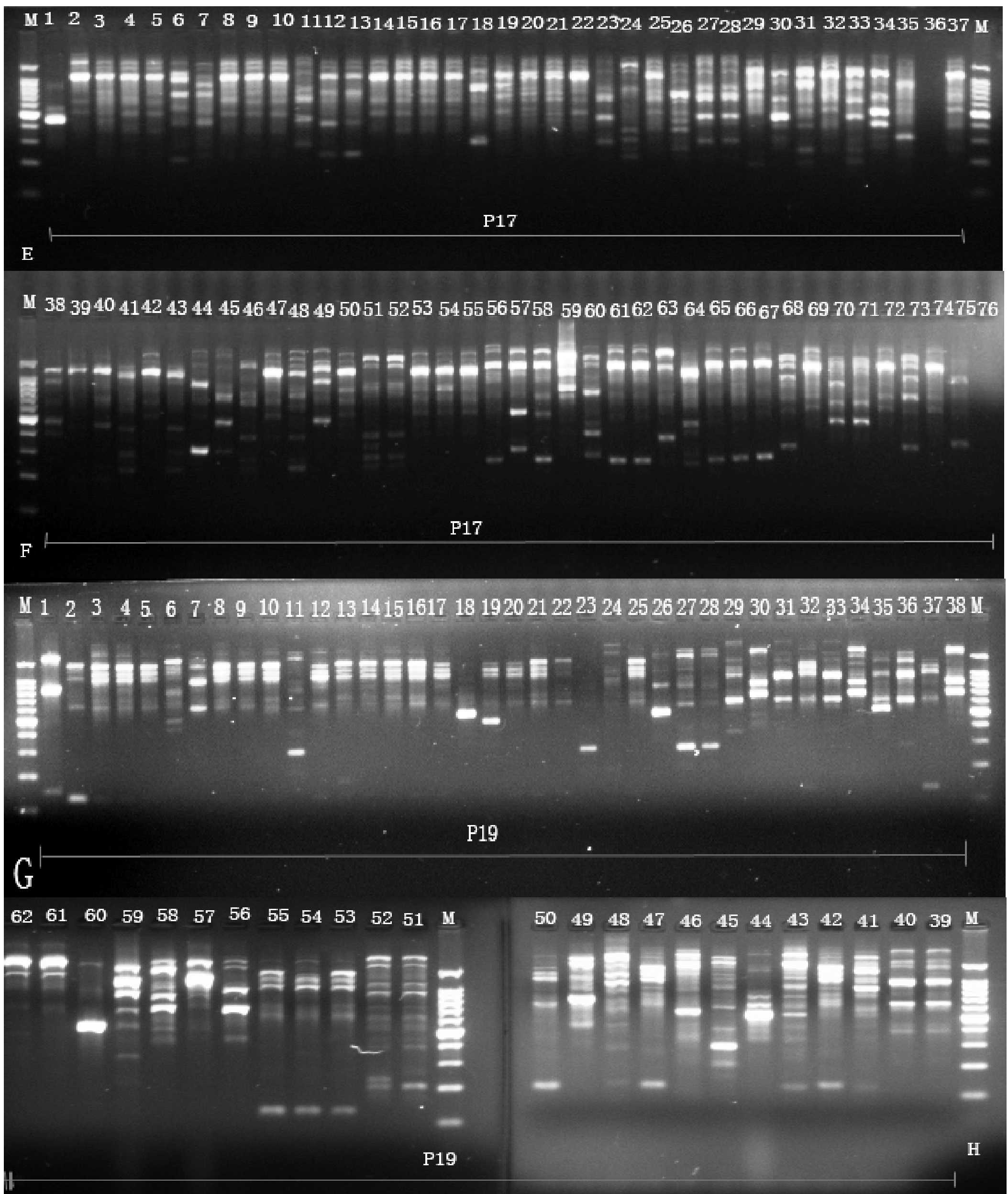
1. [1] Wang Y L, Lu G Z, Chai T J, Shun X D,2005.Diversity and concentration of airborne fungi in chicken house. *Acta Mycologica Science*(Mycosystema), 24(4): 510–516
2. [2] Wang Y L,2006. Detection and identification of fungal aerosol and related mycotoxins in animal raising environment. *Superexcellence Doctor Dissertation of Shandong Agriculture University*, 11–75
3. [3] Wang Y L, Chai T J, Lu G Z, 2006.The fungal aerosol in farming environments. *Journal of Domestic Animal Ecology*, 26(6): 51–56 (in Chinese)
4. [4] Wang Y L, Lu G Z, Chai T J, 2006.Quantificational evaluation for the potential harm of fungal aerosol in chicken house. *Journal of Domestic Animal Ecology*, 27(1): 66–72
5. [5] O’Donnell, *et al*, 2004: J Clin Microbiol 42: 5109–5120
6. [6] Wang G C, Zheng Z, Ye Q M, Zhang C L, 1996. Identify guide of common *Fusarium*.*Peking Chinese Agriculture Science Press*, 3–45
7. [7] Gao Q Y, Chen D W, Gan M H, 1986.Comparative observation of morphologic-pathology for manual inoculability three aspergillus species in ducks. Chinese Veterinary Journal, 12(8): 5–11Hu
8. [8] Q X, Xu X Z, Liu M X, et al, 1996. Research of Indoor Aerosol fungal partical in Shen-yang) Yun-nan Environment Science, 15(1): 16–19
9. [9] Hu Q X, Li B J, Ye B Y, et al, 1994.granularity distribution and rainfall influence of air fungi in Peking. Si-chuan Environment, 13(3): 52–55
10. [10] Chen H W, 1999. Airborne microbiological survey in Jinan Tai’an and Qufu. *Environment and Exploitation*. 14 (4): 43–45
11. [11] Andersen A. A. New sampler for collection, sizing and enumeration of viable airborne particles[J].*J Bacteriol*, 1958, 76: 471
12. [12] Wu S X, 1998.*Modern Medical Inspection Mannul*. Beijing: Beijing Medical UniversityBeijing Consonancy Medical University Joint Publish, 300–326
13. [13] Booth C, 1971.The Genus *Fusarium*[M]. *CMI, Kew, England*, 1–237
14. [14] Gerlach W, Nirenberg H, 1982.The genus Fusarium-A pictorial atlas[M]. *Mitteilungen aus der Biologischen Bundesanstalt Fuer Landund Forstwirtschaft (Berlin-Dahlem)*, 209: 1–405
15. [15] Nelson P E, Toussoun T A, Marasas W F O, 1983. *Fusarium species: An Illustrated Manual for Identification*[M]. Pennsylvania State University Press, 1–193
16. [16] Joffe A Z, 1986. *Fusarium Species*: *Their Biology and Toxicology*[M]. John Wiley and Sons, New York City, 1–588
17. [17] Cenis J L, 1992. Rapid extraction of fungal DNA for PCR amplification[J]. *Nucleic Acids Research*, 20: 2380
18. [18] Li G Q, Cao G R,1999. Development on pathogeny and cause of avian aspergillosis [J]. Progress in Veterinary Medicine, 20(3): 12–14
19. [19] Li G Q, Zhao B Y, Cao G R, 1997.Development on animal mycosis in China[J]. Progress in Veterinary Medicine, 18(3): 13–16
20. [20]Carlnick B W, Gao F, Su J L(translation), 1999. Avian Disease (No.10t) [M]. Bejing: Chinese Agriculture Press, 448

21. [21] Sun H L, 1987. Elementary Edition of Medicine Fungi[M]. Bejing: Chinese Science Press, 259–260
22. [22] Richard J L, Cutlip R C, Thurston J R, *et al*. Response of turkey poults to aerosolized spores of *Aspergillus fumigatus* and aflatoxigenic and nonaflatoxigenic strains of *Aspergillus flavus*[J].Avian Dis,1981,25(5): 53–67
23. [23] Zhang H, Wu J, Li J L,1995.The unstability of moniliformin production by *Fusarium subglutinans*. *ACTA of Mycology*, 14(2): 116–122, 199
24. [24] Yu X H, Che F X,1998. Moden Technique of Sampling and Detection of Air-Microbes. Bejing: Military Medicine Science Press,1–10, 341–319
25. [25]Che F X, Yu X H,1998.Principle and Technique Application of Sampling and Detection of Air-Microbes. Chinese Encyclopedia Press, 1,15

Table 1. Oligonucleotide sequences of 30 primers for RAPD-PCR

code	No.	Sequence	code	No.	sequence	code	No.	sequence
P1	S23	agtcagccac	P11	S22	tgccgagctg	P21	S130	ggaagcttgg
P2	S125	ccgaattccc	P12	S24	aatcgggctg	P22	S326	gtgccgttca
P3	S329	caccccagtc	P13	S30	gtgatcgcag	P23	S324	aggctgtgct
P4	S25	aggggtcttg	P14	S28	gtgacgtagg	P24	S129	ccaagcttcc
P5	S26	ggtccctgac	P15	S330	ccgacaaacc	P25	S322	cctacgggga
P6	S124	ggtgatcagg	P16	S21	caggcccttc	P26	S126	gggaattcgg
P7	S27	gaaacgggtg	P17	S29	gggtaacgcc	P27	S328	gggtgggtaa
P8	S123	cctgatcacc	P18	S327	ccaggaggac	P28	S325	tcccatgctg
P9	S127	ccgatatccc	P19	S122	gaggatccct	P29	S121	acggatcctg
P10	S321	tctgtgccac	P20	S323	cagcaccgca	P30	S128	gggatatcgg

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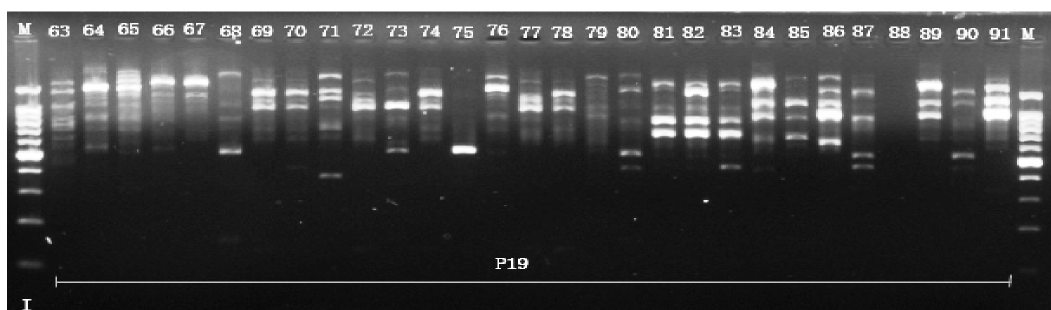
P17
E
M 38 39 40 41 42 43 44 45 46 47 48 49 50 51 52 53 54 55 56 57 58 59 60 61 62 63 64 65 66 67 68 69 70 71 72 73 74 75 76
P17
F
M 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 25 26 27 28 29 30 31 32 33 34 35 36 37 38 M
P19
G
62 61 60 59 58 57 56 55 54 53 52 51 M
50 49 48 47 46 45 44 43 42 41 40 39 M
P19
H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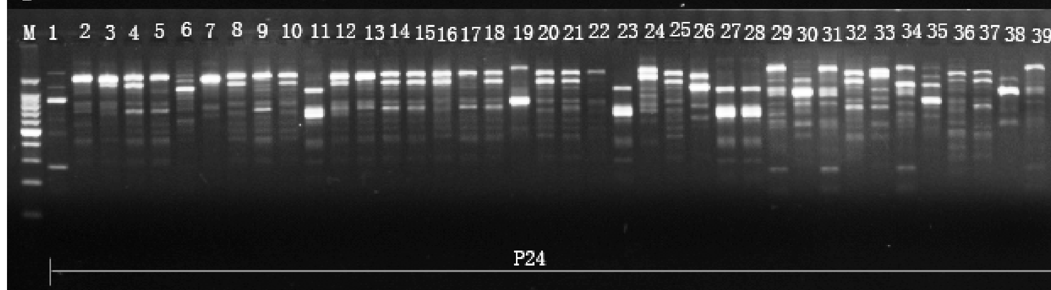
P19
I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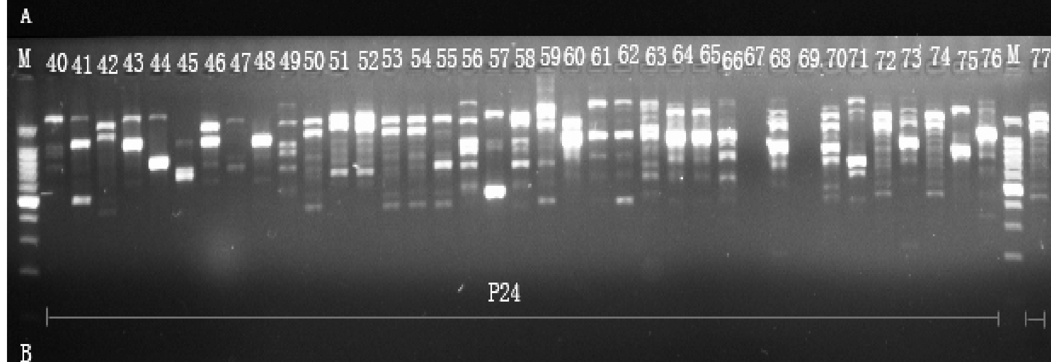
P24
A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P24
B

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P29
L

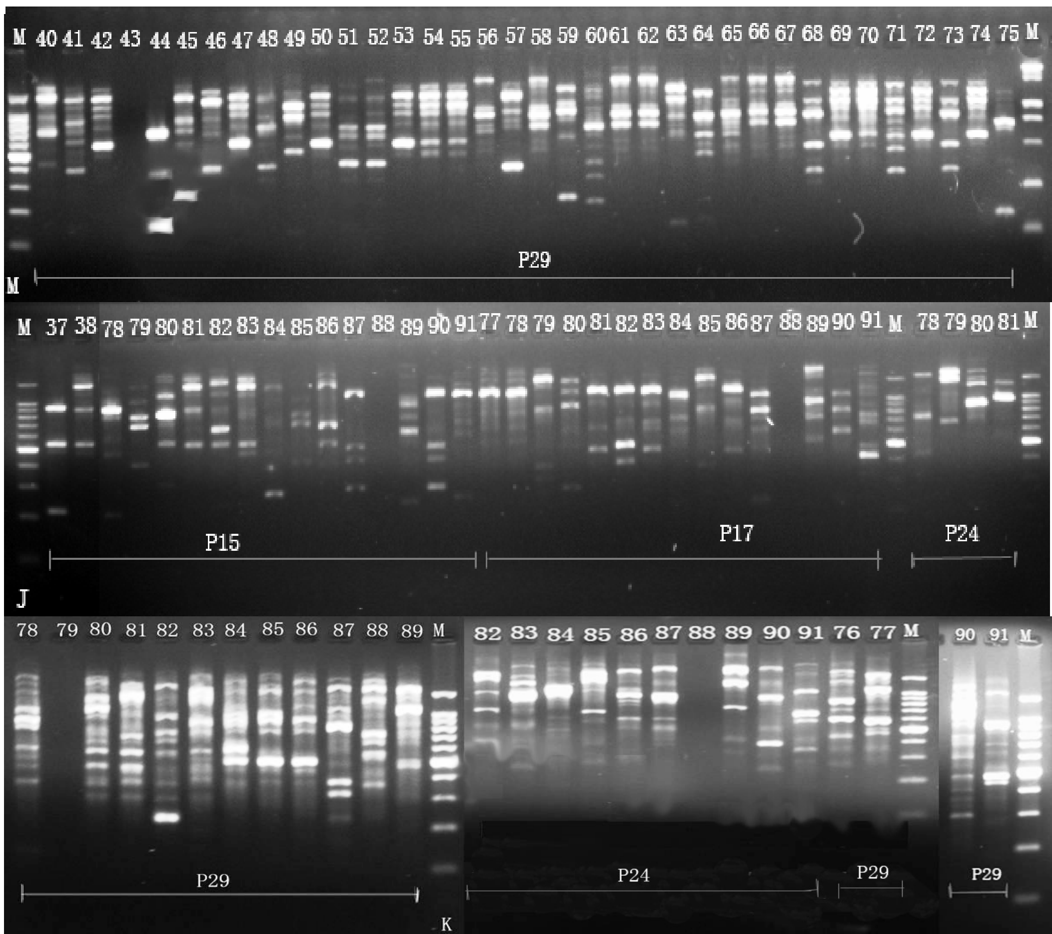

Figure 1. RAPD profiles for 91 strains using 5 optimized RAPD primers

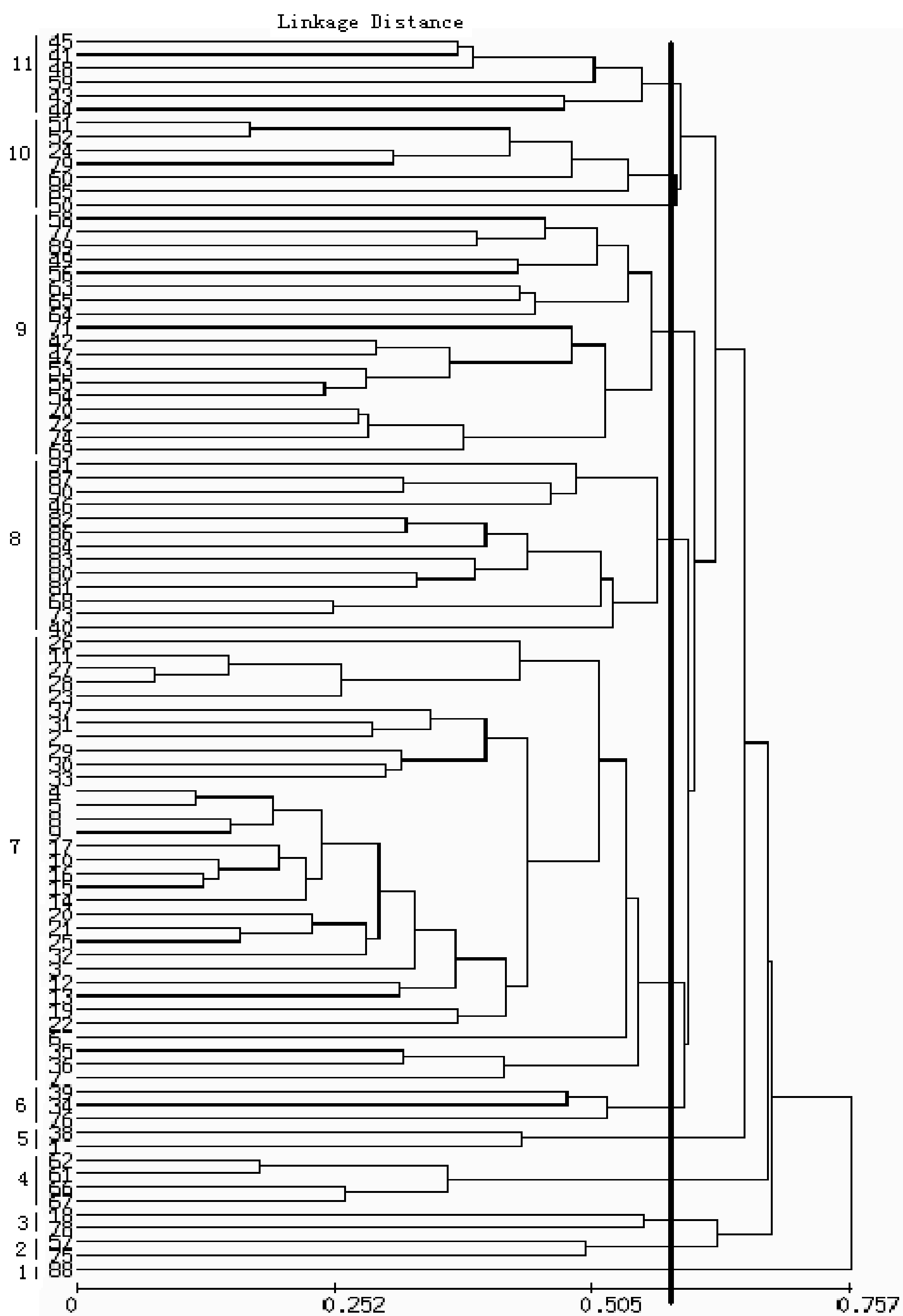

Figure 2. Dendrogram for 91 *Fusarium* strains based on RAPD pattern