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A Study on the RAPD and SCAR Molecular Markers of Piper Species

Shi Jiang [*1], **Xin Jianhua** [2] and **Xin Li** [3]

Abstract

In order to compare the genetic relationships among Kava, Pepper and it's wild relatives and to distinguish Kava from Pepper and it's wild relatives, we conducted research on Kava by using RAPD and SCAR molecular markers. 20 random primers selected from 80 random primers were used for RAPD amplification to identify the genetic relationships among Kava, Pepper and it's wild relatives. Total 170 bands were amplified by 20 random primers, in which 20 bands were polymorphic (12%). Cluster analysis grouped the 28 accessions into six groups at similarity coefficient of 0.36, where 6 materials of Kava formed a group, indicating that Kava was distantly relation to Pepper and its wild relatives. Kava had 562 bp and 355 bp specific fragments amplified by primers OPQ-02 and OPQ-03, respectively, were recycled for cloning and sequencing analysis, and then converted to SCAR markers. Two pairs of specific SCAR primers for Kava, P4.1 and P4.2, P8.1 and P8.2 were designed. PCR amplification of 28 test materials were performed using the two pairs of the specific primers respectively, the specific bands of 562 bp and 355 bp with expected sizes were amplified in 6 Kava materials but not in other materials. The results showed that primers P4.1 and P4.2, P8.1 and P8.2 might be used as specific SCAR primers for Kava germplasm resources identification. This research provided the basis for selecting rootstocks, molecular identification and the fingerprint construction of Kava.

Keywords: Kava (*Piper methysticum* Forst. f., *Piperaceae*), Pepper (*Piper nigrum* L., *Piperaceae*), Random Amplified Polymorphic DNA (RAPD), Sequence-characterized amplified region (SCAR)

1 Introduction

First: The history of the use of Kava has 3000 years, which form a unique "Kava culture" in the local (Vincent *et al.*, 1992). The geographical distribution of Kava is limited

* corresponding author

[1] Associate Prof. Dr. Shi Jiang, College of Agriculture, Department of Biotechnology, Henan University of Science & Technology, 471003 Tianjin Road, Jianxi District, Luoyang, Henan province, China. E-mail: shijiang66@21cn.com; shijiang66@126.com; shijiang@mail.haust.edu.cn, Phone: 86-379-64280115, Fax: 86-379-64282340

[2] College of Agriculture, Shihezi University, Shihezi, 832000, China;

[3] College of Food and Bioengineering, Henan University of Science & Technology, Luoyang, 471003, China

to the South Pacific island nation. Because Kava plays a decisive role in the export, it is regarded as national treasure material by the local nation. Kava has a significant effect in treatment of anxiety disorder, depression, to promote and improve the sleep quality, and also has a very good role for ease the mental stress and relax the body. And the poison or negative effects are small (LEBOT and LEVESQUE, 1989). Second *Piper methysticum* Forst. f. commonly known as Kava or Kawa, which is a wild or planted perennial medicinal bush plants of *Piper* L., *Piperaceae* in Vanuatu, Fiji, Tonga, Papua new Guinea and the Solomon Islands and other South Pacific islands. South Pacific island nation's residents prefer to use Kava roots and stems to prepare drinks which could relax the body and emotions, improve sleep and restore the strength of the body. They often set up Kava feast especially in the religious activities, a major festival celebrations and banquet guests. Kava is the indispensable beverages in the ceremonial and social activities, and it also is an essential everyday goods. Kava was praised as the Pacific longevity medicine by the local people. Third: In 2001, Kava cultivation technology had been listed as China's "Tenth Five-Year" Scientific and Technological Project. In the process of introduction and cultivation of Kava, it was difficult to distinguish among the seedlings of Kava, Pepper (*Piper nigrum* L., *Piperaceae*) and it wild relatives which was similar in morphology. How to distinguish among them was a problem. Kava belongs to a Pepper plant, but the genetic relationships among Kava, Pepper and it wild relatives was not clear. The molecular tagging technology such as RAPD and SCAR could solve these problems.

RAPD markers have been widely used in many plants and crops to assess phylogenetic relationships such as potato, *Dalbergia*, olives, *Phytophthora sojae*, *Leymus*, Safflower, *Rehmannia glutinosa*, etc. RAPD technology became popular because of its simplicity and ease of use in a moderately equipped laboratory and the assay does not require any sequence information. However, RAPDs are often criticized for their lack of reliability among laboratories (GOSSELIN *et al.*, 2002). SCAR markers were widely used in some morphology which were difficult to distinguish from the different economic value of species, identification and evaluation of germplasm such as to distinguish between *M. boninensis*, *M. acidosa* and their hybrids (WANG *et al.*, 2001), for *Bambusa balcooa* and *Bambusa tulda* identification (NAOKI *et al.*, 2003), for identification in bamboo (DAS *et al.*, 2005), in genotype identification of 26 olive cultivars (MATTEO *et al.*, 2006). Compared with the RAPD markers, SCAR primers are longer, and primer sequence and the template DNA fully complementary to each other, so the amplification results possess good stability and strong repeatability. But very little work has been done about these markers in Kava (*Piperaceae*), Pepper (*Piperaceae*) and it wild relatives (*Piperaceae*). The objectives of this study were to compare the genetic relatedness among Kava, Pepper and it wild relatives and to distinguish them during the seedling period. To achieve this goal, we first analysed genetic relatedness among Kava, Pepper and it wild relatives using the RAPD technique, and then converted Kava-specific fragments into SCAR markers suitable for identifying Kava.

2 Materials and Methods

2.1 Plant Materials

Twenty-eight accessions of Kava, Pepper and it wild relatives were studied. Five Kava accessions from the Pacific island countries were provided by the State Key Biotechnology Laboratory for Tropical Crops at Hainan (China). *Peperomia pellucida* kunth.(*Piperaceae*) was provided by South China University of Tropical Agriculture at Hainan (China). Other Pepper and it wild relatives accessions were provided by Xinglong tropical botanical garden at Hainan (China). All samples used in this study were planted in greenhouse and authenticated in the State Key Biotechnology Laboratory for Tropical Crops, Hainan Province, China (Table 1).

2.2 DNA extraction

Total genomic DNA was extracted from freshly young leaves of individual seedlings by using CTAB method (WANG and FANG, 2002). DNA concentration was assessed by taking absorbance at 260 nm. Working samples were prepared to a concentration of 20 ng μl^{-1}.

2.3 RAPD assay

All RAPD primers used in this study were random sequence, 10-base, oligonucleotide primers with C+G contents ranging from 50 to 80%. One set of primers was obtained from Operon Technologies Inc. (Almada, CA, USA) and an additional set from Shanghai Sangon Company(Shanghai, China). Eighty RAPD primers were chosen for preliminary amplification of 28 test materials. In primer screening, DNA amplifications were repeated at least twice for each primer. Twenty primers (Table 2) that gave always reproducible and scoreable amplifications were selected for the analysis of all the 28 accessions. Protocol for PCR was optimised by varying the concentration of $MgCl_2$, dNTP, primers, *Taq* polymerase and template genomic DNA (XIN and SHI, 2005). PCR reactions (20 μl) contained 20 ng of genomic DNA, 1 unit of *Taq* DNA polymerase (TAKARA Biotechnology (Dalian) Company LTD.), 2 μl of 10$\times$ *Taq* DNA polymerase buffer, 2 mM $MgCl_2$, 200 μM of each dNTPs and 0.2 μM of random primer. DNA amplification was carried out in a GeneAmp PCR System 2400 or 2700 (Perkin-Elmer) for 40 cycles. The thermal profile was as follows: denaturation at 94°C for 5 min, followed by 40 cycles of denaturation at 94°C for 1 min, annealing at 37 °C for 1min and extension at 72 °C for 2 minutes, finished with final extension at 72 °C for 7 minutes and a subsequent incubation at 4°C followed. Approximately 10 μl of completed amplification reaction was run in 1.5% agarose gels containing ethidium bromide (0.5 μg ml^{-1}) and photographed under UV light using a computer printer. Reproducibility of each experiment was confirmed at least twice.

2.4 Data analysis

Only the RAPD primers which gave consistent profiles across the populations and also those that appeared to have diagnostic markers were chosen for further analysis. The presence and absence of bands were scored as 1 or 0 respectively. Faint bands were

Table 1

Species	*Registration No.*	*Origin*
Piper hancei Maxim	2003-P-01	Hainan Bawangling (Hainan, China)
Peperomia pellucida kunth.	2003-P-02	South China University of Tropical Agriculture (Hainan, China)
Piper betle L.	2003-P-03	South China University of Tropical Agriculture (Hainan, China)
Piper sarmentosum Roxb.	2003-P-04	South China University of Tropical Agriculture (Hainan, China)
Piper methysticum Forst.f. No.1	2003-K-01	Fiji
Piper methysticum Forst. f. No.2	2003-K-02	Fiji
Piper methysticum Forst.f. No.3	2003-K-03	Fiji
Piper methysticum Forst.f. No.4	2003-K-04	Fiji
Piper methysticum Forst. f. No.5	2003-K-05	Fiji
Piper methysticum Forst.f. No.6	2003-K-06	Fiji
Lampong Type	2003-P-011	Hainan Xinglong tropical botanical garden
Banniyueer-1	2003-P-12	Hainan Xinglong tropical botanical garden
Kuching	2003-P-13	Hainan Xinglong tropical botanical garden
Dashan	2003-P-14	Hainan Xinglong tropical botanical garden
Piper hancei	2003-P-15	Hainan Xinglong tropical botanical garden
Hybrid 1	2003-P-16	Hainan Xinglong tropical botanical garden
Hybrid 3	2003-P-17	Hainan Xinglong tropical botanical garden
Hybrid 5	2003-P-18	Hainan Xinglong tropical botanical garden
Hybrid 6	2003-P-19	Hainan Xinglong tropical botanical garden
Hybrid 7	2003-P-20	Hainan Xinglong tropical botanical garden
Hybrid 8	2003-P-21	Hainan Xinglong tropical botanical garden
Yinjian 45	2003-P-226	Hainan Xinglong tropical botanical garden
Ban 293	2003-P-23	Hainan Xinglong tropical botanical garden
Banjianni	2003-P-24	Hainan Xinglong tropical botanical garden
Banyunda	2003-P-25	Hainan Xinglong tropical botanical garden
Yuanxuan 1	2003-P-26	Hainan Xinglong tropical botanical garden
Dashan×Yinni	2003-P-27	Hainan Xinglong tropical botanical garden
Jianyin 93	2003-P-28	Hainan Xinglong tropical botanical garden

Table 2

Primer	*Sequence (5′→3′)*	*Primer*	*Sequence (5′→3′)*
OPA—08	GTGACGTAGG	OPQ—07	CCCCGATGGT
OPA—10	GTGATCGCAG	OPQ—10	TGTGCCCGAA
OPA—14	TCTGTGCTGG	OPQ—11	TCTCCGCAAC
OPA—15	TTCCGAACCC	OPQ—13	GGAGTGGACA
OPA—16	AGCCAGCGAA	OPQ—14	GGACGCTTCA
OPA—17	GACCGCTTGT	OPQ—15	GGGTAACGTG
OPA—20	GTTGCGATCC	S265	GGCGGATAAG
OPQ—02	TCTGTCGGTC	S273	CACAGCGACA
OPQ—03	GGTCACCTCA	S278	TTCAGGGCAC
OPQ—06	GAGCGCTTG	S279	CAAAGCGCTC

not recorded for analysis and the data were analyzed using the method of Nei and Li (1979). Cluster analysis was then performed to create a dendrogram using UPGMA by the software MVSP3.13f.

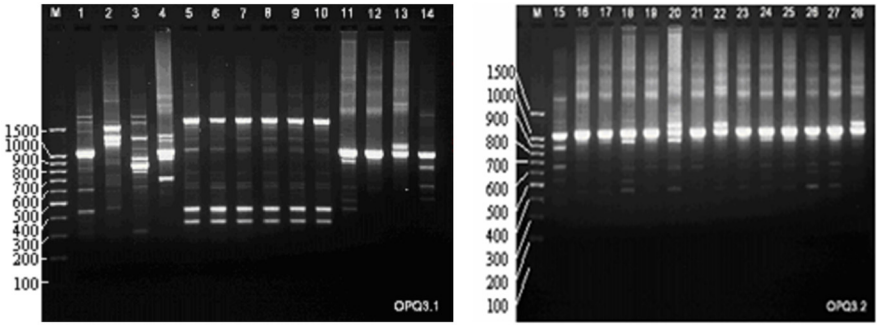
2.5 Cloning and sequencing

The target DNA fragments ($OPQ-02_{562}$ and $OPQ-03_{355}$) in the RAPD reactions were extracted from agarose gels using the QIAquick^{Gel} Extraction kit (Qiagen). The fragments were cloned into the pMD18-T vector (Promega) . *Escherichia coli* XL-1 blue competent cells were transformed with the recombinant vectors and were then plated onto LB/ampicillin/IPTG/X-Gal plates. Positive colonies were determined by blue/white screening. Plasmids from randomly selected white colonies were extracted using the lysis by alkali protocol from Joseph and David (2001) and *Hin*dIII and *Eco*RI double digestion was conducted to confirm the size of the inserted fragment. The vectors containing the fragments of the correct size were sequenced. For each sequence, a pair of target primers flanking the insert region was designed and synthesized. Each primer contained the original 10 bases of the RAPD primer plus the next 10-18 internal bases from the end. The primer pairs were used to amplify Kava, Pepper and it wild relatives DNA to identify Kava-specific SCAR markers.

3 Results

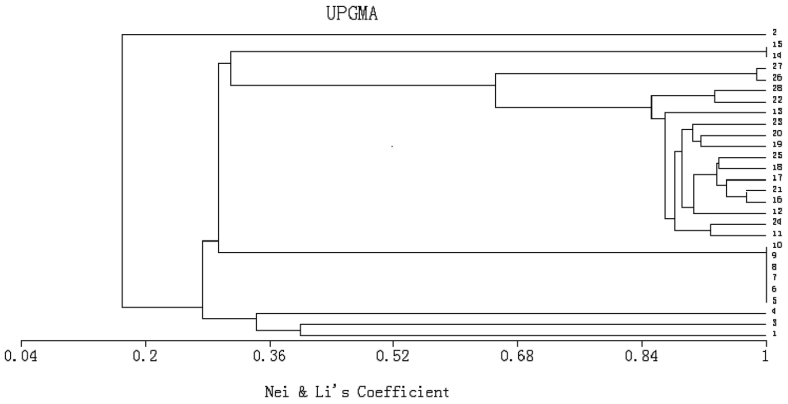
While 80 random primers were screened, out of them 20 primers produced clear and distinct patterns across all samples (Table 2, Fig. 1). PCR amplification with each of these 20 primers was done twice before scoring them. These primers generated a total of 170 bands ranging in size from roughly 300 bp to 2000 bp whereas the range with individual primers was 2–15 bands. This gave an average of 8 bands per primer. Out of 170 bands, 20(12%) of which were polymorphic for one or more species, 16 bands were monomorphic and rest 4 bands were Kava unique.

Figure 1: Agarose gel electrophoresis of OPQ-03 showing Kava's specific band at 355 bp. M is molecular marker lane (see table 1 = here include).



The data analysis and cluster analysis were conducted by using software MVSP3.13f. The clustering result of 28 accessions by using RAPD markers was shown in Figure 2.

Figure 2: Dendrogram of the genetic relationships among Kava, Pepper and it wild relatives (see table 1 = here include).



The similarity index values ranged from 0.125 to 1 indicating the presence of enormous genetic diversity at molecular level. The tested materials were classified into two clusters in similarity coefficient of 0.2. The first group had the 2nd material *Peperomia pellucida* kunth., which belonged to *Peperomia*, *Piperaceae*. The second group 2 contained the 27 materials of *Piper* L., *Piperaceae*, which meant that the materials of the different genera in the same family could be distinguished at the similarity coefficient of 0.2. It showed that the intergeneric difference was greater than that of intragenus. The 28 test materials were classified into six clusters at similarity coefficient of 0.36. Group 1 had *Piper hancei* Maxim (*Piperaceae*) and *Piper betle* L. (*Piperaceae*), Group 2 contained *Piper sarmentosum* Roxb. (*Piperaceae*), Group 3 was Kava, Group 4 was

Pepper, Group 5 was Dashan (*Piperaceae*) and *Piper hancei* (*Piperaceae*), Group 6 was *Peperomia pellucida* kunth. (*Piperaceae*), indicating a higher degree of genetic divergence among Kava and Pepper, Kava and Pepper wild relatives.

In order to increase the specificity and reproducibility of RAPD markers, the two RAPD markers ($OPQ-02_{562}$, $OPQ-03_{355}$) were converted to SCARs. Two SCAR markers were designed based on the sequences of $OPQ-02_{562}$, $OPQ-03_{355}$. The SCAR primers had been designed for $OPQ-02_{562}$ with a forward sequence of 5'-TCT GTC GGT CGT GAA CAA AAA GAA TG-3' and a reverse sequence of 5'-TCT GTC GGT CAT TTA ATT GGT TAA TTG T-3' and for $OPQ-03_{355}$ with a forward sequence of 5'-GGT CAC CTC AAA CCA AGC TTA ATC AAG-3' and a reverse sequence of 5'-GGT CAC CTC ATA ATA CAA ACT TGC AAG C-3'. The validity of the two SCAR markers were confirmed by PCR amplification of the 28 DNA samples (Fig. 3,4). The SCAR primer pairs designed for $OPQ-02_{562}$, $OPQ-03_{355}$ amplified the target fragments(562 bp, 355 bp) exclusively, which indicated that the two SCAR primers were kava-specific SCAR markers which could be used for the molecular identification of Kava germplasm resources.

Figure 3: Agarose gel showing SCAR markers amplified for the primer $OPQ-02_{562}$. M is molecular marker lane. Lanes 5-10 are Kava accessions.

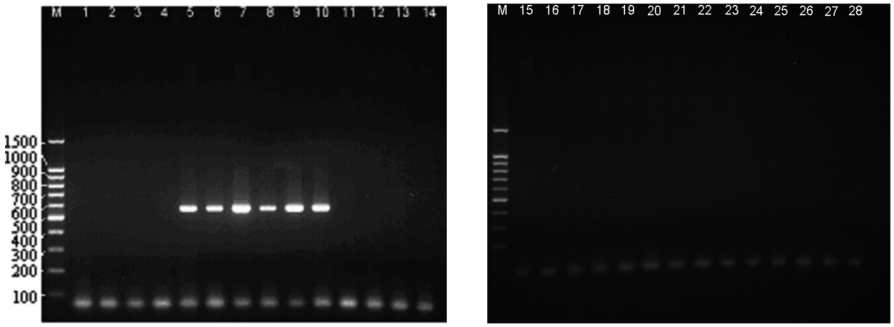
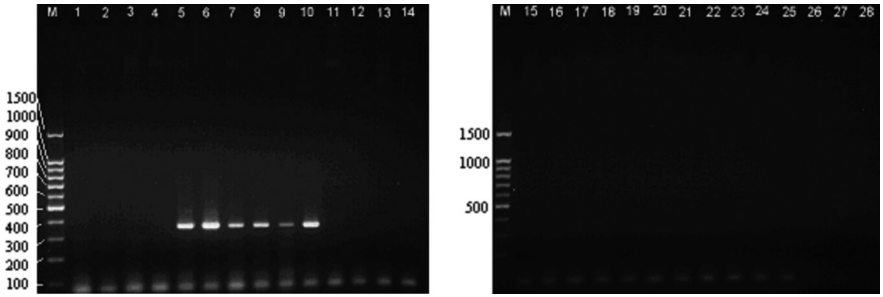


Figure 4: Agarose gel showing SCAR markers amplified for the primer $OPQ-03_{355}$. M is molecular marker lane. Lanes 5-10 are Kava accessions.



4 Discussion

Although Kava and Pepper belong to Pepper genus, the similarity coefficient between Kava and Pepper in our study was 0.36 indicating a higher degree of genetic divergence among Kava and Pepper. Jaramillo and Manos (2001) have suggested that, based on a phylogenetic analysis of sequences of the internal transcribed spacers (ITS) of nuclear ribosomal DNA, the genus Piper could potentially form three monophyletic groups: Asia clade, the South Pacific clade, and the Neotropics clade, and Kava could belong to the South Pacific clade which indicated that the level of genetic relatedness appears to be high between Kava and Asia clade. We obtained the same conclusion on this point. Kava's populations No. 5 to 10 grouped in the same cluster all the time and its similarity coefficients were 1. However, from appearance, 6 plants of Kava could be classified into three kinds of types. The 1st Kava and the 2nd Kava were green stems, the 3rd Kava and the 4th Kava were also green stems but slightly swelling internode, the 5th Kava and the 6th Kava were red stems. Why there was no reflected in the RAPD analysis of three kinds Kava appearance differences. We considered that whether the testing sites were enough to detect the genome by only using 20 random primers selected from 80 random primers. Or might be due to that Kava had no seeds, and took a long time cutting propagation, which caused the relevant unduly narrow of it genetic basis.

The similarity coefficients of Pepper population ranged from 0.612 to 0.989. This showed that genetic difference within Pepper population was relatively small, which related that Pepper was non-origin of my country, few introduction resources, limited geographical cultivation, as well as the long-term adoption of cutting propagation mainly.

SCAR markers have obvious advantages over RAPD markers because the band signals are more prominent and their amplification is less sensitive to reaction conditions. The SCAR markers developed in this study allowed us to distinguish among Kava, Pepper and it wild relatives. This research could provide references for the rootstocks selection in the grafting of *P. methysticum*, the molecular identification on its authenticity and the construction of its fingerprints.

It was reported that *Piper wichmannii* C. DC. and Kava are very similar in morphology, it is a common thing to confuse the two in the laboratory and field (Lebot and Levesque, 1989). Whether Kava-specific SCAR primers could be used to distinguish between *Piper wichmannii* C. DC. and Kava need to be further studied.

In our SCAR study of Kava and its related species, a total of four pairs of Kava specific SCAR markers were designed in which there were two pairs of primers could be used for molecular identification of Kava species and the success rate was of 50%. This loss of polymorphism during RAPD to SCAR conversion was most likely due to flanking site difference at the original RAPD primer binding site.

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