African Journal of Biotechnology Vol. 10(66), pp. 14731-14737, 26 October, 2011
Available online at http://www.academicjournals.org/AJB
DOI: 10.5897/AJB11.2342
ISSN 1684–5315 © 2011 Academic Journals

Full Length Research Paper

Analysis of genetic diversity of *Piper* spp. in Hainan Island (China) using inter-simple sequence repeat ISSR markers

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Accepted 28 September, 2011

Inter-simple sequence repeat (ISSR) analysis was used to evaluate the genetic variation of *Piper* spp. from Hainan, China. 247 polymorphic bands out of a total of 248 (99.60%) were generated from 74 individual plants of *Piper* spp. The overall level of genetic diversity among *Piper* spp. in Hainan was high, with the mean Shannon information index (I) of 0.2843 and the mean Nei's genetic diversity (H) of 0.1904. The genetic similarity (GS) coefficient ranged from 0.548 to 0.976 within 74 individual plants of *Piper* spp., and the within-species genetic distance ranged from 0.104 to 0.28. Unweighted pair group method with arithmetic mean (UPGMA) dendrogram showed that *P. kadsura* is the most divergent and the most distant of the 11 species, and that *P. hainanense* and *P. bonii*, are closely related as well as *P. sarmentosum* and *P. betle*. The diversity analysis unambiguously distinguished all *Piper* spp. The high levels of genetic diversity in Jianfengling and Diaoluoshan demonstrate that conservation of wild resources of *Piper* in these two localities is more effective than that in Limushan, Wuzhishan, Xinglong Tropical Botanical Garden and Danzhou.

Key words: Black pepper, *Piper*, genetic variation, molecular marker, inter-simple sequence repeat.

INTRODUCTION

The genus *Piper* is the largest in the family Piperaceae and contains more than 3000 species reported from the tropical and subtropical regions around the world (Rahiman and Nair, 1983). About 60 species were recorded in China, and 11 to 12 species in Hainan Island (Cheng et al., 1999; Chen 1964; Chen et al., 1992; Zheng, 1999). Black pepper (*P. nigrum* L.) the most important commercial species, was introduced into Hainan, China, in 1947 from Indonesia. Black pepper production in Hainan is estimated over 80% in China, one of the main pepper producing countries in the world. Wild *Piper* spp. possibly has resistant traits such as the resistance to foot rot disease caused by *Phytophthora capsici* (*Phytophthora palmivora* MF4), which may be useful in variety improvement of black pepper. Moreover, the fruits and roots of most *Piper* species are widely used in Chinese medicines and indigenous medicines. The leaves of *P. betle* along with other ingredients like areca nut and lime are used as "chewing gum" by local people and the leaves of *P. sarmentosum* are used as vegetable and spice (Liu, 2010). In recent years, expansion of rubber tree cultivation resulted in a decline of wild pepper resources in this region; hence investigation on the diversity of *Piper* spp. using molecular markers is of practical importance in programs of black pepper's breeding, local use and conservation of *Piper* genetic resources.

Inter-simple sequence repeat (ISSR) was first employed by Zietkiewicz et al. (1994) and Gupta et al. (1994) and has been proved to be a highly useful tool for estimating genetic diversity and assessing genetic relationships because it is simple, fast, cost-effective, reliable and highly discriminating (Ci et al., 2008; Crespe et al., 2009; Zhang and Dai, 2010; Uysal et al., 2010; Petros et al., 2008). A few genetic studies using

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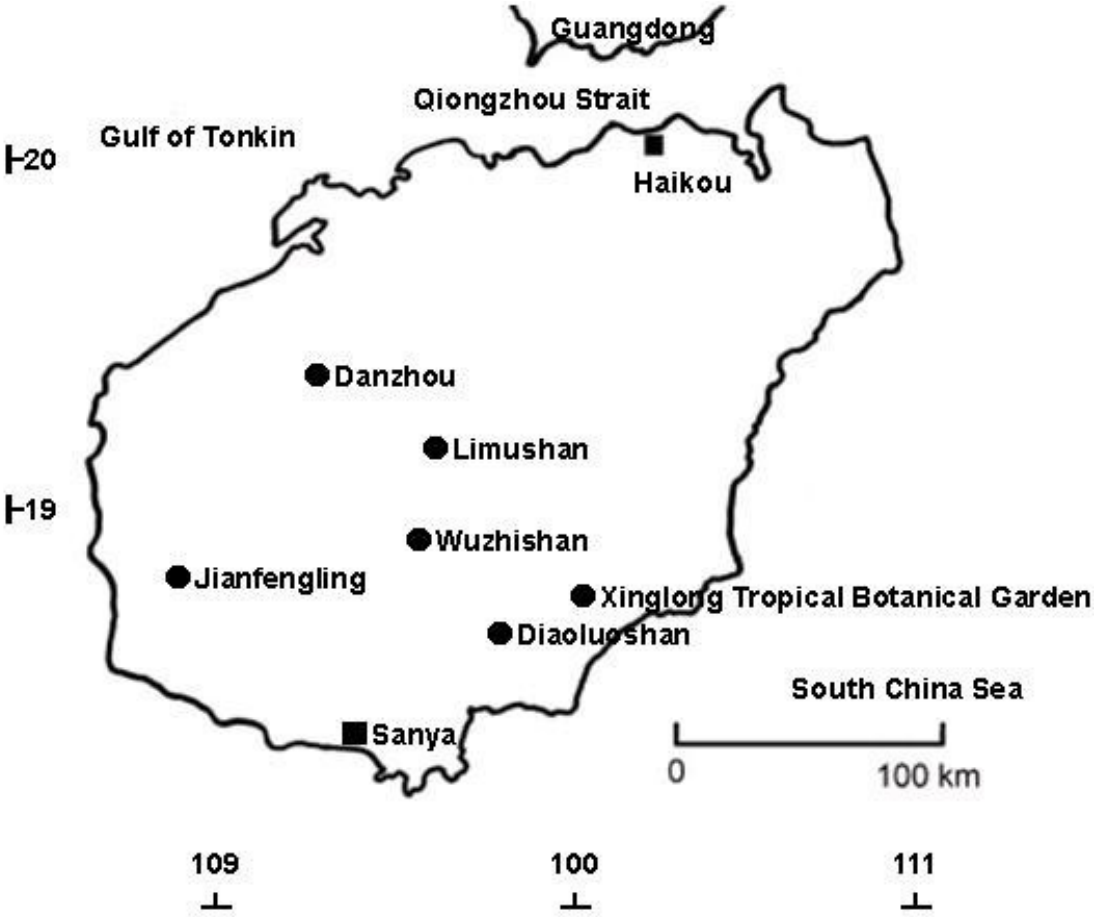

Figure 1. Locations of *Piper* spp. examined in this study. ■ Major cities in Hainan Island; ●, sites of the 74 accessions collected for which latitude and longitude coordinates were available.

molecular markers such as random amplified polymorphic DNA (RAPD) and amplified fragment length polymorphism (AFLP) have been conducted on the analysis of genetic diversity within Indian cultivars of black pepper (*P. nigrum* L.) (Pradeepkumar et al., 2003; Joy et al., 2007). However, there is no report on the use of any molecular marker technique in the genus *Piper* in China. The objective of this study therefore was to evaluate the genetic diversity among the *Piper* spp. using ISSR markers.

MATERIALS AND METHODS

Plant materials and DNA extraction

71 accessions representing ten wild *Piper* species (*P. hainanense*, *P. bonii*, *P. laetispicum*, *P. curtipedunculum*, *P. austrosinense*, *P. kadsura*, *P. puberulum*, *P. boehmeriaefolium*, *P. sarmentosum* and *P. betle)* were collected from five sites in Hainan Island and propagated in our greenhouse. Three cultivated black pepper (*P. nigrum*) varieties/accessions (Kuching, Lampong Type and Panniyur-1) were collected from plantations in Hainan Island. The collecting origins of the 74 accessions investigated are given in Figure 1 and Table 1. Genomic DNA from leaves of ten plants per accession studied was extracted and purified as described by Jiang and Liu (2009) and diluted to 50 ng/µl working solution for use.

Optimization of ISSR-PCR reaction conditions and selection of primers

The conditions of ISSR-PCR reaction were optimized using the method of single-factor experiment. The optimal ISSR-PCR amplification was established as follows: 20 µl reaction mixture containing 1.0 U Taq DNA polymerase, 0.8 mM of each dNTP, 0.2 mM primer, 1.8 mM $MgCl_2$, and 50 ng template DNA for 35 thermal cycles. Amplifications were performed in a Biometra T Gradient Thermal Cycler using the following conditions: initial denaturation at 95°C for 5 min; denaturation at 94°C for 30 s; annealing at the optimum anneal temperature selected for 30 s; extension at 72°C for 45 s; 35 cycles; the last cycle followed by 7 min extension at 72°C.

For primer selection, and 90 ISSR primers (University of British Columbia, UBC) were employed for amplification with the template DNA of *P. sarmentosum* Roxb., *P. betle* Linn., and *P. nigrum* L. var. Kuching. Out of the 90 primers tested, 19 that gave satisfactory results were used for the genetic diversity analysis of all plant materials. The optimum annealing temperatures for each primer are shown in Table 2.

Table 1. Species (and variety name), collecting region and accession number (Acc. no.) of plant materials analyzed.

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

Data analysis

These primers were selected on the basis of the robustness of amplification, clarity and scorability of banding patterns. ISSR analyses were repeated twice and only clear bands produced twice were recorded for all samples. The ISSR bands were visually scored as either present (1) or absent (0) for each accession and each primer combination. The binary matrix was then used to measure pair-wise genetic distance using Nei's (1978) unbiased genetic distance with POPGENE version 1.32 (Yeh et al., 1997). Genetic similarity (GS) coefficients were calculated by the numerical taxonomy and multivariate analysis system (NTSYS-pc) version 2.0 (Rohlf, 1998). Clustering analysis was conducted using the UPGMA.

RESULTS

Analysis of genetic diversity

The 19 selected primers generated 248 bands of different sizes for 74 accessions of genus *Piper* with an average of 13.1 polymorphic bands per primer. Maximum number of fragments (16 bands) was found with primer UBC835, whereas the smallest number (ten bands) was generated by primer UBC864. 247 out of 248 fragments were polymorphic, with 99.60% of mean percentage of polymorphic bands (PPB) (Figure 2). The mean Shannon's information index (I) was 0.2843, the mean Nei's genetic diversity (H) was 0.1904, the average number of alleles per locus (Na) was 1.5376 and the effective number of alleles per locus (NE) was 1.3256.

The calculated GS coefficients of all accessions ranged from 0.548 to 0.976, with a mean value of 0.762. The maximum coefficients of genetic similarity was between two specimens of *P. hainanense* (A10 and A12), the next was between two *P. puberulum* (D8 and D9) and the minimum was between *P. betle* (F2) and *P. hainanense* (A7) (data not shown). In further details, genetic similarity values were 0.775 to 0.976 in *P. hainanense* with the genetic distance of 0.201, 0.685 to 0.965 in *P. curtipedunculum* with the genetic distance of 0.28, 0.757 to 0.953 in *P. laetispicum* with the genetic distance of 0.196, 0.819 to 0.976 in *P. puberulum* with the genetic distance of 0.157, 0.863 to 0.963 in *P. kadsura* with the genetic distance of 1, 0.831 to 0.935 in *P. nigrum* with the

Table 2. ISSR primer sequences, their PCR annealing temperature and the polymorphism of their products.

Primer order	Sequence	Annealing temperature (℃)	Total band	Polymorphic band	PPB (%)
UBC808	(AG)8C	53.6	15	15	100
UBC809	(AG)8G	53.6	13	13	100
UBC818	(CA)8G	51.5	12	11	91.8
UBC826	(AC)8C	51.5	13	13	100
UBC827	(AC)8G	51.5	12	12	100
UBC829	(TG)8C	51.5	15	15	100
UBC834	(AG)8YT	53.6	13	13	100
UBC835	(AG)8YC	53.6	16	16	100
UBC836	(AG)8YA	51.5	13	13	100
UBC842	(GA)8YG	53.6	14	14	100
UBC844	(CT)8RC	49.2	11	11	100
UBC846	(CA)8RT	53.6	14	14	100
UBC848	(CA)8RG	49.2	12	12	100
UBC854	(TC)8RG	50.1	13	13	100
UBC856	(AC)8YA	50.1	14	14	100
UBC857	(AC)8YG	50.1	12	12	100
UBC864	(ATG)6	50.1	10	10	100
UBC868	(GAA)6	50.1	14	14	100
UBC880	(GGAGA)3	48	12	12	100
Total			248	247	99.60

Y = C/T; R = A/G; PPB: the mean percentage of polymorphic bands.

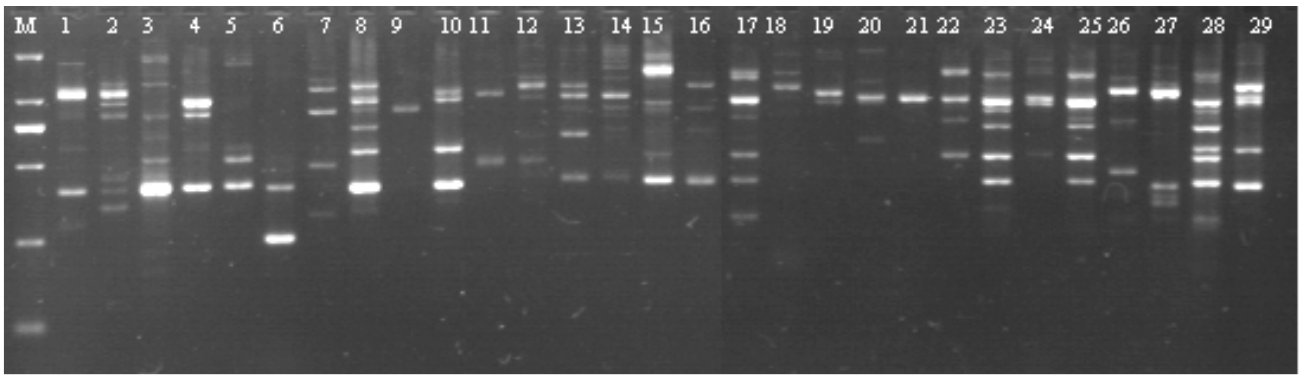

Figure 2. Examples of molecular diversity of some accessions of *Piper* spp. in Hainan determined by ISSR markers obtained with primer UBC844. M, Marker (DL2000); 1 – 23, A1 - 23; 24 – 29, B1-6.

genetic distance of 0.104, and finally 0.703 to 0.863 in *P. austrosinense* with the genetic distance of 0.16. UPGMA dendrogram generated from the similarity matrix is depicted in Figure 3. The dendrogram clustered ten (*P. hainanense*, *P. bonii*, *P. laetispicum*, *P. curtipedunculum*, *P. austrosinense*, *P. puberulum*, *P. boehmeriaefolium*, *P. sarmentosum*, *P. betle*, and *P. nigrum*) out of the 11 species of *Piper* in one major group, with *P. kadsura* the first to branch out at a genetic distance of 0.63. Within the main group, three sub-cluster groups were defined at a genetic distance of 0.65 with *P. hainanense*, *P. Bonii* and *P. curtipedunculum* located in one sub-cluster group, *P. laetispicum* and *P. austrosinense* in another, and *P. puberulum*, *P. boehmeriaefolium*, *P. sarmentosum*, *P. betle* and *P. nigrum* in the last one. At a genetic distance of 0.67, 74 species were clustered into seven subgroups, except for *P. hainanense* and *P. bonii* that were placed in one, *P. sarmentosum* and *P. betle* in another, while the remaining clustered in separate subgroups. In addition, *P. hainanense* and *P. bonii* were distinctly divided from each other at a genetic distance of 0.74, while *P. betle*, and *P. nigrum* were distinguished from each other at a genetic distance of 0.85.

The observed number of alleles (NA) within the localities ranged from 1.0645 to 1.8024, with the overall observed number of alleles been 1.9960 (Table 3). The

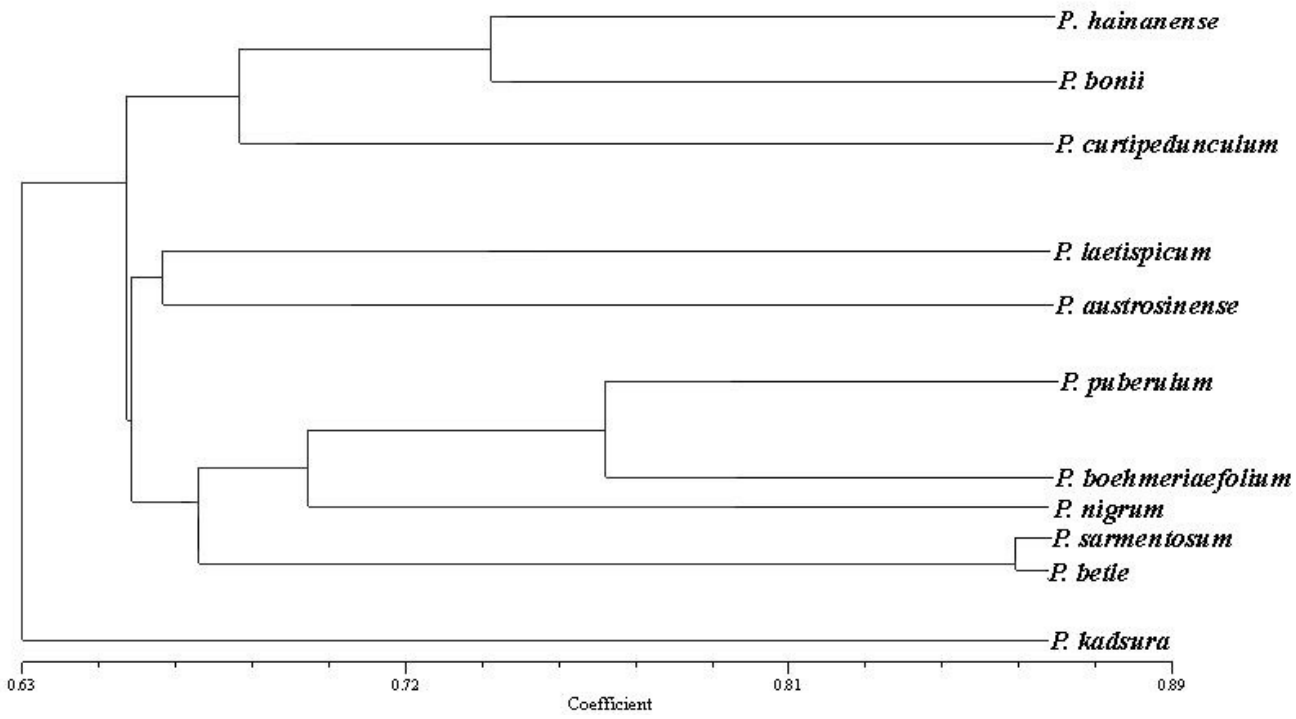

Figure 3. UPGMA dendrogram illustrating the genetic relationships between the 11 species based on ISSR analysis.

Table 3. Descriptive statistics based on ISSR data for the six localities from which the *Piper* accessions were collected in Hainan Island, China.

Localities	Acession size	NA	NE	H	I
Jianfenglinling	23	1.8024	1.4514	0.2678	0.4047
Diaoluoshan	19	1.7903	1.4454	0.2655	0.4014
Limushan	5	1.4960	1.3648	0.2052	0.2983
Wuzhishan	22	1.7016	1.3304	0.2064	0.3209
Xinglong Tropical Botanical Garden	2	1.0645	1.0645	0.0323	0.0447
Pop6	3	1.3710	1.2968	0.1649	0.2361
Overall/Mean	74	1.9960	1.5211	0.3121	0.4744
St.Dev		0.0635	0.3135	0.1507	0.1913

* NA = Observed number of alleles; NE = effective number of alleles; H = Nei's gene diversity; I = Shannon's information index.

effective number of alleles (NE) within the localities ranged from 1.0645 to 1.4514, with the overall effective number of alleles of 1.5211. The Nei's gene diversity (H) within the localities ranged from 0.0323 to 0.26787, with the overall Nei's gene diversity being 0.3121. Also, the Shannon's Information index (I) within the localities ranged from 0.0447 to 0.4047, with the overall Shannon Information index being 0.4744. The highest value of within-locality variation was observed in Jianfenglinling, and the lowest in Xinglong Tropical Botanical Garden. Jianfenglinling showed the highest level of genetic diversity because four species (*P. hainanense*, *P. bonii*, *P. laetispicum* and *P. curtipedunculum*) were collected from that locality, while in Xinglong Tropical Botanical Garden, only two black pepper cultivars were collected. Overall, the localities included in this study showed relatively high levels of genetic diversity. The dendrogram generated according to geographic locations where the accessions collected indicate that Jianfenglinling, Diaoluoshan, Limushan and Wuzhishan formed a group, with Jianfenglinling and Diaoluoshan being closely clustered together (Figure 4), while Tropical Botanical Garden and Danzhou formed another group.

DISCUSSION

Herein, we presented the optimization of PCR

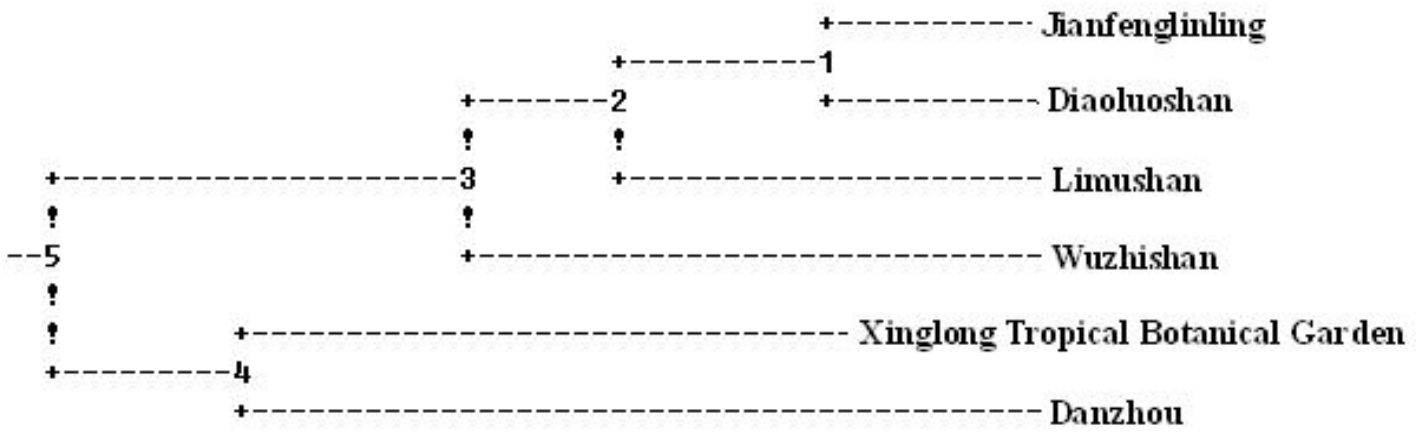

Figure 4. Dendrogram illustrating genetic distances between *Piper* spp of the six localities in Hainan Island based on ISSR analysis.

components and conditions for ISSR analysis of *Piper* spp. This allows a more precise estimate of genetic variation than that based on morphological characters. The 19 selected primers generated 248 bands of different sizes among the 74 accessions of genus *Piper*, with an average of 13.1 polymorphic bands per primer. 247 out of 248 fragments were detected to be polymorphic with the mean percentage of polymorphic bands (PPB) of 99.60%. The relatively high level of polymorphism observed and their reproducibility in this study are evidences to the robustness of the ISSR technique.

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. In this study, *P. hainanense*, *P. bonii*, *P. laetispicum*, *P. curtipedunculum*, *P. austrosinense*, *P. puberulum*, *P. boehmeriaefolium*, *P. sarmentosum*, *P. betle* and *P. kadsura* were naturally distributed in Hainan, China, and three cultivars (Lampong Type, Panniyur-1 and Kuching) of *P. nigrum* were introduced from abroad. Pradeepkumar et al. (2003) reported the analysis of genetic diversity and relationships in *Piper* (*P. longum*, *P. colubrinum* and 22 Indian cultivars of *P. nigrum*) using RAPD markers, but none of the wild populations from China were included.

ISSR analysis showed that *P. hainanense* was more closely related to *P. bonii*, and *P. sarmentosum* was most closely related to *P. betle,* but *P. kadsura* was distantly related to the rest (Figure 3). The genetic distance within each species naturally distributed was higher than that within *P. nigrum* of three main cultivars in China. This is in accordance with the conclusions of Pradeepkumar et al. (2003), since cultivated varieties of black pepper propagated by cutting and modern breeding practice tend to narrow the genetic base of the species. Generally, the genetic diversity of species is influenced largely by the reproductive mode of the species. The wild species of *Piper* are dioecious (Krishnamurthi, 1996) and under natural conditions, long-term outcrossing may lead to a gradual increase in the genetic variation among individuals of wild *Piper* spp.

Furthermore, evaluation of genetic variation levels of the plants from the six localities indicate that the total genetic diversity was relatively high, and the statistical parameters such as the effective number of alleles (NE), the Nei's gene diversity (H) and the Shannon information index (I) were significantly higher than those for the general plants (Nybom, 2004).

Relatively, the effective NE, the H and the I within Jianfenglinling and Diaoluoshan were higher than those within other localities, and this showed that conserving maximum diversity in *Piper* in these two localities is more effective than that in other localities. The major reason for the loss of genetic diversity of *Piper* may be attributed to the accelerated deforestation driven by the demand for monoculture of commercial plantations, especially rubber plantation. Natural rubber acreage in Hainan Island increased from 368,900 ha in 2002 to 415,100 ha in 2006 (Huang, 2007), and it is estimated that the planting area of natural rubber trees in Hainan Island exceeded 480,002 ha in 2010. Most wild plants of *Piper* are climbers and adapted to the moist and shaded habitats. Thus, rubber plantation replaced the natural forests and destroyed wild *Piper* resources found in the natural forests. In addition, intemperate consumption of wild plants of *Piper* as Chinese medicines and indigenous medicines by local people may be another reason. More also, the fact that Jianfenglinling and Diaoluoshan were clustered in ISSR dendrogram demonstrated that the accessions from these localities has a close genetic relationship.

This may be attributed to the fact that Jianfenglinling and Diaoluoshan share similar habitats and sufficient numbers of accessions with greater species coverage were collected there. In conclusion, the results of this study clearly demonstrate that ISSR markers can be reliably used for quantification of genetic diversity and relationships, species identification and evaluation of conservation status of wild resources of *Piper*.

ACKNOWLEDGEMENTS

The authors thank Dr. Wu of the Xinglong Tropical Botanical Garden for providing the plant materials used in this study. This research was funded by the National Natural Science Foundation of China (31060204).

REFERENCES

Cheng Y-Q, Xia N-H, Gilbert MG (1999). Piperaceae. In: Wu Z, Raven PH. eds. Flora of China Vol. 4. Beijing: Science Press; St Louis: Missouri Botanical Garden Press, pp. 110-131.

Chen F-B, Zhang J-X, Lin Y-E, Wang Q-H, Pan X-F (1992). Survey reports of Black pepper, coffee, cocoa germplasm resources in Hainan Island. In: South China Institute of Tropical Crop Science, Institute of Crop Germplasm Resources (Chinese Academy of Agricultural Sciences) eds. The Investigation Collection of Hainan Island Crop (Plant) Germplasms. Beijing: Agriculture Press, pp. 67-85.

Chen H-Y (1964). Flora of Hainan. Beijing, Science Press, I: 330-338.

Ci X-Q, Chen J-Q, Li Q-m, Li J (2008). AFLP and ISSR analysis reveals high genetic variation and inter-population differentiation in fragmented populations of the endangered *Litsea szemaois* (Lauraceae) from Southwest China. Plant Syst. Evol. 273: 237-246.

Crespe L, Pernet A, Bris ML, Gudin S, Hibrand-Saint Oyant L (2009). Application of ISSRs for cultivar identification and assessment of genetic relationships in rose. Plant Breeding 128: 501-506.

Gupta M, Chyi Y-S, Romero-Severson J, Owen JL (1994). Amplification of DNA markers from evolutionarily diverse genomes using single primers of simple-sequence repeats. Theor. Appl. Genet. 89: 998-1006.

Huang X-J (2007). Domestic and international market conditions of natural rubber (a). China Rubber, 14: 18-20.

Jiang Y, Liu J-P (2009). A comparison of extraction methods of genomic DNA from black pepper. Biotechnology, 19: 41-44.

Joy N, Abraham Z, Soniya EV (2007). A preliminary assessment of genetic relationships among agronomically important cultivars of black pepper. BMC Genet. 8: p. 42.

Liu J-P (2010). Development and utilization of wild pepper resources in Hainan. China Trop. Agric. 2: 35-36

Nei M (1978). Estimation of average heterozygosity and genetic distance from a small number of individuals. Genetics, 89: 533-590.

Petros Y, Merker A, Zeleke H (2008). Analysis of genetic diversity and relationships of wild *Guizotia* species from Ethiopia using ISSR markers. Genet. Resour. Crop Evol. 55: 451-458

Pradeepkumar T, Karihaloo JL, Archak S, Baldev A (2003). Analysis of genetic diversity in *Piper nigrum* L. using RAPD markers. Genet. Resour. Crop Evol. 50: 469-475.

Rohlf FJ (1998). NTSYS-pc: Numerical Taxonomy and Multivariate Analysis System, vers. 2.0. Applied Biostatistics Inc., New York.

Uysal H, Fu Y-B, Kurt O, Peterson GW, Diederichsen A, Kusters P (2010). Genetic diversity of cultivated flax (*Linum usitatissimum* L.) and its wild progenitor pale flax (*Linum bienne Mill.*) as revealed by ISSR markers. Genet. Resour. Crop Evol. 57: 1109-1119.

Yeh FC, Yang RC, Boyle TBJ, Ye Z-H, Mao JX (1997). POPGENE, the User-Friendly Shareware for Population Genetic Analysis. Molecular Biology and Biotechnology Centre, University of Alberta, Canada. http://www.ualberta.ca/~fyeh/

Zhang L-J, Dai S-L (2010). Genetic variation within and among populations of *Orychophragmus violaceus* (Cruciferae) in China as detected by ISSR analysis. Genet. Resour. Crop Evol. 57: 55-64.

Zheng W-Q (1999). Germplasm resources of wild pepper and their use. Guangxi Sci. Technol. Trop. Crops 73: 12-13, 35.

Zietkiewicz E, Rafalski A, Labuda D (1994). Genome fingerprinting by simple sequence repeat (SSR)-anchored polymerase chain reaction amplification. Genomics, 20: 176-183.