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*Full Length Research Paper*

# Variations in the isolates of *Macrophomina phaseolina* from sesame in China based on amplified fragment length polymorphism (AFLP) and pathogenicity

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**Charcoal rot that is caused by *Macrophomina phaseolina*, the most damaging disease in sesame in China. Variations in the 35 isolates of *M. phaseolina* collected from the main sesame producing regions in central China comprising of Hubei, Henan, Anhui, Jiangxi province were studied based on morphology, pathogenicity and amplified fragment length polymorphism (AFLP). The results showed that the morphological characteristics, including density of aerial mycelia, sclerotia quantity, sclerotium size and growth speed of colony, had rich variations. The pathogenicity index of these isolates ranged from 0.03 to 4.64 with an average of 2.10. Four isolates (17, 21, 28 and 35) with pathogenicity index more than 4.0 from the locations alongside Yangtze River were found here. AFLP analysis indicated that the paired genetic similarity coefficients of these isolates ranged from 0.65 to 0.97 with an average of 0.83. These isolates could be divided into seven genotypic groups based on unweighted pair group method with arithmetic mean (UPGMA) dendrogram. However, no clear relationships among the groups, geographic regions and PI were found. These results may be helpful to understanding the population structure of the fungus and contribute to the control of sesame charcoal rot in China.**

**Key words:** *Macrophomina phaseolina*, pathogenicity, charcoal rot, sesame, amplified fragment length polymorphism (AFLP).

## INTRODUCTION

Sesame (*Sesamum indicum* L.), characterized with its short growing period, low water requirements, wide adaptability to soil condition, high oil content (about 55%), and special function compositions to health such as sesamin and sesamolin, is one of the major oilseed crops following soybean, peanut and oilrape in China. It was planted mainly in the Yangtze river and the Yellow river valley in China comprising Hubei, Henan, Anhui, Jiangxi province, and accounts for 90% of total sesame (620 million kg, 2009) of China. Compared to other oilseed crops, the yield potential of sesame is very low. Major factors that limit its productivity besides narrow genetic base are extreme susceptibility to biotic and abiotic stresses.

Sesame charcoal rot have been proved to be caused by *Macrophomina phaseolina* (Tassi) Goid. (Mihail, 1992). The fungus can infect about 500 plant species in more than 100 families throughout the world (Kunwar et al., 1986; Mihail and Taylor, 1995; Purkayastha et al., 2006), is one of the most damaging seed and soil borne pathogen in both agricultural and natural ecosystems. The diversity of host species and the geographic range have suggested that the *M. phaseolina* is quite heterogeneous. Its variability has been confirmed by reports demonstrating differences in pathogenicity of isolates

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obtained from both a single plant and a single host species (Babu et al., 2010; Cloud and Rupe, 1991; Jana et al., 2005; Saleh et al., 2010). Despite having a wide host range, only one species is recognized within *Macrophomina* (Mihail and Taylor, 1995) and efforts to divide *M. phaseolina* into sub-species were unsuccessful largely due to the extreme intra-specific variations in morphology and pathogenicity (Das et al., 2008; Fernández et al., 1991; Khan, 2007).

Molecular techniques like random amplified polymorphic DNA (RAPD) and amplified fragment length polymorphism (AFLP) analysis are useful in revealing genetic variability in the fungal population used to study genetic diversity in populations of *M. phaseolina* isolated from varied plants in different countries like Mexican (Mayék-Pérez et al., 2001) Australia (Fuhlbohm, 1997) Brazil (Almeida et al., 2003) and India (Das et al., 2008; Jana et al., 2003). RAPD studies generate a large number of markers, but their reproducibility is often poor (Aarts et al., 1998; Mueller and Wolfenbarger, 1999). AFLP is a reliable genotyping method with a high degree of reproducibility and discriminatory power with typically 10 to 20 and more fragments analyzed simultaneously (Savelkoul et al., 1999; Vos et al., 1995).

Sesame charcoal rot is the most damaging disease in sesame in China. It induces more than 30% or even complete losses under favorable conditions in most planting seasons and brings about 300 million dollars loss every year, especially, in Hubei province, as it is alongside Yangtze River with high temperature and moisture during the growth period of sesame. However, no detailed reports demonstrated pathogenic and genetic variability of the isolates of *M. phaseolina* from sesame in China. The present study, therefore, was conducted to investigate the kind of genetic relatedness among the *M. phaseolina* isolates from sesame in China. This was for a better understanding of the population structure of the fungus and its pathogenicity on sesame in order to contribute to the strategies for reducing the loss from sesame charcoal rot.

## MATERIALS AND METHODS

### Origin of isolates

A total of 35 sesame stalks showing typical charcoal rot symptoms were collected from 32 geographical locations in the main sesame producing region in central China comprising Hubei, Henan, Anhui, Jiangxi province in 2009, covering longitude 114.017° to 116.241° East, and latitude 28.377° to 33.581° North (Table 1). In the four provinces, Jiangxi was a typical black sesame production region, and the other three belonged to white sesame. The fungus *M. phaseolina* was isolated from each sample by placing a bit of infected stalk tissue on Potato Dextrose Agar (PDA) plate followed by incubation at 28°C for 4 days. Pure cultures were developed by single spore culture and maintained on PDA at 28°C and their morphological characteristics including density of aerial mycelia of 4 days, sclerotia quantity of 24 h, sclerotia quantity of 67 h, sclerotium size (length + width)/2 (mm) of 7 days, growth speed of colony (mm/h) of these isolates were tested.

### Pathogenicity study

The pathogenicity of the 35 isolates was tested on Ezhi No. 2, a cultivar susceptive to charcoal rot, according to the method of Reyes-Franco et al. (2006) with little revision. In detail, the isolates were cultured in PDA Petri dishes and incubated in darkness at 30°C for 7 days, and then each isolate was inoculated on the centre of 5 culture bottles with PDA respectively. Afterwards, 20 germinating seeds were placed along the medium fringe of each culture bottle. Seeds were previously surface-sterilized using 0.1% $HgCl_2$ for 4 to 5 min and rinsed thrice in sterile tap water. The treatments were randomized using a completely randomized design with five replications. After the culture bottles were incubated at 30°C for 5 days, seedlings were evaluated for symptoms caused by the pathogen using one 0 to 5 rating scale. Where 0 = healthy seedling; 1 = discoloration of 1/3 of the seedling root; 2 = discoloration of 1/3 to 2/3 of the seedling root; 3 = discoloration of 2/3 to whole of the seedling root; 4 = discoloration of whole of the seedling root and 1/2 of the sprout; 5 = discoloration of whole of the seedling root and more than 1/2 of the sprout discolored or rotted. Pathogenicity Index (PI) was calculated for each isolate using the following formula according to Manici et al. (1995):

$$PI=\sum_{i=0}^{n} (I \times Xi) / \sum_{i=0}^{n} Xi$$

I = disease grade; Xi = numbers of seeds in grade I; PI ≤ 2 was taken as low pathogenicity, 2 < PI ≤ 4 as middle pathogenicity, and 4 < PI ≤ 5 as high pathogenicity.

### Extraction of genomic DNA

The mycelium developed from single microsclerotia was transferred on to cellophanes with PDA below and incubated at 28°C. Mycelia from PDA were harvested and used to extract genome DNA with Cetyl Trimethyl Ammonium Bromide (CTAB) extraction buffer (50 mM Tris–HCl, pH 8.0, 700 mM NaCl, 10 mM EDTA, 10% CTAB). According to Dasa et al. (2008) the pure quantified DNA samples were stored at 4 °C for further use.

### ITS sequencing

The Internal Transcribed Spacer (ITS) region was amplified with primers ITS1 (5'-TCCGTAGGTGAACCTGCGG-3') and ITS4 (5'-TCCTCCGCTTATTGATATGC-3') (White et al., 1990). The amplified product was electrophoresed on 1% agarose gel and the fragment was extracted and purified using the Prepagene kit (Bio-Rad). All the sequencings were performed by the Shanghai Sangon Biological Engineering Technology and Services Co., Ltd (Shanghai, China). The resulting ITS sequences were analyzed for homologies to the sequences deposited in the GenBank databases (http://www.ncbi.nlm.nih.gov/). Sequence analysis was performed using the software DNAMAN (http://www.lynnon.com).

### AFLP analysis

DNA from 35 isolates was subjected to genetic diversity analysis by the AFLP method following the protocol reported by Vos et al. (1995) and Mayek-perez et al. (2001) Oligonucleotide primers used for the preamplification step were:

EcoRI (EcoRI . A) 5'-AGACTGCGTACCAATTC/A-3',
MseI (MseI . A) 5'-GACGATGAGTCCTGAGTAA/A-3'.

**Table 1.** Origin and morphological variation of *M. phaseolina* isolates.

| No. | Origin | Longitude (East) | Latitude (North) | Pathogenicity index | Sclerotium size of 7d (mm)* | Growth speed of colony (mm/h) |
|---|---|---|---|---|---|---|
| 1 | AHHefei | 117.29 | 31.87 | 0.03 | 0.11 | 0.48 |
| 2 | HNHuangchuan | 115.05 | 32.13 | 2.34 | 0.13 | 0.50 |
| 3 | HNLuohe | 114.02 | 33.58 | 3.40 | 0.14 | 0.42 |
| 4 | HNPingyu | 114.64 | 32.96 | 2.58 | 0.10 | 0.68 |
| 5 | HNPingyu | 114.64 | 32.96 | 3.48 | 0.12 | 0.66 |
| 6 | HNZhumadian | 114.03 | 32.98 | 0.58 | 0.12 | 0.62 |
| 7 | HBEzhou | 114.90 | 30.39 | 0.51 | 0.13 | 0.68 |
| 8 | HBEzhou | 114.90 | 30.39 | 3.64 | 0.11 | 0.53 |
| 9 | HBHanchuan | 113.84 | 30.66 | 0.33 | 0.12 | 0.44 |
| 10 | HBHonghu | 113.47 | 29.81 | 1.68 | 0.14 | 0.68 |
| 11 | HBHuangpi | 114.38 | 30.88 | 0.60 | 0.11 | 0.52 |
| 12 | HBHuangpi | 114.38 | 30.88 | 1.46 | 0.14 | 0.75 |
| 13 | HBHuangmei | 115.94 | 30.07 | 0.46 | 0.12 | 0.62 |
| 14 | HBHuangshi | 115.04 | 30.20 | 2.29 | 0.15 | 0.65 |
| 15 | HBJiayu | 113.93 | 29.97 | 2.65 | 0.16 | 0.66 |
| 16 | HBJianli | 112.90 | 29.81 | 1.06 | 0.12 | 0.54 |
| 17 | HBJiangxia | 114.31 | 30.35 | 4.64 | 0.13 | 0.70 |
| 18 | HBJingzhou | 112.24 | 30.34 | 1.35 | 0.12 | 0.60 |
| 19 | HBMacheng | 115.01 | 31.17 | 3.99 | 0.15 | 0.68 |
| 20 | HBSuizhou | 113.38 | 31.69 | 3.03 | 0.14 | 0.65 |
| 21 | HBTianmen | 113.17 | 30.66 | 4.51 | 0.13 | 0.67 |
| 22 | HBWuchang | 114.32 | 30.55 | 1.64 | 0.13 | 0.50 |
| 23 | HBWuchang | 114.32 | 30.55 | 2.43 | 0.13 | 0.61 |
| 24 | HBWuxue | 115.56 | 29.84 | 1.14 | 0.10 | 0.58 |
| 25 | HBXiantao | 113.46 | 30.36 | 1.78 | 0.11 | 0.65 |
| 26 | HBXiangyang | 112.21 | 32.09 | 1.46 | 0.13 | 0.56 |
| 27 | HBXiaogan | 113.92 | 30.93 | 0.31 | 0.13 | 0.44 |
| 28 | HBXinzhou | 114.80 | 30.84 | 4.22 | 0.14 | 0.65 |
| 29 | HBYangxin | 115.22 | 29.83 | 2.40 | 0.11 | 0.70 |
| 30 | HBYicheng | 112.26 | 31.72 | 0.13 | 0.14 | 0.57 |
| 31 | HBZaoyang | 112.77 | 32.13 | 0.50 | 0.14 | 0.58 |
| 32 | HBZhongxiang | 112.59 | 31.17 | 3.90 | 0.12 | 0.56 |
| 33 | JXJinxian | 116.24 | 28.38 | 1.61 | 0.13 | 0.69 |
| 34 | JXJiujiang | 116.00 | 29.71 | 3.10 | 0.14 | 0.63 |
| 35 | JXNanchang | 115.86 | 28.68 | 4.16 | 0.14 | 0.72 |

* Sclerotium size was calculated by (length + width)/2 (mm).

The preamplification step was followed by a second selective amplification step using two selective nucleotides, A plus A, T, G or C.

#### Data analysis

Data of the phenotype of the 35 isolates including density of aerial mycelia of 4 days, sclerotia quantity of 24 h, sclerotia quantity of 67 h, sclerotium size (length + width)/2 (mm) of 7 days, growth speed of colony (mm/h), PI and their location (longitude and latitude) were analyzed with SPSS 13.

For AFLP, the presence or absence of each band was determined and designated 1, present or 0, absent. The genetic similarity between individuals was estimated by using the simple matching coefficient (Hair et al., 1992; Sokal and Michener, 1958). The similarity matrix generated was then used to produce a dendrogram by the UPGMA using NTSYSpc-2.2 (Hair et al., 1992).

## RESULTS

### Morphological analysis

In morphology, rich variations were observed in the 35 isolates. In detail, 3 grades were observed in the density of aerial mycelia after culturing for 4 days from low to

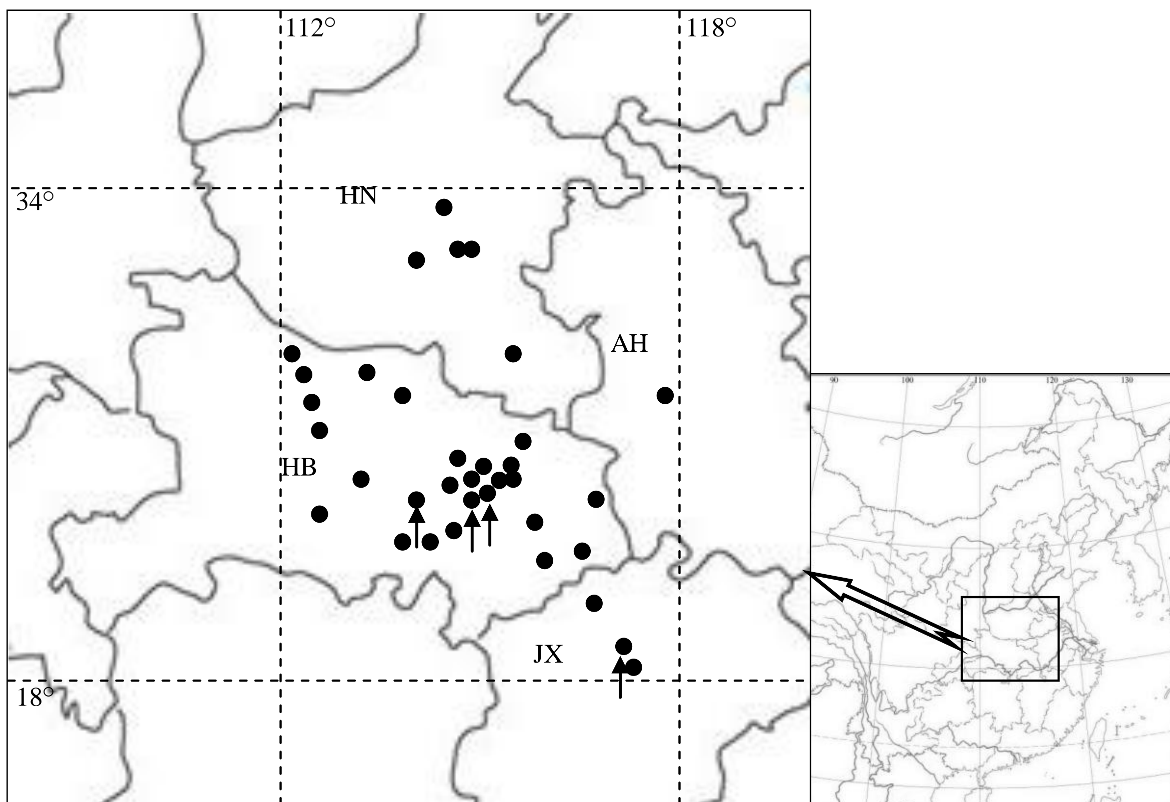


**Figure 1.** Map of China showing the collection region of *M. phaseolina* isolates. AH—Anhui, HB—Hubei, HN—Henan, JX—Jiangxi. Filled circle indicated the sites of the collection of 35 isolates. Filled arrow indicated the locations of the isolates with PI more than 4.0.

high; the sclerotia quantity of 24 and 67 h culturing showed that these isolates were different in propagating sclerotia, especially, No. 4, 5 and 27 producing no sclerotia after 24 h of culturing; the sclerotium size of 7 days culturing varied between 0.10 (No.24 from Wuxue) and 0.16 (No.15 from Jiayu) with average 0.13 mm; the growth of colony of the 35 isolates ranged in speed from 0.42 (No.3 from Luohe) to 0.75 (No.12 from Huangpi) with 0.60 mm/h in average (Table 1).

## Pathogenic variation

Five days after artificial inoculation, water-soaked spots or parts were observed in the root or stalk tissue of most seedlings, and some seedlings became soft and shrunken. The PI of these isolates, which was calculated according to the disease severity of the inoculated seedlings, ranged from 0.03 (No.1 from Hefei) to 4.64 (No.17 from Jiangxia) with an average of 2.10. There were four isolates (No. 17, 21, 28 and 35) exhibited PI more than 4.0 from the locations alongside Yangtze River, 3 of them were collected from the Southern and Eastern part of Hubei province, and 1 belonged to Jiangxi province, which also was in the South and East direction of Hubei (Figure 1). However, no clear association between the PI of the isolates and their locations was found here (Figure 2), especially, the isolates from the same location, for example, No.7 and No.8, also varied in PI.

## ITS rDNA polymorphism

Primers ITS1 and ITS 4 were used to amplify part of the nuclear rDNA operon spanning the 3’ end of the 18S rDNA gene, the first internal transcribed spacer, the 5.8S rDNA gene, the second ITS region and the 5’ end of the 28S rDNA gene. Sequences of the ITS region of the 35 *M. phaseolina* isolates collected from different geographic locations showed 100% identity to each other, and were 99% similar when compared to a *M. phaseolina* ITS sequence reported in GenBank. It indicated that all the 35 isolates were *M. phaseolina.*

## AFLP fingerprint of the isolates

The variability of the isolates at the molecular level was analyzed using AFLP. In total, sixteen primer

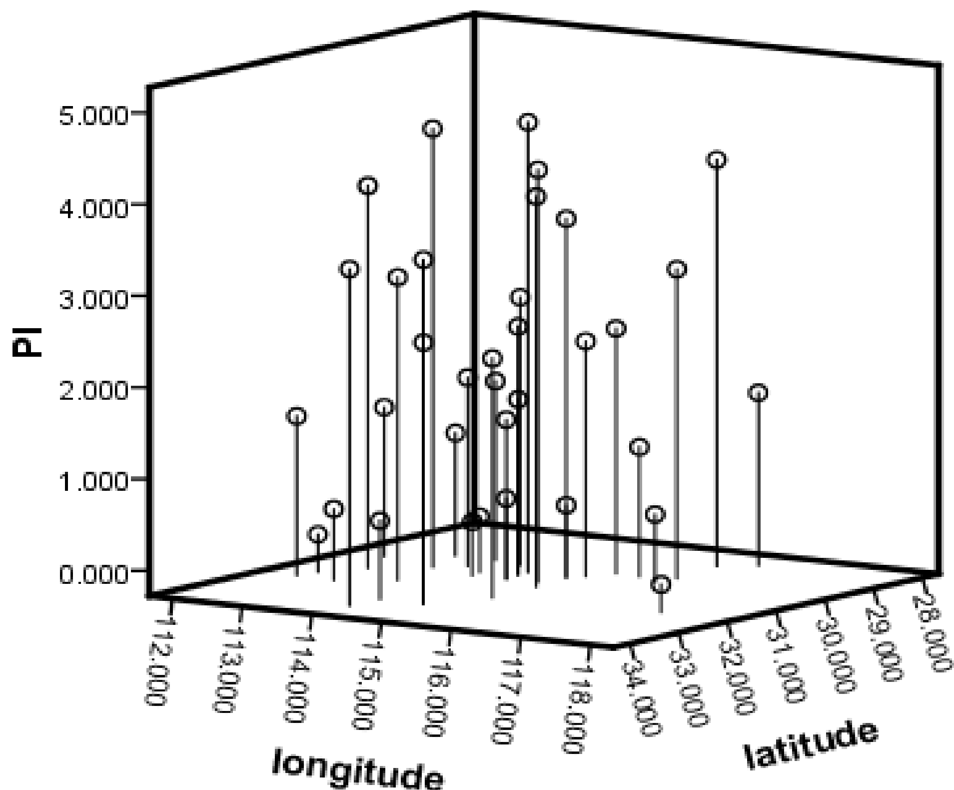


**Figure 2.** Distribution of the 35 isolates with different PI in longitudes and latitudes. Little circle indicated the 35 isolates.

combinations were used and produced a total of 402 bands, 297 (73.9%) of which were polymorphic. In general, 6 to 32 polymorphic bands were produced by the primer combinations, and seventy percent of the bands ranged from 100 to1000 nucleotides in size. The primer combinations EcoRI-AAA/MseI-AAA, EcoRI-AAT/MseI-AAT, EcoRI-AAG/MseI-AAG, and EcoRI-AAC plus MseI-AAA, MseI-AAT, MseI-AAG and MseI-AAC showed better polymorphic and produced more than 20 bands, respectively. The paired genetic similarity of these isolates based on AFLP ranged from 0.65 to 0.97 with an average of 0.83.

According to AFLP analysis, 35 isolates were divided into seven genotypic groups by UPGMA at an arbitrary level of 82.2% similarity (Figure 3). The first genotypic group contained a single isolate No.1 from Anhui province. Group 2 contained 23 isolates, consisting of 3 distinct subgroups; the first subgroup was composed of 1 isolated from Henan, 6 isolates from Hubei and all the 3 isolates from Jiangxi province; the second subgroup had 12 isolates from Hubei and 1 from Henan province; No.7 was in the third subgroup solely. The third and the fourth groups included 4 isolates respectively. No. 23, 24 and 22 represented group five, six and seven, respectively. The clustered results showed no clear correlation within the group geographic regions and PI.

## DISCUSSION

This study was the first to evaluate the morphological, pathogenic and genetic variability of *M. phaseolina* isolates from sesame in China. It showed great variation in the morphological and pathogenic traits, but no differences were detected in the ITS region. To gain further insight into the population structure and genetic diversity of the pleomorphic fungus *M. phaseolina*, AFLP was analyzed in the present study, and it revealed considerable genetic similarity (0.83 in average similarity coefficient) among the isolates from different geographic regions. These results indicated that AFLP was powerful in analysis of the variation of *M. phaseolina*, and suggested that these isolates may evolve from the same ancestral population. Similar results also were reported among the isolates from the hosts as sorghum, soybean, chickpea and corn (Almeida et al., 2003; Babu et al., 2010; Das et al., 2008).

Generally, different planting regions could characterize boundary for the fungus spreading and help build ecological types. An example here was the three isolates from Jiangxi province showing high genetic similarity and clustered together earlier. However, it was just a typical exception in these isolates, as no obvious relationships were detected among morphology, pathogenicity,

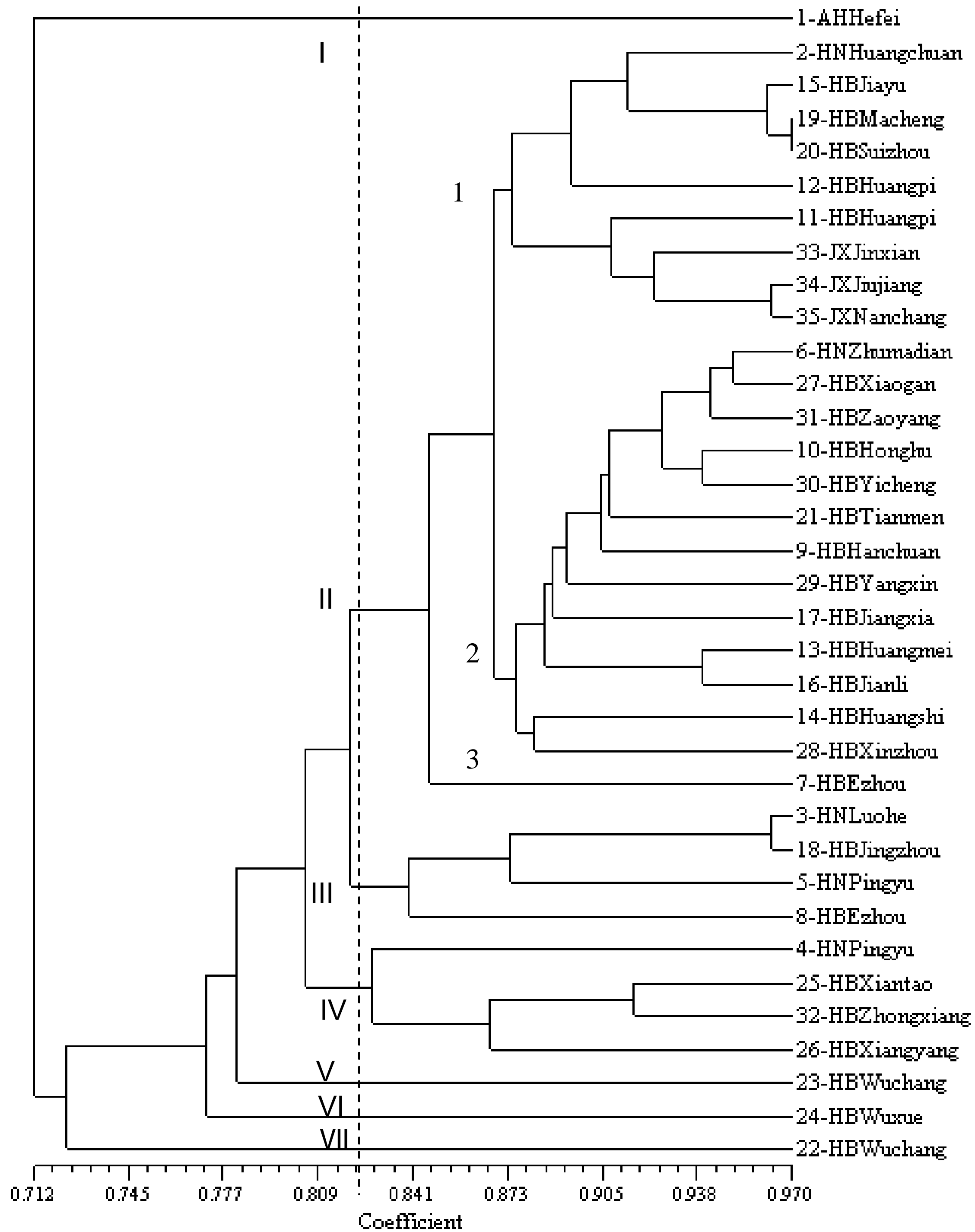


**Figure 3.** Dendrogram obtained from 35 isolates of *M. phaseolina* with UPGMA based on AFLP.

genotypic variability and the geographic regions, and it even showed no visible boundary among the regions alongside Yangtze River. The results supported the studies of Vandemark et al. (2000) and Reyes-Franco et al. (2006), but conflicted the reports of Mayék-Pérez et al. (2001), Purkayastha et al. (2008), Reyes-Franco et al. (2006) and Su et al. (2001).

Given the isolates with high PI distributed around Hubei province, the center of main sesame producing regions in China, and no obvious relationships among those isolates between their clustered groups and geographic regions, the worrying information may be noticed that

these isolates with high PI will scatter easily to larger regions including Henan, Anhui and Hunan provinces under appropriate climate conditions for their similar genetic basis. Of course, these isolates with high PI may be used to select sesame germplasm to facilitate breeding resistant cultivars.

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