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 cathavana 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: *EcoR* I, *Mse* I, T4 DNA ligase were purchased from USA Biolab Company, *Taq* enzyme was purchased from USA Gene Company, 5'-FAM fluorescently labelled *MseI* 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-Na₂ (pH value of 8.0)] that had been preheated up to 60-65°C were added and blended mildly. Then 50µl 1% Na₂S₂O₅ 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 / isoamvl 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 CH₃COONa 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 20 μ l TE solution and preserved for use at -20°C.

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11.Lampong type (Piper nigrum L.)GardenPiper L.12.Banniyueer-1(Piper nigrum L.)Hainan Xinglong Tropical Botanical Garden $Piper L., original name of 'Panniyur-1'13.Kuching (Piper nigrum L.)Hainan Xinglong Tropical BotanicalGardenPiper L., original name of 'Ruching'14.Dashan (Piper sp.)Hainan Xinglong Tropical BotanicalGardenPiper L., original name of 'Ruching'15.Piper hancei (Piper sp.)Hainan Xinglong Tropical BotanicalGardenPiper L.,16.Hybrid 3 (Piper nigrum L.)Hainan Xinglong Tropical BotanicalGardenPiper L., 'Banniyueer-1' × 'Lampong Type'18.Hybrid 5 (Piper nigrum L.)Hainan Xinglong Tropical BotanicalGardenPiper L., 'Banniyueer-1' × 'Lampong Type'20.Hybrid 5 (Piper nigrum L.)Hainan Xinglong Tropical BotanicalGardenPiper L., 'Banniyueer-1' × 'Sheng 20'21.Hybrid 8 (Piper nigrum L.)Hainan Xinglong Tropical BotanicalGardenPiper L., 'Banniyueer-1' × 'Sheng 20'22.Yinjian 45 (Piper nigrum L.)Hainan Xinglong Tropical BotanicalGardenPiper L., 'Banniyueer-1' × 'Sheng 20'23.Ban 293 (Piper nigrum L.)Hainan Xinglong Tropical BotanicalGardenPiper L., 'Banniyueer-1' × 'Sheng 20'24.Banjianni (Piper nigrum L.)Hainan Xinglong Tropical BotanicalGardenPiper L., 'Banniyueer-1' × 'Yamong Type' × 'Banniyueer-1'25.Banyunda (Piper nigrum L.)Hainan Xinglong Tropical BotanicalGardenPiper L., 'Banniyueer-1' × 'Yamong Type'26.Yuanxuan 1(Piper nigrum L.)$, , , ,	5					
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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' 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' × 'Lampong Type' 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' × 'Cambodia' 24. Banjianin (Piper nigrum L.) Hainan Xinglong Tropical Botanical Garden Piper L. 25. <td>10</td> <td></td> <td>Hainan Xinglong Tropical Botanical</td> <td></td>	10		Hainan Xinglong Tropical Botanical					
15. Kuching (Piper nigrum L.) 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 5 (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' × 'Sheng 20' 21. Hybrid 8 (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' × 'Cambodia' × Lampong Type' 25. Banyunda (Piper nigrum L.) Hainan Xinglong Tropical Botanical Garden Piper L., 'Banniyueer-1' × 'Yuanxuan 1' × 'Dashan' Garden	12.	Banniyueer-1(<i>Piper nigrum</i> L.)		Piper L., original name of 'Panniyur-1'				
14.Dashan (Piper sp.)GardenPiper L.15.Piper hancei (Piper sp.)Hainan Xinglong Tropical Botanical GardenPiper L.16.Hybrid 1 (Piper nigrum L.)Hainan Xinglong Tropical Botanical GardenPiper L.17.Hybrid 3 (Piper nigrum L.)Hainan Xinglong Tropical Botanical GardenPiper L., 'Banniyueer-1' × 'Lampong Type'18.Hybrid 5 (Piper nigrum L.)Hainan Xinglong Tropical Botanical GardenPiper L., 'Banniyueer-1' × 'Lampong Type'19.Hybrid 6 (Piper nigrum L.)Hainan Xinglong Tropical Botanical GardenPiper L., 'Banniyueer-1' × 'Lampong Type'20.Hybrid 7 (Piper nigrum L.)Hainan Xinglong Tropical Botanical GardenPiper L., 'Banniyueer-1' × 'Lampong Type'21.Hybrid 8 (Piper nigrum L.)Hainan Xinglong Tropical Botanical GardenPiper L., 'Banniyueer-1' × 'Sheng 20'22.Yinjian 45 (Piper nigrum L.)Hainan Xinglong Tropical Botanical GardenPiper L., 'Banniyueer-1' × 'Sheng 20'23.Ban 293 (Piper nigrum L.)Hainan Xinglong Tropical Botanical GardenPiper L., 'Lampong Type' × 'Cambodia'24.Banjianni (Piper nigrum L.)Hainan Xinglong Tropical Botanical GardenPiper L., 'Banniyueer-1' × 'Dashan'25.Banyunda (Piper nigrum L.)Hainan Xinglong Tropical Botanical GardenPiper L.26.Yuanxuan 1(Piper nigrum L.)Hainan Xinglong Tropical Botanical GardenPiper L.27.Dashan (Piper nigrum L.)Hainan Xinglong Tropical Botanical GardenPiper L.28.Jianyin 93 (Piper nigrum L.)Haina	12	Variation (Dimensionen I.)	Hainan Xinglong Tropical Botanical	Dia I animinal mana of 'Weahing'				
14. Dashan (Piper sp.) 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' 20. Hybrid 7 (Piper nigrum L.) Hainan Xinglong Tropical Botanical Garden Piper L., 'Banniyueer-1' × 'Lampong Type' 21. Hybrid 8 (Piper nigrum L.) Hainan Xinglong Tropical Botanical Garden Piper L., 'Banniyueer-1' × 'Seng 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' × 'Cambodia' 24. Banjianni (Piper nigrum L.) Hainan Xinglong Tropical Botanical Garden Piper L., 'Banniyueer-1' × 'Yuanxuan 1' × 'Dashan' 25. Banyunda (Piper nigrum L.) Hainan Xinglong Tropical Botanical Garden Piper L., 'Banniyueer-1' × 'Yuanxuan 1' × 'Dashan'	13.	Kuching (Piper nigrum L.)		Piper L., original name of Kuching				
15.Piper hancei (Piper sp.)GardenPiper L.16.Hybrid 1 (Piper nigrum L.)Hainan Xinglong Tropical Botanical GardenPiper L., 'Banniyueer-1' × 'Lampong Type'17.Hybrid 3 (Piper nigrum L.)Hainan Xinglong Tropical Botanical GardenPiper L., 'Banniyueer-1' × 'Lampong Type'18.Hybrid 5 (Piper nigrum L.)Hainan Xinglong Tropical Botanical GardenPiper L., 'Banniyueer-1' × 'Lampong Type'19.Hybrid 6 (Piper nigrum L.)Hainan Xinglong Tropical Botanical GardenPiper L., 'Banniyueer-1' × 'Lampong Type'20.Hybrid 7 (Piper nigrum L.)Hainan Xinglong Tropical Botanical GardenPiper L., 'Banniyueer-1' × 'Lampong Type'21.Hybrid 8 (Piper nigrum L.)Hainan Xinglong Tropical Botanical GardenPiper L., 'Banniyueer-1' × 'Seng 20'22.Yinjian 45 (Piper nigrum L.)Hainan Xinglong Tropical Botanical GardenPiper L., 'Banniyueer-1' × 'Seng 20'23.Ban 293 (Piper nigrum L.)Hainan Xinglong Tropical Botanical GardenPiper L., 'Lampong Type' × 'Cambodia'24.Banjianni (Piper nigrum L.)Hainan Xinglong Tropical Botanical GardenPiper L., 'Banniyueer-1' × 'Yuanxuan 1' × 'Dashan'25.Banyunda (Piper nigrum L.)Hainan Xinglong Tropical Botanical GardenPiper L.26.Yuanxuan 1(Piper nigrum L.)Hainan Xinglong Tropical Botanical GardenPiper L.27.Dashan (Piper nigrum L.)Hainan Xinglong Tropical Botanical GardenPiper L.28.Jianyin 93 (Piper nigrum L.)Hainan Xinglong Tropical Botanical GardenPiper L. <t< td=""><td>14</td><td>Dechen (Bingram)</td><td>Hainan Xinglong Tropical Botanical</td><td>DinerI</td></t<>	14	Dechen (Bingram)	Hainan Xinglong Tropical Botanical	DinerI				
15. Piper hancei (Piper sp.) 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' × 'Song 20' 21. Hybrid 8 (Piper nigrum L.) Hainan Xinglong Tropical Botanical Garden Piper L., 'Lampong Type' × 'Cambodia' 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., 'Banniyueer-1' × 'Yuanxuan 1' × 'Dashan' 24. Banjianni (Piper nigrum L.) Hainan Xinglong Tropical Botanical Garden Piper L., 'Banniyueer-1' × 'Yuanxuan 1' × 'Dashan' 25. Banyunda (Piper nigrum L.) Hainan Xinglong Tropical Botanical Garden Piper L., 'Banniyuee	14.	Dashan (1 iper sp.)	Garden	Tiper L.				
16.Hybrid 1 (<i>Piper nigrum</i> L.)Hainan Xinglong Tropical Botanical Garden <i>Piper</i> L., 'Banniyueer-1' × 'Lampong Type'17.Hybrid 3 (<i>Piper nigrum</i> L.)Hainan Xinglong Tropical Botanical Garden <i>Piper</i> L., 'Banniyueer-1' × 'Lampong Type'18.Hybrid 5 (<i>Piper nigrum</i> L.)Hainan Xinglong Tropical Botanical Garden <i>Piper</i> L., 'Banniyueer-1' × 'Lampong Type'19.Hybrid 6 (<i>Piper nigrum</i> L.)Hainan Xinglong Tropical Botanical Garden <i>Piper</i> L., 'Banniyueer-1' × 'Lampong Type'20.Hybrid 7 (<i>Piper nigrum</i> L.)Hainan Xinglong Tropical Botanical Garden <i>Piper</i> L., 'Banniyueer-1' × '293'21.Hybrid 8 (<i>Piper nigrum</i> L.)Hainan Xinglong Tropical Botanical Garden <i>Piper</i> L., 'Banniyueer-1' × '293'22.Vinjian 45 (<i>Piper nigrum</i> L.)Hainan Xinglong Tropical Botanical Garden <i>Piper</i> L., 'Lampong Type' × 'Cambodia'23.Ban 293 (<i>Piper nigrum</i> L.)Hainan Xinglong Tropical Botanical Garden <i>Piper</i> L., 'Lampong Type' × 'Cambodia'24.Banjianni (<i>Piper nigrum</i> L.)Hainan Xinglong Tropical Botanical Garden <i>Piper</i> L., 'Lampong Type' × 'Banniyueer-1'25.Banyunda (<i>Piper nigrum</i> L.)Hainan Xinglong Tropical Botanical Garden <i>Piper</i> L., 'Banniyueer-1' × 'Yuanxuan 1' × 'Dashan' Garden26.Yuanxuan 1(<i>Piper nigrum</i> L.)Hainan Xinglong Tropical Botanical Garden <i>Piper</i> L.27.Dashan (<i>Piper nigrum</i> L.)Hainan Xinglong Tropical Botanical Garden <i>Piper</i> L.28.Jianyin 93 (<i>Piper nigrum</i> L.)Hainan Xinglong Tropical Botanical Garden <i>Piper</i> L.	15	Piner hancei (Piner sp.)	Hainan Xinglong Tropical Botanical	PinerI				
16. Hybrid 1 (Piper nigrum L.) Forden 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'×'Lampong Type' 21. Hybrid 8 (Piper nigrum L.) Hainan Xinglong Tropical Botanical Garden Piper L., 'Banniyueer-1'×'Lampong Type' 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' × 'Cambodia' 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 (Piper nigrum L.) Hainan Xinglong Tropical Botanical Garden	15.	Tiper nuncer (Tiper sp.)		Tiper E.				
17.Hybrid 3 (Piper nigrum L.)Hainan Xinglong Tropical Botanical GardenPiper L., 'Banniyueer-1' × 'Lampong Type'18.Hybrid 5 (Piper nigrum L.)Hainan Xinglong Tropical Botanical GardenPiper L., 'Banniyueer-1' × 'Lampong Type'19.Hybrid 6 (Piper nigrum L.)Hainan Xinglong Tropical Botanical GardenPiper L., 'Banniyueer-1' × 'Lampong Type'20.Hybrid 7 (Piper nigrum L.)Hainan Xinglong Tropical Botanical GardenPiper L., 'Banniyueer-1' × 'Lampong Type'21.Hybrid 8 (Piper nigrum L.)Hainan Xinglong Tropical Botanical GardenPiper L., 'Banniyueer-1' × 'Seng 20'22.Yinjian 45 (Piper nigrum L.)Hainan Xinglong Tropical Botanical GardenPiper L., 'Lampong Type' × 'Cambodia'23.Ban 293 (Piper nigrum L.)Hainan Xinglong Tropical Botanical GardenPiper L., 'Lampong Type' × 'Cambodia'24.Banjianni (Piper nigrum L.)Hainan Xinglong Tropical Botanical GardenPiper L., 'Banniyueer-1' × 'Sunbodia' × 'Lampong Type'25.Banyunda (Piper nigrum L.)Hainan Xinglong Tropical Botanical GardenPiper L., 'Banniyueer-1' × 'Yuanxuan 1' × 'Dashan'26.Yuanxuan 1(Piper nigrum L.)Hainan Xinglong Tropical Botanical GardenPiper L.28.Jianyin 93 (Piper nigrum L.)Hainan Xinglong Tropical Botanical GardenPiper L.29.Banjianyin 20 (Piper nigrum L.)Hainan Xinglong Tropical Botanical GardenPiper L., 'Cambodia' × 'Lampong Type'29.Banjianyin 43 (Piper nigrum L.)Hainan Xinglong Tropical Botanical GardenPiper L., 'Cambodia' × 'Lampong Type'	16	Hybrid 1 (<i>Piper nigrum</i> L.)		<i>Piper</i> L., 'Bannivueer-1' × 'Lampong Type'				
17. Hybrid 3 (Piper nigrum L.) 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' × '293' 22. Yinjian 45 (Piper nigrum L.) Hainan Xinglong Tropical Botanical Garden Piper L., 'Banniyueer-1' × '293' 23. Ban 293 (Piper nigrum L.) Hainan Xinglong Tropical Botanical Garden Piper L., 'Lampong Type' × 'Cambodia' 24. Banjianni (Piper nigrum L.) Hainan Xinglong Tropical Botanical Garden Piper L., 'Banniyueer-1' × 'Yuanxuan 1' × 'Dashan' 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. 28. Jianyin 93 (Piper nigrum L.) Hainan Xinglong Tropical Botanical Garden Piper L., 'Cambodia' × 'Lampong Type'								
18.Hybrid 5 (Piper nigrum L.)Hainan Xinglong Tropical Botanical GardenPiper L., 'Banniyueer-1' × 'Lampong Type'19.Hybrid 6 (Piper nigrum L.)Hainan Xinglong Tropical Botanical GardenPiper L., 'Banniyueer-1' × 'Lampong Type'20.Hybrid 7 (Piper nigrum L.)Hainan Xinglong Tropical Botanical GardenPiper L., 'Banniyueer-1' × 'Lampong Type'21.Hybrid 8 (Piper nigrum L.)Hainan Xinglong Tropical Botanical GardenPiper L., 'Banniyueer-1' × '293'22.Yinjian 45 (Piper nigrum L.)Hainan Xinglong Tropical Botanical GardenPiper L., 'Banniyueer-1' × '293'23.Ban 293 (Piper nigrum L.)Hainan Xinglong Tropical Botanical GardenPiper L., 'Lampong Type' × 'Cambodia'24.Banjianni (Piper nigrum L.)Hainan Xinglong Tropical Botanical GardenPiper L., 'Banniyueer-1' × 'Yuanxuan 1' × 'Dashan'26.Yuanxuan 1(Piper nigrum L.)Hainan Xinglong Tropical Botanical GardenPiper L., 'Banniyueer-1' × 'Yuanxuan 1' × 'Dashan'27.Dashan (Piper sp.) × Yinni (Piper nigrum L.)Hainan Xinglong Tropical Botanical GardenPiper L., 'Banniyueer-1' × 'Yuanxuan 1' × 'Dashan'26.Yuanxuan 1(Piper nigrum L.)Hainan Xinglong Tropical Botanical GardenPiper L., 'Cambodia' × 'Lampong Type'28.Jianyin 93 (Piper nigrum L.)Hainan Xinglong Tropical Botanical GardenPiper L., 'Cambodia' × 'Lampong Type'29.Banjianyin 20 (Piper nigrum L.)Hainan Xinglong Tropical Botanical GardenPiper L., 'Cambodia' × 'Lampong Type'30.Banjianyin 43 (Piper nigrum L.)State Key BiotechnologyFipe	17.	Hybrid 3 (<i>Piper nigrum</i> L.)		<i>Piper</i> L., 'Banniyueer-1' × 'Lampong Type'				
18. Hybrid S (Piper nigrum L.) 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' × 'Lampong Type' 21. Hybrid 8 (Piper nigrum L.) Hainan Xinglong Tropical Botanical Garden Piper L., 'Banniyueer-1' × '293' 22. Yinjian 45 (Piper nigrum L.) Hainan Xinglong Tropical Botanical Garden Piper L., 'Banniyueer-1' × '293' 23. Ban 293 (Piper nigrum L.) Hainan Xinglong Tropical Botanical Garden Piper L., 'Lampong Type' × 'Cambodia' 24. Banjianni (Piper nigrum L.) Hainan Xinglong Tropical Botanical Garden Piper L., 'Banniyueer-1' × 'Yuanxuan 1' × 'Dashan' 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., 'Banniyueer-1' × 'Yuanxuan 1' × 'Dashan' 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 Gard								
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30 Banjianyin 43 (Piper nigrum L.) 31 Tobacoo (Nicotiana tabacum) 31 State Key Biotechnology	.							
31 Tobacoo (Nicotiana tabacum)	30	Banjianyin 43 (Piper nigrum L.)						
Laboratory for Tropical Crops	21	Tobacco (Nigotiana tabacum)	State Key Biotechnology					
	51		Laboratory for Tropical Crops					

Note: "×"indicates "hybrid".

Linker preparations of EcoRI and MseI: Linker 1 of EcoRI was 5'-CTCGTA GAC TGC GTA CC-3'and linker 2 of EcoRI was 5'- AAT TGG TACGCA GTC TAC-3'. Linker 1 of MseI was 5'- GAC GAT GAG TCC TGAG-3'and linker 2 of MseI was 5'- TAC TCA GGA CTC AT-3'. The designed linkers were synthesized by Shanghai Sangon Company. Linker 2 of EcoRI and linker 2 of MseI were phosphorylated at the 5'end at the same time of synthesis. After synthesizing, linker 1, 2 of EcoRI and linker 1, 2 of MseI were annealed to form EcoRI linker and MseI linker (Sambrook et al., 2002). The primer sequence of EcoRI pre-amplification (Eo) was 5'- GAC TGC GTA CCA ATT CA-3' and the primer sequence of MseI 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/ μ l 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. AFLF	EcoRI/Mse	l primer	combinations.
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S. No.	Serial number of primers													
5. INO.	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: 5.0μ l samples that had completed linkers, 1.0μ l E₀ (10 pmol/ μ l), 1.0μ l M₀ (10 pmol/ μ l), 5.0μ l 10× PCR buffer, 5.0μ l MgCl₂ (25 mmol/L), 4.0μ l dNTP (2.5 mmol/L), 0.5μ l 10 mg/ml BSA, 1.0μ l *Taq* enzyme (5 U/ μ l), 27.5 μ l double distilled water, the whole volume was 50.0 μ l. 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 preamplification 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 MgCl₂ (25 mmol/L), 2.0µl dNTP (2.5 mmol/L), 0.5µl EcoRI primer (10 pmol/µl), 0.5µl MseI 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 40μ l 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. Preelectrophoresis was made for 30 min with the constant power of 80 W. The loading groove was flush out, the comb was inserted and 5μ l 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

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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

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 \hat{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 4^{th} group was Kava 4 with green stems and slightly swelling internode, the 5^{th} 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.

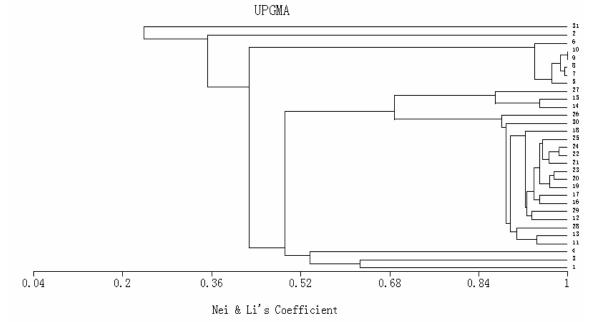


Fig.2.Fluorescent AFLP clustering result of 31 materials.

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 bandtype 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.

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