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Evaluation of genetic diversity in *Piper* spp using RAPD and SRAP markers

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ABSTRACT. Random amplified polymorphic DNA (RAPD) and sequence-related amplified polymorphism (SRAP) analysis were applied to 74 individual plants of *Piper* spp in Hainan Island. The results showed that the SRAP technique may be more informative and more efficient and effective for studying genetic diversity of *Piper* spp than the RAPD technique. The overall level of genetic diversity among *Piper* spp in Hainan was relatively high, with the mean Shannon diversity index being 0.2822 and 0.2909, and the mean Nei's genetic diversity being 0.1880 and 0.1947, calculated with RAPD and SRAP data, respectively. The ranges of the genetic similarity coefficient were 0.486-0.991 and 0.520-1.000 for 74 individual plants of *Piper* spp (the mean genetic distance was 0.505 and 0.480) and the within-species genetic distance ranged from 0.063 to 0.291 and from 0.096 to 0.234, estimated with RAPD and SRAP data, respectively. These genetic indices indicated that these species are closely related genetically. The dendrogram generated with the RAPD markers was topologically different from the dendrogram based on SRAP markers, but the SRAP technique clearly distinguished all *Piper* spp from each other. Evaluation of genetic variation levels of six populations showed that

the effective number of alleles, Nei's gene diversity and the Shannon information index within Jianfengling and Diaoluoshan populations are higher than those elsewhere; consequently conservation of wild resources of *Piper* in these two regions should have priority.

Key words: *Piper*; Genetic diversity; Molecular marker; RAPD; SRAP

INTRODUCTION

The genus *Piper* is widely distributed in pan-tropical areas, with black pepper (*Piper nigrum*) being the most important commercial spice crop (known as "King of Spices"). China is one of the main producers of black pepper. Hainan Island accounts for over 80% of annual production and is one of the most important biodiversity-rich areas in China, with 11 *Piper* spp being recorded (Cheng, 1964; Zheng, 1999). Some wild relatives naturally distributed in Hainan have been used locally for spice, medicine and as vegetables before black pepper was introduced from abroad (Liu, 2010). Molecular characterization of these wild species is needed for the development, utilization and conservation of the wild pepper resources; this type of information will help us understand the level of genetic diversity and geographical distribution of genetic variation.

DNA markers offer many advantages over morphological characters for the determination of genetic diversity and the identification of species, such as not being influenced by the environment and detection directly at the DNA level. There have been few studies on genetic diversity of Indian black pepper using molecular markers (Pradeepkumar et al., 2003; Joy et al., 2007). However, the *Piper* spp that are distributed naturally in China have not been investigated to determine their genetic diversity and genetic relationships with each other.

The utility of RAPD technique or sequence-related amplified polymorphism (SRAP) as genetic markers for assessing genetic variation among plants has been clearly established (Williams et al., 1990; Welsh and McClelland, 1990; Morgante and Olivieri, 1993; Gupta et al., 1994; Thormann et al., 1994; Ferriol et al., 2003a,b). We analyzed the genetic diversity of the *Piper* spp in Hainan employing RAPD and SRAP markers. To our knowledge, this is the first report on the characterization of genetic variation present among species in *Piper* naturally distributed in Hainan Island, China.

MATERIAL AND METHODS

Plant materials and DNA extraction

Seventy-four accessions of the species *P. hainanense*, *P. bonii*, *P. laetispicum*, *P. curtipedunculum*, *P. austrosinense*, *P. kadsura*, *P. puberulum*, *P. boehmeriaefolium*, *P. sarmentosum*, *P. betle*, and three black pepper (*P. nigrum*) varieties Kuching, Lampong Type (Lampung Broad Leaf) and Panniyur-1 collected from Jianfengling (A1-A23, thus designated as Pop1), Diaoluoshan (B1-B19, Pop2), Limushan (C1-C5, Pop3), Wuzhishan (D1-D22, Pop4), Xinglong tropical botanical garden (E1-E2, Pop5), and Danzhou (F1-F3, Pop6) were included in our study (Table 1). Genomic DNA from leaves of 10 plants per accession was extracted and purified, as described by Jang and Liu (2009), and diluted to 50 ng/μL for use.

Table 1. Accession number (Acc. No.), species (and variety name) and sources of the *Piper* spp.

Acc. No.	Species	Source	Acc. No.	Species	Source
A1	*P. hainanense*	Jianfengling	B15	*P. curtipedunculum*	Diaoluoshan
A2	*P. hainanense*	Jianfengling	B16	*P. curtipedunculum*	Diaoluoshan
A3	*P. bonii*	Jianfengling	B17	*P. curtipedunculum*	Diaoluoshan
A4	*P. laetispicum*	Jianfengling	B18	*P. curtipedunculum*	Diaoluoshan
A5	*P. laetispicum*	Jianfengling	B19	*P. curtipedunculum*	Diaoluoshan
A6	*P. curtipedunculum*	Jianfengling	C1	*P. austrosinense*	Limushan
A7	*P. hainanense*	Jianfengling	C2	*P. curtipedunculum*	Limushan
A8	*P. curtipedunculum*	Jianfengling	C3	*P. curtipedunculum*	Limushan
A9	*P. laetispicum*	Jianfengling	C4	*P. austrosinense*	Limushan
A10	*P. hainanense*	Jianfengling	C5	*P. curtipedunculum*	Limushan
A11	*P. laetispicum*	Jianfengling	D1	*P. puberulum*	Wuzhishan
A12	*P. hainanense*	Jianfengling	D2	*P. kadsura*	Wuzhishan
A13	*P. laetispicum*	Jianfengling	D3	*P. puberulum*	Wuzhishan
A14	*P. laetispicum*	Jianfengling	D4	*P. kadsura*	Wuzhishan
A15	*P. bonii*	Jianfengling	D5	*P. kadsura*	Wuzhishan
A16	*P. hainanense*	Jianfengling	D6	*P. puberulum*	Wuzhishan
A17	*P. curtipedunculum*	Jianfengling	D7	*P. kadsura*	Wuzhishan
A18	*P. hainanense*	Jianfengling	D8	*P. puberulum*	Wuzhishan
A19	*P. hainanense*	Jianfengling	D9	*P. puberulum*	Wuzhishan
A20	*P. curtipedunculum*	Jianfengling	D10	*P. puberulum*	Wuzhishan
A21	*P. hainanense*	Jianfengling	D11	*P. puberulum*	Wuzhishan
A22	*P. hainanense*	Jianfengling	D12	*P. puberulum*	Wuzhishan
A23	*P. hainanense*	Jianfengling	D13	*P. puberulum*	Wuzhishan
B1	*P. curtipedunculum*	Diaoluoshan	D14	*P. puberulum*	Wuzhishan
B2	*P. hainanense*	Diaoluoshan	D15	*P. puberulum*	Wuzhishan
B3	*P. hainanense*	Diaoluoshan	D16	*P. puberulum*	Wuzhishan
B4	*P. hainanense*	Diaoluoshan	D17	*P. puberulum*	Wuzhishan
B5	*P. curtipedunculum*	Diaoluoshan	D18	*P. puberulum*	Wuzhishan
B6	*P. curtipedunculum*	Diaoluoshan	D19	*P. puberulum*	Wuzhishan
B7	*P. curtipedunculum*	Diaoluoshan	D20	*P. puberulum*	Wuzhishan
B8	*P. laetispicum*	Diaoluoshan	D21	*P. boehmeriaefolium*	Wuzhishan
B9	*P. curtipedunculum*	Diaoluoshan	D22	*P. austrosinense*	Wuzhishan
B10	*P. hainanense*	Diaoluoshan	E1	*P. nigrum* var. Lampong Type	Xinglong Tropical Botanical Garden
B11	*P. hainanense*	Diaoluoshan	E2	*P. nigrum* var. Panniyur-1	Xinglong Tropical Botanical Garden
B12	*P. curtipedunculum*	Diaoluoshan	F1	*P. sarmentosum*	Danzhou
B13	*P. curtipedunculum*	Diaoluoshan	F2	*P. betle*	Danzhou
B14	*P. curtipedunculum*	Diaoluoshan	F3	*P. nigrum* var. Kuching	Danzhou

RAPD fingerprinting

Nine selected 10-mer primers (Life Technologies, Shanghai) were used for PCR amplification (Table 2). The reaction mixture, with a 20-μL total volume, contained 1.0 U Taq DNA polymerase (TaKaRa Biotechnology Co., Ltd., Dalian, China), 6X PCR buffer, 0.4 mM dNTP, 0.25 μM primer, 1.5 mM Mg^{2+}, and 25 ng template DNA. PCR was performed in a thermocycler (Biometra T1 Thermocycle, German) with the following program: 5 min at 94°C for initial denaturing, followed by 6 cycles of 30 s at 94°C, 30 s at 32°C and 45 s at 72°C. The last cycle was followed by a 7-min extension at 72°C. Amplified products were analyzed on 2% (w/v) gels and visualized on Vilber Gel Documentation System (Viber Lourmat).

SRAP fingerprinting

Nine selected pairs of primers (Life Technologies) from 121 different primer combinations were used for detecting polymorphism in open reading frames (ORFs) (Table 3). Each 20-μL PCR mixture consisted of 1.0 U Taq DNA polymerase (TaKaRa Biotechnology),

6X PCR buffer, 0.6 mM dNTP, 0.35 µM primer, 1.5 mM Mg^{2+}, and 25-200 ng template DNA. Thermal cycling (Biometra T1 Thermocycle) started with 5 min at 94°C for initial denaturing, and 5 cycles of 30 s at 94°C, 30 s at 35°C, and 45 s at 72°C, followed by 40 cycles of 30 s at 94°C, 30 s at 4°C and 45 s at 72°C. The last cycle was followed by a 7-min extension at 72°C. Amplified products were analyzed on 2% (w/v) gels and visualized with the Vilber Gel Documentation System.

Data analysis

RAPD and SRAP analyzes were repeated twice and only clear bands produced both times were recorded for all samples. For each primer (RAPD) or primer combination (SRAP), the presence or absence of bands in each individual plants was visually scored. Data were set in a binary matrix, genetic similarities were calculated with NTSYS-pc (Numerical Taxonomy and Multivariate Analysis System) version 2.0 (Rohlf, 1998). Clustering analysis was conducted using the unweighted pair group method with arithematic mean (UPGMA). The mean Shannon diversity index (*I*), the mean Nei's (1978) genetic diversity (*H*), the mean number of alleles per locus (N_A), and the effective number of alleles per locus (N_E) were estimated by POPGENE version 1.32 (Yeh et al., 1997).

RESULTS

The 9 selected RAPD primers generated 111 amplified products, with a mean of 11.1 bands per primer, ranging from 9 (ba45, ba106 and s02) to 14 (ba01 and s20) (Figure 1, Table 2). Of the bands, 110 were polymorphic (99.1%) with the size of the bands ranging from 250 to 1000 bp. The mean *I* was 0.2822, the mean *H* was 0.1880, the mean N_A was 1.6436, and N_E was 1.3186.

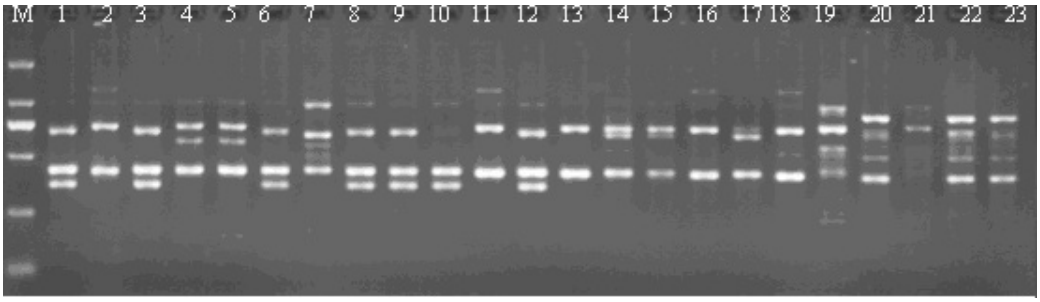

Figure 1. Gel electrophoresis of amplification products obtained with primer ba45. *Lane M* = molecular marker (DL2000); *lanes 1-23* = samples.

Analysis of the 74 individual plants of *Piper* spp with nine SRAP primer combinations identified 127 reproducible fragments, with an average of 14.1 bands per primer (Figure 2, Table 3). The highest number of fragments was 16 for the primers me9em1 and me8em10, and the lowest number of fragments was 11 for the primer me7em10. Amplified DNA fragments varied in size from 100 to 2000 bp. All 127 bands were polymorphic. The mean *I* was 0.2909, the mean *H* was 0.1947, the mean N_A was 1.5499, and N_E was 1.3331.

Table 2. RAPD primer sequences and the polymorphism of their products.

Primer order	Sequence	Total bands	Polymorphic bands	PPB (%)
ba01	CCGTCGGTAG	14	14	100
ba38	AGGTGACCGT	13	13	100
ba45	TCAGAGCGCC	9	8	88.9
ba106	ACACGTGGTC	9	9	100
s02	TGATCCCTGG	9	9	100
s08	GTCCACACGG	12	12	100
s14	TCCGCTCTGG	12	12	100
s20	GGACCCTTAC	14	12	100
s1172	CACTCTCCTC	10	10	100
Total		111	110	99.1

PPB = percentage of polymorphic bands.

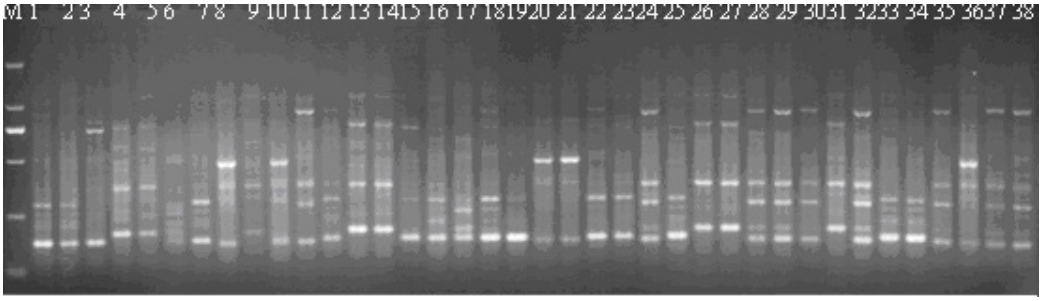

Figure 2. Gel electrophoresis of amplification products obtained with the primer combination me3em6. *Lane M* = molecular marker (DL2000); *lanes 1-38* = samples.

Table 3. SRAP primer sequences and the polymorphism of their products.

Primer order	Sequence	Total bands	Polymorphic bands	PPB (%)
me3em6	me3 TGAGTCCAAACCGGACC em6 GACTGCGTACGAATTGAC	15	15	100
me3em9	me3 TGAGTCCAAACCGGACC em9 GACTGCGTACGAATTGCA	13	13	100
me7em10	me7 TGAGTCCAAACCGGAAG em10 GACTGCGTACGAATTCAA	11	11	100
me8em1	me8 TGAGTCCAAACCGGTAA em1 GACTGCGTACGAATTTGC	14	14	100
me8em10	me8 TGAGTCCAAACCGGTAA em10 GACTGCGTACGAATTCAA	16	16	100
me9em1	me9 TGAGTCCAAACCGGTCC em1 GACTGCGTACGAATTTGC	16	16	100
me2em9	me2 TGAGTCCAAACCGGAGC em9 GACTGCGTACGAATTGCA	14	14	100
me5em4	me5 5TGAGTCCAAACCGGTGT em4 GACTGCGTACGAATTCCA	14	14	100
me4em8	me4 TGAGTCCAAACCGGTAG em8 GACTGCGTACGAATTAAC	14	14	100
Total		127	127	100

PPB = percentage of polymorphic bands.

The calculated genetic similarity coefficient for RAPD and SRAP markers ranged from 0.486 to 0.991, with a mean of 0.7385 and from 0.5197 to 1.000, with an average of 0.7599, respectively. These results indicated that these species are closely related. In more

detail, genetic similarity values based on RAPD and SRAP markers ranged from 0.806-0.939 and 0.784-0.978 in *P. hainanense* (with a genetic distance of 0.133 and 0.194), 0.664-0.955 and 0.708-0.942 in *P. curtipedunculum* (0.291 and 0.234), 0.664-0.930 and 0.769-1.000 in *P. laetispicum* (0.266 and 0.231), 0.0.806-0.991 and 0.821-0.959 in *P. puberulum* (0.185 and 0.138), 0.874-0.971 and 0.822-0.951 in *P. kadsura* (0.097 and 0.129), 0.892-0.955 and 0.822-0.918 in *P. nigrum* (0.063 and 0.096), and 0.841-0.912 and 0.708-0.886 in *P. austrosinense* (0.071 and 0.178), respectively.

The dendrogram generated with the RAPD markers was topologically different from the dendrogram based on SRAP markers (Figures 3 and 4). The dendrogram based on the RAPD data grouped the species into five clusters at a genetic similarity of 0.66: *P. hainanense* and *P. bonii* in one cluster, *P. austrosinense*, *P. puberulum* and *P. boehmeriaefolium* in one, *P. nigrum*, *P. sarmentosum* and *P. betle* in one, *P. laetispicum* and *P. curtipedunculum* in one, and *P. kadsura* in one. In the dendrogram generated with SRAP markers, at genetic similarity of 0.68, *P. hainanense*, *P. bonii* and *P. curtipedunculum* shared a group, *P. puberulum*, *P. boehmeriaefolium*, *P. sarmentosum*, *P. betle* and *P. nigrum* were placed in a group, and *P. laetispicum*, *P. kadsura* and *P. austrosinense* grouped separately in three different groups. Both dendrograms separated *P. nigrum*, *P. sarmentosum* and *P. betle* from the rest, but the dendrogram based on the RAPD data did not differentiate *P. curtipedunculum* from *P. laetispicum*. In contrast, the dendrogram based on SRAP markers unambiguously distinguished all *Piper* spp from each other. The high level of genetic variation detected by SRAP was consistent with the observed morphological variability, and the results obtained

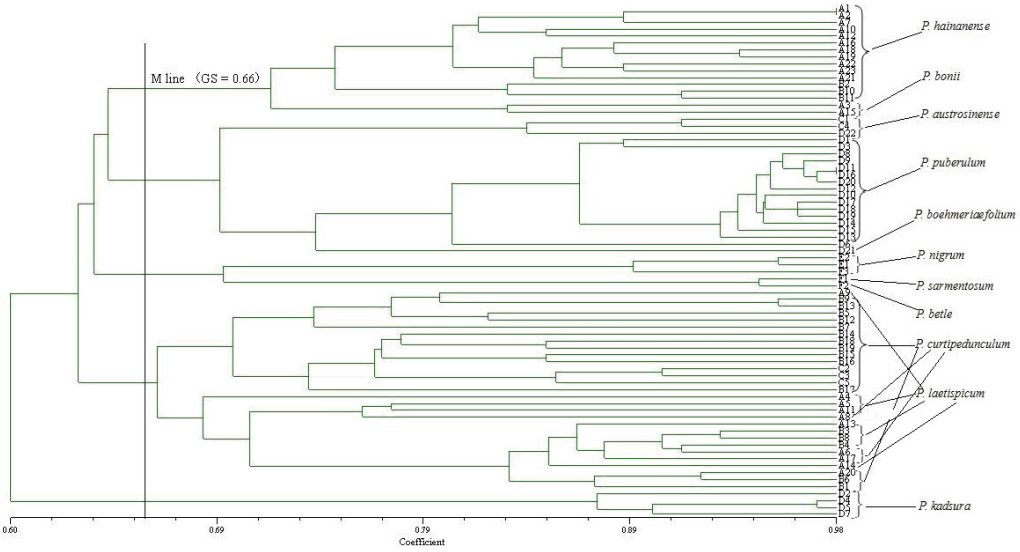

Figure 3. The UPGMA dendrogram illustrating the genetic relationships between the 74 accessions based on RAPD analysis. *Piper hainanense* (A1, A2, A7, A10, A12, A16, A18, A19, A21, A22, A23, B2, B3, B4, B10, B11), *P. bonii* (A3, A15), *P. laetispicum* (A4, A5, A9, A11, A13, A14, B8), *P. curtipedunculum* (A6, A8, A17, A20, B1, B5, B6, B7, B9, B12, B13, B14, B15, B16, B17, B18, B19, C2, C3, C5), *P. austrosinense* (C1, C4, D22), *P. puberulum* (D1, D3, D6, D8, D9, D10, D11, D12, D13, D14, D15, D16, D17, D18, D19, D20), *P. kadsura* (D2, D4, D5, D7), *P. boehmeriaefolium* (D21), *P. nigrum* (E1, E2, F3), *P. sarmentosum* (F1), and *P. betle* (F2).

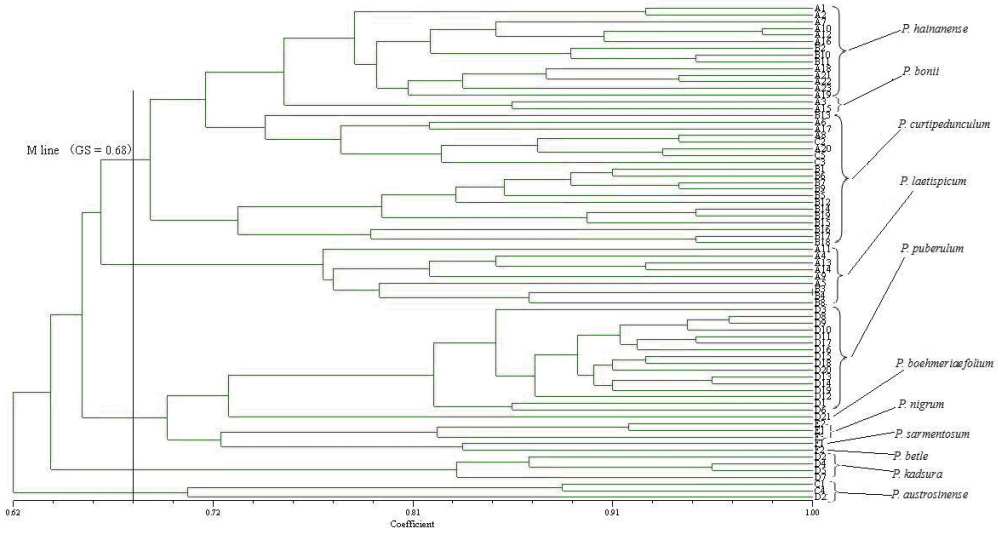

Figure 4. The UPGMA dendrogram illustrating the genetic relationships between the 74 accessions based on SRAP analysis. *Piper hainanense* (A1, A2, A7, A10, A12, A16, A18, A19, A21, A22, A23, B2, B3, B4, B10, B11), *P. bonii* (A3, A15), *P. laetispicum* (A4, A5, A9, A11, A13, A14, B8), *P. curtipedunculum* (A6, A8, A17, A20, B1, B5, B6, B7, B9, B12, B13, B14, B15, B16, B17, B18, B19, C2, C3, C5), *P. austrosinense* (C1, C4, D22), *P. puberulum* (D1, D3, D6, D8, D9, D10, D11, D12, D13, D14, D15, D16, D17, D18, D19, D20), *P. kadsura* (D2, D4, D5, D7), *P. boehmeriaefolium* (D21), *P. nigrum* (E1, E2, F3), *P. sarmentosum* (F1), and *P. betle* (F2).

with SRAP analysis were in accordance with the morphological classification. In addition, the dendrograms clearly indicated that there was no correlation between the marker-based grouping pattern and geographical origin.

Seventy-four accessions were collected from Jianfengling (A1-A23, designated as Pop1), Diaoluoshan (B1-B19, Pop2), Limushan (C1-C5, Pop3), Wuzhishan (D1-D22, Pop4), Xinglong Tropical Botanical Garden (E1-E2, Pop5), and Danzhou (F1-F3, Pop6). To estimate genetic variation levels of each population, all the statistical analyses were performed using the POPGENE program (version 1.32) (Tables 4 and 5). Based on RAPD and SRAP data, the observed N_A within the populations ranged from 1.0450 to 1.8739 and from 1.0866 to 1.7795, with the mean observed N_A being 1.9910 and 2.0000, respectively. The N_E within the populations ranged from 1.0450 to 1.4983 and from 1.0866 to 1.4425, with the mean N_E being 1.5718 and 1.5202, respectively. The *H* within the populations ranged from 0.0225 to 0.2947 and from 0.0433 to 0.2632, with the mean *H* being 0.3327 and 0.3123, respectively. The *I* within the populations ranged from 0.0312 to 0.4445 and from 0.0600 to 0.3977, with the mean *I* being 0.4994 and 0.4752, respectively. Estimation with both RAPD and SRAP markers showed that the lowest value of within-population variation was in Pop5. Overall, the populations included in this study showed a relatively high level of genetic diversity. The fact that Pop1 and Pop2 clustered together in the dendrograms demonstrated that they are closely related, while the genetic distance between Pop1 and Pop6 was the largest (Figures 5 and 6).

Table 4. Descriptive statistics based on RAPD data for 6 *Piper* populations in Hainan, China.

Populations	Sample size	N_A	N_E	H	I
Pop1	23	1.8739	1.4983	0.2947	0.4445
Pop2	19	1.7748	1.4391	0.2651	0.4008
Pop3	5	1.4414	1.3463	0.1903	0.2738
Pop4	22	1.7748	1.3016	0.1994	0.3193
Pop5	2	1.0450	1.0450	0.0225	0.0312
Pop6	3	1.3514	1.2811	0.1562	0.2236
Mean	74	1.9910	1.5718	0.3327	0.4994
SD		0.0949	0.3301	0.1514	0.1883

N_A = observed number of alleles; N_E = effective number of alleles; H = Nei's gene diversity; I = Shannon information index; Pop1 = population from Jianfengling; Pop2 = population from Diaoluoshan; Pop3 = population from Limushan; Pop4 = population from Wuzhishan; Pop5 = population from Xinglong Tropical Botanical Garden; Pop6 = population from Danzhou.

Table 5. Descriptive statistics based on SRAP data for 6 *Piper* populations in Hainan, China.

Populations	Sample size	N_A	N_E	H	I
Pop1	23	1.7795	1.4358	0.2593	0.3924
Pop2	19	1.7795	1.4425	0.2632	0.3977
Pop3	5	1.5512	1.4055	0.2280	0.3315
Pop4	22	1.7559	1.3512	0.2204	0.3434
Pop5	2	1.0866	1.0866	0.0433	0.0600
Pop6	3	1.3465	1.2772	0.1540	0.2205
Mean	74	2.0000	1.5202	0.3123	0.4752
SD		0.0000	0.3121	0.1491	0.1886

For abbreviations, see legend to Table 4.

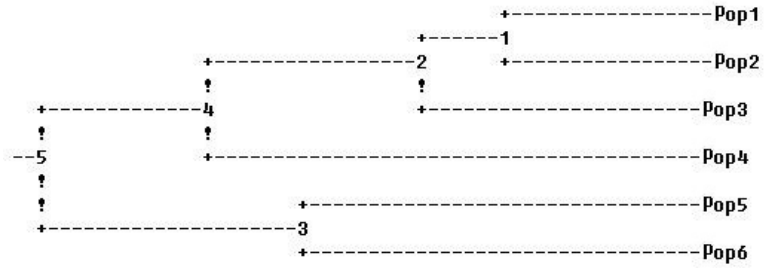

Figure 5. Dendrogram illustrating the genetic distance between six populations of *Piper* spp in Hainan, China, based on RAPD analysis.

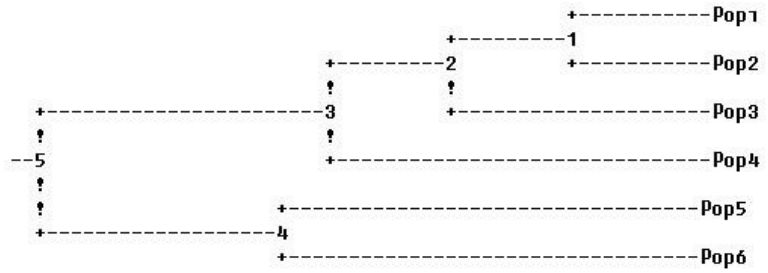

Figure 6. Dendrogram illustrating the genetic distance between six populations of *Piper* spp in Hainan, based on SRAP analysis.

DISCUSSION

RAPD is one of the most widely used techniques for analysis of plant genetic diversity. Pradeepkumar et al. (2003) assessed genetic variation in 22 Indian cultivars of black pepper, including *P. longum* and *P. colubrinum*, using RAPD markers. In our study, the PPB (percentage of polymorphic bands) and the mean number of bands per primer detected with SRAP (100 and 14.1%) were higher than those detected with RAPD (99.1 and 11.1%). Moreover, the dendrogram based on the RAPD data did not discriminate *P. curtipedunculum* from *P. laetispicum*, but the dendrogram based on SRAP markers clearly distinguished all *Piper* spp from each other. The results show that the SRAP technique can be more informative and more efficient and effective for studying genetic diversity than the RAPD technique. The ability to evaluate genetic variation among species at the molecular level is directly related to the number of polymorphisms detected and their reproducibility (Nguyen et al., 2004). The RAPD technique involves the amplification of random segments of genomic DNA using random primers and a low annealing temperature, which allows non-specific binding to the DNA. SRAP markers preferentially amplify ORFs, which may include coding regions of the genome involved in morphological and agronomic traits, and likely reflect differences in coding sequences, which are thought to be relatively conserved among species (Ferriol et al., 2003b).

The high mean I values (0.2822 and 0.2909), the mean H (0.1880 and 0.1947), the mean N_A (1.6436 and 1.5499), and the N_E (1.3186 and 1.3331) estimated with RAPD and SRAP data, respectively, compared with those for plants in general, indicated that the level of genetic diversity among individual plants of *Piper* spp in Hainan is high and conservation of wild pepper germplasm has been effective (Zheng, 1999; Nybom, 2004). The genetic similarity among three cultivars (Lampong Type, Panniyur-1, Kuching) of *P. nigrum* introduced from abroad was higher than that of the *Piper* spp naturally distributed in Hainan, demonstrating that breeding practices tend to narrow the genetic base.

Evaluation of genetic variation levels of six populations (Pop1 from Jianfengling, Pop2 from Diaoluoshan, Pop3 from Limushan, Pop4 from Wuzhishan, Pop5 from Xinglong Tropical Botanical Garden, and Pop6 from Danzhou) showed that the effective number of alleles, Nei's gene diversity and Shannon information index within Pop1 and Pop2 were higher than those elsewhere; consequently, conservation of wild resources of *Piper* in these two regions should take priority. The high genetic variability observed in these regions confirms their distinction as centers of diversity of *Piper* (Zheng, 1999).

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