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ANALYSIS OF THE GENETIC RELATIONSHIP BETWEEN *PIPER METHYSTICUM* AND PEPPER BY AFLP

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Abstract

The aim of the research was to discuss the genetic relationships among *Piper methysticum*, Pepper and it wild relatives. With 28 germplasms including 6 materials of *P. methysticum*, 21 materials of cultivated and wild pepper, 1 material of *Peperomia pellucida* belonged to different genus as tested materials, DNA was extracted from leaves and primers with good band-type and high polymorphism and resolving power were screened from 64 pairs of primers for AFLP amplification and the clustering analysis was conducted with MVSP3.13f software. 191 bands were amplified by 4 pairs of primers, 189 of which had polymorphism being 98.6%. 28 germplasms were clustered into 6 different groups at the genetic similarity coefficient of 0.52 by silver staining AFLP, in which 6 materials of *Piper methysticum* were clustered into a single group, indicating that *P. methysticum* belonged to *Pipper* L., *Piperaceae* and were distantly related to the others. The research provided the basis for selecting rootstocks, molecular identification and the fingerprint construction of *P. methysticum*.

Introduction

The common name of *Piper Methysticum* Forst. f. is called Kava or Kawa, a perennial shrub plant of the genus *Piper* (*Piperaceae*). It is an outstanding pharmaceutic species which naturally grows in Vanuatu, Fiji, Tonga, Papua New Guinea, Soloman Island etc. The beverage making from Kava root and stem has been used by Pacific Islanders for relaxing, recovering strength of the body and improving sleeping. Pacific Islanders consume Kava drink in social, ritual and ceremonial activities. Kava banquet is specially arranged in the ceremonies to welcome honored guests and also is an indispensible drink in daily-life. The Pacific elixir-Kava famed by local people has 3000 years history for using and the unique "Kava Culture" has been formed in the local islands. Kava has a significant effect to treat anxiety, depression and has the function for promoting sleep, improving sleeping quality (Mullar, *et al*., 1999; Wheatley, 2001; Smith, 2001). The pharmacological poisonous action and side effects of Kava have not been found significantly in the optimum dose condition (Pittler, 2000; Cropley, *et al*., 2002).The growing of Kava as a cash crop in South Pacific island countries which is considered as a national treasure from natural resources. Scientific observation of the use of Kava dates to British explorer James Cook's exploration, a voyage to the Pacific Ocean in 1769, the first time from the perspective of taxonomic description of the plant (Forster, 1786). Lewin (1886) was the first scientist to write a monograph on Kava and believed that he was the first person made a detailed study of kava from the perspective of botany. Steinmetz (1960) wrote a book named "Kava Kava (*Piper methysticum*): Famous drug plant of the South Sea Islands", which made kava familiar to the outside world. Vincent Lebot *et al*., (1992) published the latest monograph

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on Kava and a large number of references were listed in the book, which enabled us to further deepen the understanding and comprehensive on Kava. *Piper methysticum* belongs to Pepper plants, but the genetic relationship between *Piper methysticum* and Pepper is not very clear. The purpose of this study was to make clear the genetic relationship between *Piper methysticum* and Pepper by AFLP analysis.

Amplified fragment length polymorphism (AFLP) is a new molecular marker technology which can amplify genomic DNA restricted fragments based on PCR technology. Genomic DNA was cut by restriction endonuclease, the linkers of double-strand was connected with the end of DNA fragment and the linker sequences and adjacent restriction site sequence were taken as the linked locations of primer. Compared with random amplified polymorphic DNA (RAPD), AFLP has better stability and stronger repeatability. AFLP not only has the advantages of RFLP such as stable results and good repeatability, but it also has the advantages of RAPD such as simple to operate and high detection efficiency of polymorphism. While AFLP also has some drawbacks including that the kits are expensive, it demands high experimental conditions, it is strict to the DNA quality of samples and the experimental results could be affected by incomplete digestion of the genome. So AFLP has higher demands for the purity of DNA and the quality of endonuclease. AFLP has been widely applied in the researches on the genetic diversity and relationship of germplasm resources such as tobacco (Yang *et al*., 2006), maize (Du *et al*., 2006), poplar tree (Gao *et al.*, 2006), tea plant (Huang *et al*., 2006) and tilapia (Yang *et al*., 2006). But the researches on AFLP molecular marker of *Piper methysticum* and its close relative species have not been reported (Shi *et al*., 2007). The genetic relationships among *Piper methysticum*, *Piper nigrum* and its wild relatives were discussed by AFLP molecular marker technology 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.

Materials and Methods

Test materials: Test materials are listed in Table 1.

Reagents: *Eco*RI *Mse*I T_4 DNA ligase were purchased from USA Biolab Company, *Taq* enzyme was purchased from USA Gene Company, 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 (Shi *et al*., 2005): About 0.2 g leaves were ground into powders in liquid nitrogen. Adding 0.3 g aluminum powder or quartz sand was helpful to grind. 500 µl 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 60-65ºC were added and blended mildly. Then 50 µl 1% $Na_2S_2O_5$ and 25 µl β-mercaptoethanol (with the final concentration up to 1%) were added to place in warm bath at 65ºC 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, 50 µl 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 was added. The solution must be wobbled gently, otherwise it easily made DNA ruptured and degraded. The solution was mixed fully. After

mixing fully, centrifugation with the centrifugal force of 2,000 g was made for 10 min at room temperature. The temperature in the process of centrifugation should not lower than 15ºC, too 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 12,000 g. The supernatant liquid was taken to add the same volume of isopropanol and 1/ 10 volume of 3 mol/L NaAc solution or twice volume of ice-precooled anhydrous ethanol and freeze for 30 min. Then centrifugation with the centrifugal force of 12,000 g was conducted for 5 min., at 4ºC. 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 20 µl TE solution and preserved for use at –20ºC.

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 (Joseph 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 –20ºC.

AFLP enzyme digestion system and conditions: 32.5 µl water, 5.0 µl 10× NE buffer solution 2, 10.0 µl DNA (100 ng/µl), 0.5 µl 100× BSA, 1.0 µl *Eco*RI (10 U/µl), 1.0 µl *Mse*I (10 U/µl), the total volume was 50.0 µl, the enzyme digestion was made for 3-4 h at 37ºC and the enzyme digestion at 70ºC for 15 min., made the enzyme inactivated.

Connection of the enzyme digestion sample for silver-staining AFLP: 10.0 µl enzyme digestion sample, 4.8µl water, 1.0 µl *Eco*RI linker (20 pmol/µl), 1.0 µl *Mse*I linker (50 pmol/µl), 0.2 µl 100× BSA, 2 µl 10× buffer solution, 1.0 µl T_4 DNA ligase (3 U/µl). The total volume was 20 µl. Reactions at 16ºC for 20 h and at 70ºC for 10 min., made the ligase inactivated and the linkers were preserved for use at –20ºC.

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.

Preamplification: The preamplification reaction system were as follows: 5.0 µl samples that had completed linkers, 1.0 µl E_O (10 pmol/µl), 1.0 µl M_O (10 pmol/µl), 5.0 µl 10× PCR buffer, 5.0 µl $MgCl_2$ (25 mmol/L), 4. 0 µl dNTP (2.5 mmol / L), 0.5 µl 10 mg/ml BSA, 1.0 µl *Taq* enzyme (5 U/µl), 27.5 µl double distilled water, the whole volume was 50.0 µl. The preamplification reaction conditions were as follows: 94ºC 2 min; 94ºC 30 s, 56ºC 60 s, 72ºC 60 s, 35 cycles; 72ºC 5 min.

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

S. no. primers	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

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

Electrophoresis detection: 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 95ºC and then it was put into ice bath. 5% denatured gel was prepared with the thickness of the denatured gel being 0.4 mm. The preparation of 100 ml 5% denaturing polyacrylamide gel electrophoresis was: 42.042 g urea, 12.5 ml 40% PA, 20 ml 5× TBE, finally the gel volume was diluted to 100 ml. Before use, 200 μl 1% ammonium persulfate and 40 μl TEMED were added to 50 ml 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 5 μl samples were loaded. Electrophoresis was made with the content power of 40 W till bromophenol blue approached the bottom of the gel. The electrophoresis was stopped after about 4-5 h. AFLP polymorphism analysis was made by fluorescence AFLP with ABI377 sequencing machine. Sliver staining was made on the gel by sliver-staining AFLP to observe the results and the results were taken photo for preservation.

Silver staining flow: The gel was fixed for 30 min by 10% acetic acid and rinsed with distilled water for 2-3 times with 3 min per time. 0.1% silver nitrate and 0.56% formaldehyde were prepared in 10 min before use (through adding 1 g $AgNO_3$ and 1.5 ml 37% formaldehyde to 1 L water). The gel was stained for 30 min and rinsed gently with distilled water for 5 s. 0.28 mol/L Sodium carbonate, 0.56% formaldehyde and 200 μl 10 mg/ml Sodium thiosulfate prepared in 5 h before use (through adding 30 g Na_2CO_3 to 1 L water for cooling to 4ºC and adding 1.5 ml 37% formaldehyde and 200 μl 10 mg/ml sodium thiosulfate) were used to make low temperature color-reaction for 5～10 min. When the contrast ratio of band to background was clearest, the color reaction was stopped with 10% acetic acid and the gel was rinsed with distilled water for 2 min.

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: Table 3 shows that 4 pairs of primers amplified bands on 191 loci in 28 materials, the amplified loci of each pair of primer was 48 and 1-1′ primer had the most amplification loci (85). The total polymorphism site number amplified by 4 pairs of primers was 189, the average amplified site number of each pair of primer was 47 and the average proportion of polymorphism loci to total amplified loci was 98.6%. The identification rates of 4 pairs of primers on 28 materials all reached 100%. The bands amplified by 4 pairs of primers had consistent signal intensity, even distribution and good reproducibility.

AFLP clustering: Fig. 1 shows that 28 tested materials were divided into 2 clusters at the point where the similarity coefficient was 0.2. Material No. 2 *Peperomia pellucida* Kunth. belonged to the fist group, which belonged to *Peperomia* plant of *Piperaceae*. And 27 shares of materials in *Piper* L. of *Piperaceae* belonged to the second group. This meant that different genus of materials could be distinguished at the similarity coefficient of near 0.2, which showed that the intergeneric difference was greater than the intragenus difference. Twenty-eight materials were divided into 3 clusters at the point where the similarity coefficient was 0.36. The 1st group was *P. pellucida*, the 2nd group was *P. methysticum* and the 3rd group was 21 pepper plant materials except *P. methysticum*, which indicated that the differences between *P. methysticum and P. nigrum*, *P. methysticum* and other relative wild pepper species were greater. Twenty-eight materials were divided into 6 clusters at the point where the similarity coefficient was 0.68. The 1st group included *Piper hancei* Maxim and *Piper betle* L., the 2nd group included *Piper sarmentosum* Roxb., the 3rd group was *P. methysticum*, the 4th group was *P. pellucida*, the 5th group was common *Piper* species and the 6th group was Dashan, *Piper hancei* and ‘Dashan’ × ‘Yinni’, which reflected that different geographic populations in Pepper species had difference. At the point where the similarity coefficient was 0.84, spicebush and betel were only divided into different types. 6 plants of *P. methysticum* were divided into 3 groups by using sliver-staining AFLP technology. The 1st group included Kava 1 and Kava 2 with green stems, the 2nd group included Kava 3 and Kava 4 with green stems and slightly swelling internode, the 3rd group included Kava 5 and Kava 6 with red stems. The clustering results of 6 plants of *P. methysticum* by AFLP were in accordance with the morphological clustering results on the appearance of *P. methysticum*, which also indicated the reliability and stability of AFLP results. But the similarity coefficients between Kava 1 and Kava 2, Kava 3 and Kava 4, Kava 5 and Kava 6 within 3 groups were all 1 and couldn’t be distinguished.

Table 3. Amplification of 4 pairs of primers on 28 materials screened by sliver-staining AFLP.

Serial number of primers (E-M)	Selective bases (E-M)	Amplification loci (No.)	Polymorphism loci (No.)	The proportion of polymorphism loci (%)	The identification rate (%)
1-1′	AAC-CAA	85	85	100	100
1-2′	AAC-CAC	36	34	94.4	100
1-3′	AAC-CAG	27	27	100	100
1-4′	AAC-CAT	43	43	100	100

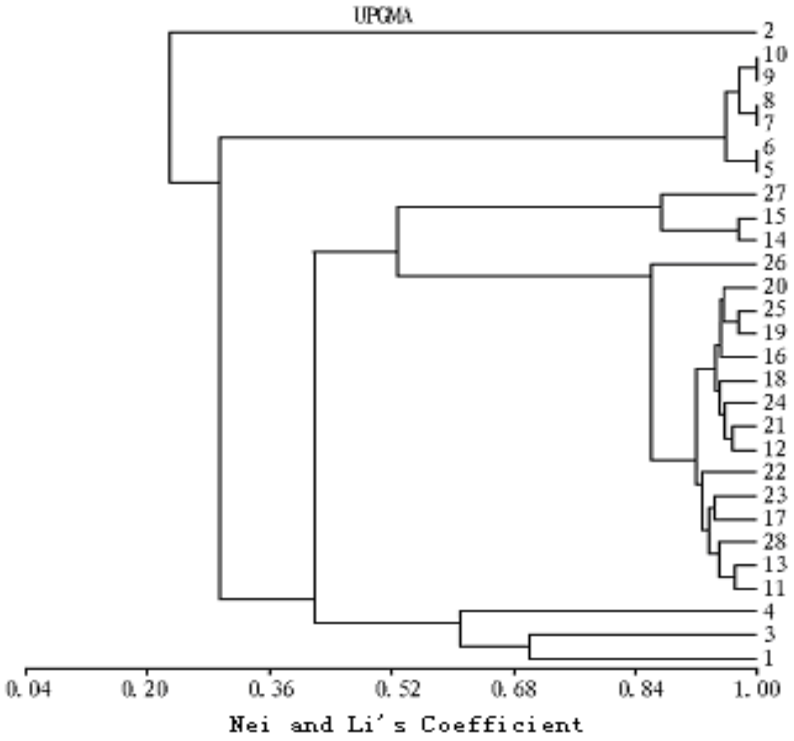

Fig. 1. Sliver-staining AFLP clustering of 28 materials.

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 28 materials, which were the high-efficient primers in AFLP detection at present and had higher application values.

As for 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.

One of the biggest advantages for AFLP marker technology was that large numbers of loci were detected by a small quantity of selective primer in shorter time. Usually in AFLP analysis, each amplification band was correspondant with one locus in the molecule of genomic DNA. In 28 materials 191 DNA bands with different molecular weights were amplified with 4 pairs of primers by using sliver-staining AFLP, which meant that 191 loci in the genomes of 28 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 sliver staining AFLP, 189 polymorphism loci were found, occupying 98.9%, which indicated that on the detected loci there were 98.9% materials existed variations. The precision and efficiency of this detection was better than any former fingerprinting technology. So it was thought that 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. The similarity coefficients between Kava 1 and Kava 2, Kava 3 and Kava 4, Kava 5 and Kava 6 were all 1 and couldn't be distinguished, which might be caused that they came from the same plant of material *P. methysticum*.

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