Pak. J. Bot., 44(2): 479-484, 2012.

A STUDY ON THE FLUORESCENT LABELED AFLP MOLECULAR MARKERS RESEARCH OF KAVA AND *PIPER NIGRUM*

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Abstract

This research used 6 materials of kava, 23 materials of cultivated pepper and wild pepper, 1 material of *Peperomia pellucida kunth.*, 1 material of tobacco, added up to 31 test materials. The 31 germplasms were divided into 5 different groups at 0.52 of the genetic similarity coefficient by fluorescent AFLP. The result showed that Kava with *Piper* and wild *Piper* were distantly related to each other, and fluorescent AFLP is applicable to DNA fingerprint of germ line identification.

Introduction

Kava (*Piper methystium*) has the usage of treating anxiety, depression and greatly improving the sleep quality, which have significant effects for spirit stress and the side effect are small (Cropley *et al*., 2002). The raw materials required for Kava preparations at home are all comes from the South Pacific island countries, whereas the Institute of Tropical Bioscience and Biotechnology, Chinese Academy of Tropical Agricultural Science in Haikou and Zhejiang Forestry Academy in Hangzhou are researching *Piper methystium* artificial cultivation nowadays. The purpose of this study was to make clear the genetic relationship among *Piper methystium*, *Piper nigrum* and its wild relatives by the technology of fluorescent AFLP molecular marker. The amplified fragment length polymorphism (AFLP) technique (Vos *et al*., 1995) is frequently used for the identification of molecular markers because of its high efficiency, reproducibility, and reliability. At present AFLP has been widely applied in the researches on the genetic diversity and relationship of germplasm resources e.g. in tobacoo (Yang *et al*., 2006), maize (Du *et al*., 2006; Du *et al*., 2003), tea plant (Huang *et al*., 2006), poplar tree (Gao *et al*., 2006), tilapia (Yang *et al*., 2006) and so on. Available AFLP protocols include different detection methods with nonlabeled, radioactive-labeled, or fluorescent-labeled selective amplification primers. Fluorescent fragment labelling applicable on automated sequencers can add an extra advantage by providing a radioactive-free system e.g. in rice (Aggarwal *et al*., 2002), evergreen azaleas (Riek *et al*., 1999), chestnut (Zhou *et al*., 2006), *Populus cathayana* Rehd (Chen *et al*., 2010), *Ribes* spp. (Ahmet *et al.*, 2010), Polygonaceae (Yasmeen *et al.*, 2010) and so on. In order to provide references for the rootstocks selection in the grafting of *P. methysticum,* the molecular identification on its authenticity and the construction of its fingerprints, we introduced six Kava materials from the South Pacific island, other pepper materials were collected from Hainan Xinglong Tropical Botanical Garden for studying the fluorescent AFLP molecule marker of them, which enabled us to further deepen the understanding and comprehension on Kava.

Materials and methods

Test Materials: Test materials used in this experiment are listed in Table 1, in which material No. 31 is common nicotiana acted as a "roadmap" role, the others belong to *Piperaceae* plant.

Reagents: *Eco*R I, *Mse* I, T4 DNA ligase were purchased from USA Biolab Company, *Taq* enzyme was purchased from USA Gene Company, 5′-FAM fluorescently labelled *Mse*I primer was purchased from Beijing Dingguo Biotechnology Co., Ltd., the reagents for polyacrylamide gel electrophoresis were purchased from USA BBI Company and other conventional chemical reagents were analytical pure reagents made in China.

DNA extraction: About 0.2g leaves were ground into powders in liquid nitrogen (Sambrook *et al*., 2002). Adding 0.3g aluminum powder or quartz sand was helpful to grind. 500μl 2× CTAB nucleic acid extraction liquid [2% (W/V) CTAB, 1.4 mol/L NaCl, 50 mmol/L Tris (pH value of 8.0), 20 mmol/L EDTA-Na_2 (pH value of 8.0)] that had been preheated up to 60-65°C were added and blended mildly. Then 50μl 1% $Na_2S_2O_5$ and 25μl β- mercaptoethanol (with the final concentration up to 1%) were added to place in warm bath at 65°C for 30 min. During the process, the bottom of tube was flicked to make the solution mixed fully. After the solution was cooled to room temperature, 50μl 20% polyvinylpyrrolidone (PVP) was added up to the final concentration of 1%. The total volume was observed by visual measurement and the same volume of the solution of chloroform / isoamyl alcohol (24:1) was added. The solution must be wobbled gently, otherwise it easily made DNA ruptured and degraded. The solution was mixed, centrifugation with the centrifugal force of 2,000g was made for 10 min at room temperature. The temperature in the process of centrifugation should not lower than 15°C, very low temperatures might cause CTAB precipitation and lose DNA. The supernatant liquid was taken to put into another centrifugal tube to add the same volume of

the solution of chloroform/isoamyl alcohol and make centrifugation with the centrifugal force of 12,000g. The supernatant liquid was taken to add the same volume of isopropanol and 1/10 volume of 3 mol/L CH_3COONa solution or twice volume of ice-precooled anhydrous ethanol and freeze for 30 min. Then centrifugation with the centrifugal force of 12,000g was conducted for 5 min at 4°C. After discarding the supernatant liquid, the precipitate was washed with 75% ethanol twice. Vacuum pumping was made for 2-3 min. The extracted DNA was dissolved in 20μl TE solution and preserved for use at -20°C.

Table 1. Test materials.

S. No.	Name of materials	Sampling location	Explanation
1.	*Piper hancei* Maxim	Hainan Bawangling	*Piperaceae*
2.	*Peperomia pellucida* kunth.	South China University of Tropical Agriculture in Danzhou of Hainan	*Peperomia* (genus name)
3.	*Piper betle* L.	South China University of Tropical Agriculture in Danzhou of Hainan	Originally planted in Indonesia，*Piper* L.
4.	*Piper sarmentosum* Roxb.	Haikou school region of South China University of Tropical Agriculture	*Piper* L.
5.	*Piper methysticum* Forst.f. No. 1 (local name: Kava)	Fiji	*Piper* L., green stems
6.	*Piper methysticum* Forst.f. No. 2 (local name: Kava)	Fiji	*Piper* L., green stems
7.	*Piper methysticum* Forst.f. No. 3 (local name: Kava)	Fiji	*Piper* L., green stems and swelling internodes
8.	*Piper methysticum* Forst.f. No. 4 (local name: Kava)	Fiji	*Piper* L., green stems and swelling internodes
9.	*Piper methysticum* Forst.f. No. 5 (local name: Kava)	Fiji	*Piper* L., red stems
10.	*Piper methysticum* Forst.f. No. 6 (local name: Kava)	Fiji	*Piper* L., red stems
11.	Lampong type (*Piper nigrum* L.)	Hainan Xinglong Tropical Botanical Garden	*Piper* L.
12.	Banniyueer-1(*Piper nigrum* L.)	Hainan Xinglong Tropical Botanical Garden	*Piper* L., original name of 'Panniyur-1'
13.	Kuching (*Piper nigrum* L.)	Hainan Xinglong Tropical Botanical Garden	*Piper* L., original name of 'Kuching'
14.	Dashan (*Piper* sp.)	Hainan Xinglong Tropical Botanical Garden	*Piper* L.
15.	*Piper hancei* (*Piper* sp.)	Hainan Xinglong Tropical Botanical Garden	*Piper* L.
16.	Hybrid 1 (*Piper nigrum* L.)	Hainan Xinglong Tropical Botanical Garden	*Piper* L., 'Banniyueer-1' × 'Lampong Type'
17.	Hybrid 3 (*Piper nigrum* L.)	Hainan Xinglong Tropical Botanical Garden	*Piper* L., 'Banniyueer-1' × 'Lampong Type'
18.	Hybrid 5 (*Piper nigrum* L.)	Hainan Xinglong Tropical Botanical Garden	*Piper* L., 'Banniyueer-1' × 'Lampong Type'
19.	Hybrid 6 (*Piper nigrum* L.)	Hainan Xinglong Tropical Botanical Garden	*Piper* L., 'Banniyueer-1' × 'Lampong Type'
20.	Hybrid 7 (*Piper nigrum* L.)	Hainan Xinglong Tropical Botanical Garden	*Piper* L., 'Banniyueer-1' × '293'
21.	Hybrid 8 (*Piper nigrum* L.)	Hainan Xinglong Tropical Botanical Garden	*Piper* L., 'Banniyueer-1' × 'Sheng 20'
22.	Yinjian 45 (*Piper nigrum* L.)	Hainan Xinglong Tropical Botanical Garden	*Piper* L., 'Lampong Type' × 'Cambodia'
23.	Ban 293 (*Piper nigrum* L.)	Hainan Xinglong Tropical Botanical Garden	*Piper* L., 'Lampong Type' × ' Banniyueer-1'
24.	Banjianni (*Piper nigrum* L.)	Hainan Xinglong Tropical Botanical Garden	*Piper* L., 'Banniyueer-1'×'Cambodia'×' Lampong Type'
25.	Banyunda (*Piper nigrum* L.)	Hainan Xinglong Tropical Botanical Garden	*Piper* L., 'Banniyueer-1' × 'Yuanxuan 1' × 'Dashan'
26.	Yuanxuan 1(*Piper nigrum* L.)	Hainan Xinglong Tropical Botanical Garden	*Piper* L.
27.	Dashan (*Piper* sp.) × Yinni (*Piper nigrum* L.)	Hainan Xinglong Tropical Botanical Garden	*Piper* L.
28.	Jianyin 93 (*Piper nigrum* L.)	Hainan Xinglong Tropical Botanical Garden	*Piper* L., ' Cambodia' × 'Lampong Type'
29	Banjianyin 20 (*Piper nigrum* L.)	Hainan Xinglong Tropical Botanical Garden	
30	Banjianyin 43 (*Piper nigrum* L.)	Hainan Xinglong Tropical Botanical Garden	
31	Tobacoo (*Nicotiana tabacum*)	State Key Biotechnology Laboratory for Tropical Crops	

Note: "×"indicates "hybrid".

Linker preparations of *Eco*RI and *Mse*I: Linker 1 of *Eco*RI was 5′-CTCGTA GAC TGC GTA CC-3′and linker 2 of *Eco*RI was 5′- AAT TGG TACGCA GTC TAC-3′. Linker 1 of *Mse*I was 5′- GAC GAT GAG TCC TGAG-3′and linker 2 of *Mse*I was 5′- TAC TCA GGA CTC AT-3′. The designed linkers were synthesized by Shanghai Sangon Company. Linker 2 of *Eco*RI and linker 2 of *Mse*I were phosphorylated at the 5′end at the same time of synthesis. After synthesizing, linker 1, 2 of *Eco*RI and linker 1, 2 of *Mse*I were annealed to form *Eco*RI linker and *Mse*I linker (Sambrook *et al*., 2002). The primer sequence of *Eco*RI pre-amplification (Eo) was 5′- GAC TGC GTA CCA ATT CA- 3′ and the primer sequence of *Mse*I pre-amplification (Mo) was 5′-GAT GAG TCC TGA GTA AC-3′. The above primers were also synthesized by Shanghai Sangon Company. After synthesis, the primers were calculated and added to the sterilized double-distilled water till the final concentration of primer was 10 pmol/µl and preserved for use at -20°C.

AFLP primers: At the time of screening AFLP primers, PCR reaction system should be optimized to screen out the optimum primer combination for preventing too much or too little bands on AFLP fingerprinting. The primers with higher polymorphism, better quality of band-type and higher resolving power were selected as AFLP primers from 64 pairs of AFLP primers listed in Table 2 with 3 repetitions to ensure its stability.

Table 2. AFLP *Eco*RI/ *Mse*I primer combinations.

S. No.	Serial number of primers							
	1′	2′	3′	4′	5′	6′	7′	8′
1.	AAC/CAA	AAC/CAC	AAC/CAG	AAC/CAT	AAC/CTA	AAC/CTC	AAC/CTG	AAC/CTT
2.	AAG/CAA	AAG/CAC	AAG/CAG	AAG/CAT	AAG/CTA	AAG/CTC	AAG/CTG	AAG/CTT
3.	ACA/CAA	ACA/CAC	ACA/CAG	ACA/CAT	ACA/CTA	ACA/CTC	ACA/CTG	ACA/CTT
4.	ACT/CAA	ACT/CAC	ACT/CAG	ACT/CAT	ACT/CTA	ACT/CTC	ACT/CTG	ACT/CTT
5.	ACC/CAA	ACC/CAC	ACC/CAG	ACC/CAT	ACC/CTA	ACC/CTC	ACC/CTG	ACC/CTT
6.	ACG/CAA	ACG/CAC	ACG/CAG	ACG/CAT	ACG/CTA	ACG/CTC	ACG/CTG	ACG/CTT
7.	AGC/CAA	AGC/CAC	AGC/CAG	AGC/CAT	AGC/CTA	AGC/CTC	AGC/CTG	AGC/CTT
8.	AGG/CAA	AGG/CAC	AGG/CAG	AGG/CAT	AGG/CTA	AGG/CTC	AGG/CTG	AGG/CTT

Preamplification: The preamplification reaction system were as follows: 5.0µl samples that had completed linkers, 1.0µl E_O (10 pmol/µl), 1.0µl M_O (10 pmol/µl), 5.0µl 10× PCR buffer, 5.0µl $MgCl_2$ (25 mmol/L), 4.0µl dNTP (2.5 mmol/L), 0.5µl 10 mg/ml BSA, 1.0µl *Taq* enzyme (5 U/µl), 27.5µl double distilled water, the whole volume was 50.0µl. The preamplification reaction conditions were as follows: 94°C 2 min; 94°C 30 s, 56°C 60 s, 72°C 60 s, 35 cycles; 72°C 5 min.

Fluorescent Selective amplification: The pre-amplification products diluted 20 times were taken as the selective amplication templates. The selective amplification reaction system were as follow: 3.0µl diluted samples for preamplification, 2.5µl 10× PCR buffer solution, 1.5µl $MgCl_2$ (25 mmol/L), 2.0µl dNTP (2.5 mmol/L), 0.5µl *Eco*RI primer (10 pmol/µl), 0.5µl *Mse*I primer (10 pmol/µl, 5′-FAM fluorescently labelled), 0.5µl *Taq* enzyme (5 U/µl), 14.5µl double distilled water, the total volume was 25.0µl. The temperature parameters for the selective amplification were as follows: 10 cycles of 94°C 40 s, 65°C 40 s, 72°C 1 min, and the annealing temperature decreased 1°C in each cycle; 30 cycles of 94°C 40 s, 55°C 40 s, 72°C 1 min; 72°C 5min.

Electrophoresis detection: Before a run on the ABI 377, the PCR amplification product was mixed with formamide loading solution (98% formamide, 10 mmol/L EDTA, 0.25% bromophenol blue) by the portion of 8:3 to denature for 8 min at 95°C and then it was put into ice bath. 5% denatured gel was prepared with the thickness of the denatured gel being 0.4mm. The preparation of 100ml 5% denaturing polyacrylamide gel electrophoresis was: 42.042g urea, 12.5ml 40% PA, 20 ml 5× TBE, finally the gel volume was diluted to 100ml. Before use, 200µl 1% ammonium persulfate and 40µl TEMED were added to 50ml 5% denatured polyacrylamide gel, the gel was poured rapidly after mixing gently and the gel was solidified for 2 h after inserting the comb. Pre-electrophoresis was made for 30 min with the constant power of 80 W. The loading groove was flush out, the comb was inserted and 5µl samples were loaded. Electrophoresis was made with the content power of 40W till bromophenol blue approached the bottom of the gel. The electrophoresis was stopped after about 4-5 h. AFLP polymorphism analysis was made by fluorescent AFLP with ABI377 sequencing machine (Perkin Elmer Applied Bio-system) and the results were taken photo for preservation.

Clustering analysis: When the data was analyzed by clustering, AFLP bands of each sample were counted. Only clear and steady bands were calculated, the markers with band were 1 and the markers without band were 0. According to the method of Nei *et al.,* (1979), the similarity coefficients among different materials were calculated and the similarity coefficient between any two materials were calculated by using formula of $S_{xy} = 2N_{xy}/(N_x+N_y)$, in which S_{xy} was the similarity coefficient between two materials, N_x and N_y were the site numbers that were amplified by material x and material y respectively and N_{xy} was the shared site number of material x and material y. Clustering analysis was made by using UPGMA method with MVSP3.13f software.

Results

Screening of AFLP Primers: 17 pairs of primers provided with higher polymorphism, better banding quality and higher resolution were screened from 64 pairs

of fluorescent AFLP primers. The number primers were as follow: 1-2′、1-3′、1-4′、1-5′、2-3′、2-4′、3-5′、3-6′、4-4′、4-6′、5-1′、5-7′、6-2′、7-4′、8-6′、8-7′、8-8′. Table 3 shows that 4 pairs of primers amplified bands on 317 loci in 31 materials, the amplified loci of each pair of primers was 79 and 2-3′primer had the most amplification loci (110). The total polymorphism site number amplified by 4 pairs of primers was 310, the average amplified site number of each pair of primer was 77.5 and the average proportion of polymorphism loci to total amplified loci was 97.28%. The identification rates of 4 pairs of primers on 31 materials all reached 100%. The bands amplified by 4 pairs of primers had consistent signal intensity, even distribution and good reproducibility. Figure 1 shows the fingerprint of fluorescent AFLP using 2-3′primers.

Table 3. Amplification of 4 pairs of primers on 31 materials screened by sliver-staining AFLP.

Serial No. of primers (E-M)	Selective bases (E-M)	Amplification loci (No.)	Polymorphism loci (No.)	Proportion of polymorphism loci (%)	Identification rate (%)
2-3′	AAG-CAG	110	109	99.09	100
2-4′	AAG-CAT	104	102	98.08	100
5-7′	ACC-CTG	61	59	96.72	100
8-7′	AGG-CTG	42	40	95.24	100

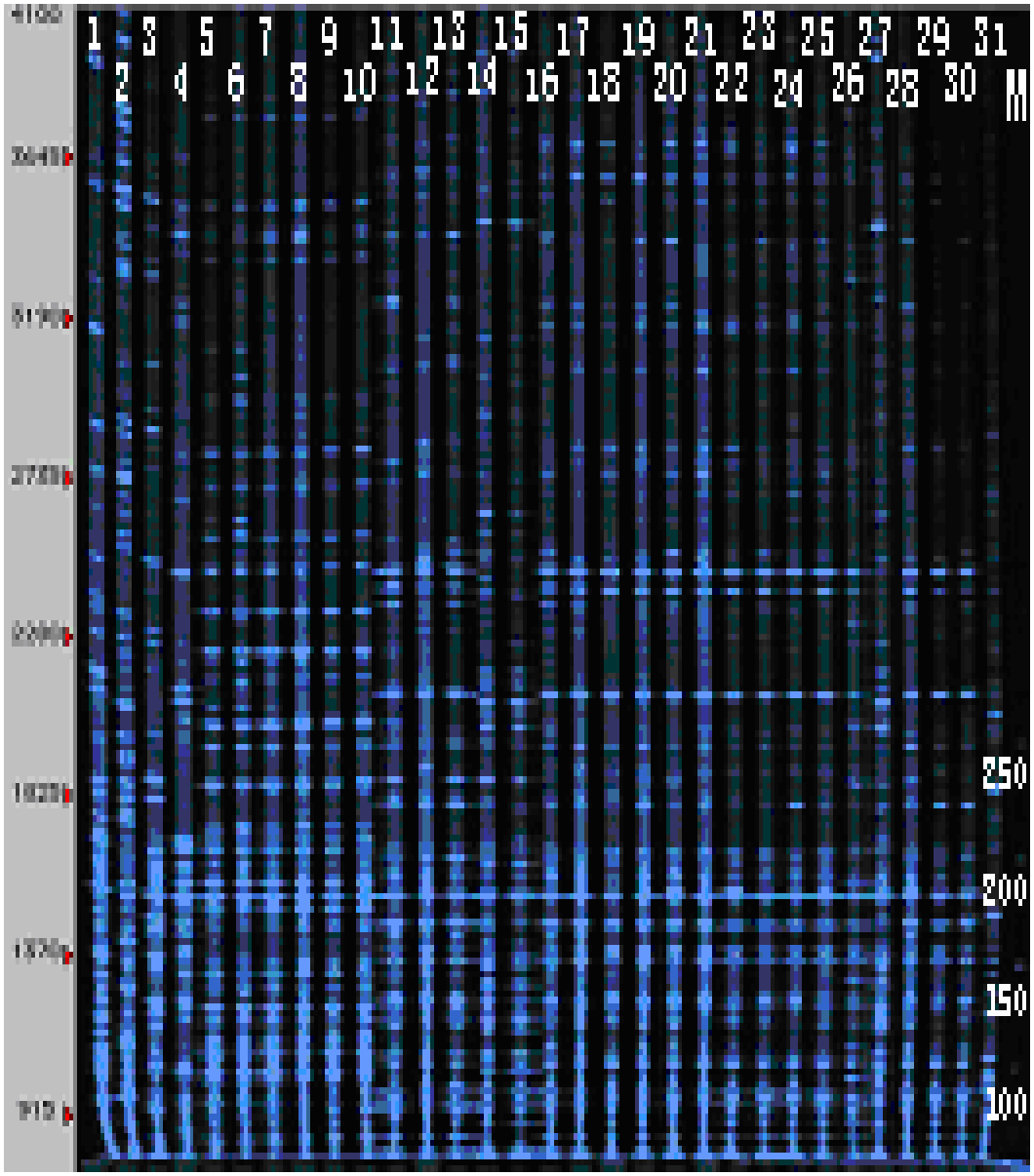

Fig.1.The fingerprint of fluorescent AFLP(2-3′).

AFLP clustering: Thirty one tested materials were divided into 2 clusters at the point where the similarity coefficient was 0.2 (Fig. 2). The 1st group was *Nicotiana tabacum* which belongs to *Solanaceae* and the 2nd group was the rest 30 materials of *Piperaceae*, which illustrated that the differences between the different families was greater than the differences between the different genus within a family. 31 tested materials were divided into 3 clusters at the point where the similarity coefficient was 0.36. The 1st group was *Nicotiana tabacum,* the 2nd group was *Peperomia pellucida* kunth. and the 3rd group was *P. methysticum*, which indicated that the intergeneric difference was greater than the intragenus difference and the differences between *P. methysticum and P. nigrum*, *P. methysticum* and other relative wild pepper species were greater. 31 materials were divided into 5 clusters at the point where the similarity coefficient was 0.52. The 1st group was *Nicotiana tabacum*, the 2nd group was *Peperomia pellucida* kunth., the 3rd group was *P. methysticum,* the 4th group was common *Piper* species and the 5th group included *Piper hancei* Maxim, *Piper betle* L. and *Piper sarmentosum* Roxb., which further indicated that the differences between *P. methysticum and P. nigrum*, *P. methysticum* and other relative wild pepper species were greater. 31 materials were divided into 7 clusters at the point where the similarity coefficient was 0.68. The 1st group was *Nicotiana tabacum*, the 2nd group was *Peperomia pellucida* kunth., the 3rd group was *P. methysticum*, the 4th group was common *Piper* species, the 5th group was *Piper sarmentosum* Roxb., the 6th group was *Piper betle* L. and the 7th group was *Piper hancei* Maxim, which reflected that *Piper sarmentosum* Roxb., *Piper betle* L. and *Piper hancei* Maxim had difference. At the point where the similarity coefficient was 0.84, spicebush and betel were only divided into different types, which reflected that different geographic populations in Pepper species had difference. At the point where the similarity coefficient was 0.99, 6 plants of *P. methysticum* were divided into 5 groups by using fluorescent AFLP technology. The 1st group was Kava 1 with green stems, the 2nd group was Kava 2 with green stems, the 3rd group was Kava 3 with green stems and slightly swelling internode, the 4th group was Kava 4 with green stems and slightly swelling internode, the 5th group included Kava 5 and Kava 6 with red stems, which showed that the resolving power of fluorescent AFLP was higher than silver staining AFLP (Shi *et al*., 2009). The similarity coefficients between Kava 5 and Kava 6 were 1 and couldn't be distinguished, which might be caused that they came from the same plant of material *P. methysticum*.

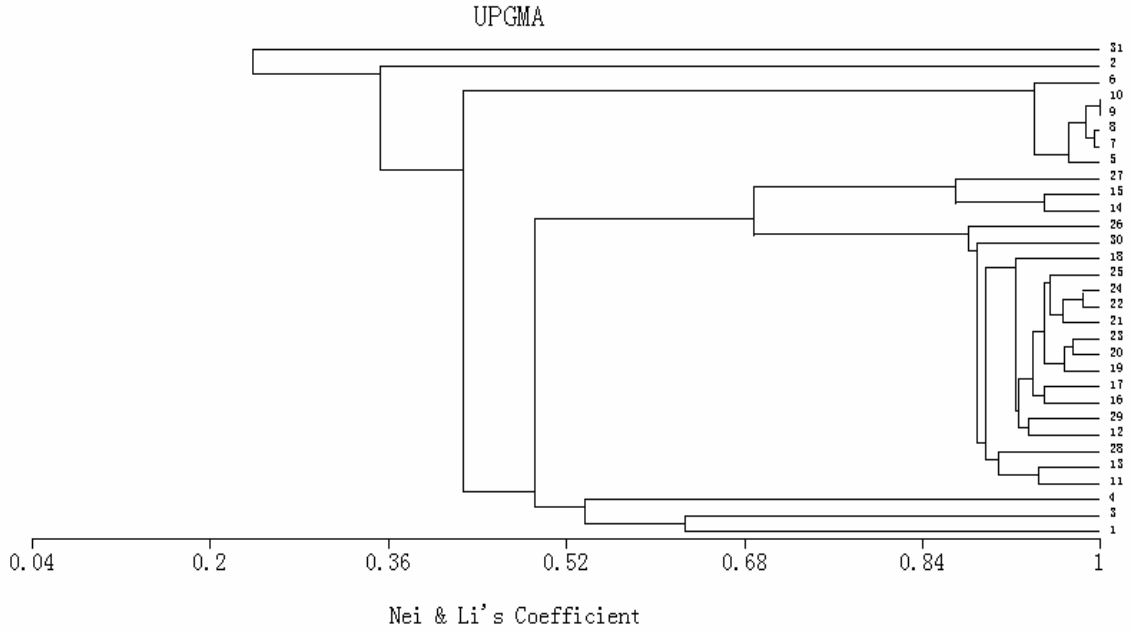

Fig.2.Fluorescent AFLP clustering result of 31 materials.

Discussion

The results showed that the band type, amount and distribution uniformity degree of the bands amplified by different primers had greater differences and their distinguishing capabilities were also different. So screening the primers was indispensable. Sixty-four pairs of AFLP primers that were used in the test almost represented all kinds of types. Four pairs of primers screened in the test had high proportion of polymorphism bands and high capability of distinguishing 31 materials, which were the high-efficient primers in AFLP detection at present and had higher application values.

As for fluorescent AFLP technical operations, 3 steps of enzyme digestion, connection and amplification are all very important. Double enzyme digestion must be completed thoroughly. So the test had higher demands for the purity of DNA and the quality of endonuclease, otherwise, the phenomenon of unstable amplification band-type would appear. For obtaining better enzyme digestion effect, the quality of template DNA should be ensured and the reaction conditions of the enzyme digestion should be optimized. In the test, template DNA had great fragment without degradation and RNA pollution, which accorded with the demands on DNA in AFLP technical operations. Amplification was a PCR process and the factors that

affected PCR would affect AFLP amplification effect. Amplification can be divided into pre-amplification and selective amplification and pre-amplification was the necessary means that verified the effects of enzyme digestion and connection. Only the DNA fragment that had broader range of pre-amplification fragment (50-1,500 bp), greater amplification amount and better coherence among samples could be taken as template DNA for AFLP selective amplification. *Taq* enzyme with high quality should be selected in selective amplification. Fluorescent AFLP was more efficient and sensitive in comparison with silver staining AFLP, and silver staining AFLP is suitable for sensitivity detection of large fragments (200-600 bp), whereas the fluorescent AFLP has the higher sensitivity of detection of small fragments (50-450 bp) (Shi *et al*., 2009).

One of the biggest advantages for fluorescent AFLP marker technology was that large numbers of loci were detected by a small quantity of selective primer in shorter time. Usually in fluorescent AFLP analysis, each amplification band was correspondant with one locus in the molecule of genomic DNA. In 31 materials 317 DNA bands with different molecular weights were amplified with 4 pairs of primers by using sliver-staining AFLP, which meant that 317 loci in the genomes of 31 materials were detected by these four pairs of primers. Appearance of polymorphism amplification bands indicated that certain material or some materials existed variations on these loci. In detecting with 4 pairs of primers by using fluorescent AFLP, 310 polymorphism loci were found, occupying 97.28%, which indicated that on the detected loci there were 97.28% materials existed variations. The precision and efficiency of this detection was better than any former fingerprinting technology. So it was thought that fluorescent AFLP technology was a kind of technology with the highest detection efficiency at present.

The research on the molecular markers of *P. methysticum, Piper nigrum* and their wild relatives showed that *P. methysticum* was confirmed as the genus *Piper*, which was in accordance with the results of Jaramillo *et al*., (2001) who suggested that the genus *Piper* should be divided into 3 big clades including Asian clade, south pacific clade and neotropical clade and *P. methysticum* belonged to south pacific clade. We obtained the same conclusion on this point. Though *P. methysticum* belonged to the genus *Piper*, it had farer genetic relationship with *Piper nigrum* and it wild relatives and had certain distance.

Acknowledgement

We thank the State Key Biotechnology Laboratory for Tropical Crops for support. Funds were provided by the "15" national key scientific and technological project of China (2001BA707B02).

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(Received for publication 20 January 2010)