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Full Length Research Paper

Population structure of *Euodia rutaecarpa* in China revealed by amplified fragment length polymorphism (AFLP) and sequence-related amplified polymorphism (SRAP)

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In the present paper, experimental materials of 33 accessions representative of the *Euodia rutaecarpa* from four Chinese provinces were analyzed using the sequence-related amplified polymorphism (SRAP) and amplified fragment length polymorphism (AFLP) techniques, focusing on their molecular discrimination and the assessment of their genetic relatedness. For the analysis, we optimized 10 pairs of SRAP primers and 6 pairs of AFLP primers, (The software package NTSYS-pc 2.1 was applied to analyze the data matrix) and cluster analysis distributed samples into two clusters, one with *E. rutaecarpa* var. *officinalis* (Dode) Huang and the other with *E. rutaecarpa* (Juss.) Benth by SRAP+AFLP markers in the same similarity coefficient of 0.53 (Genetic parameters also analyzed using POPGENE version 1.31). Genetic diversity in the species was detected with SRAP (H = 0.2260, I = 0.3341) and AFLP (H = 0.1665; I = 0.2518) markers. Genetic variability levels of E. *rutaecarpa* var. *officinalis* (Dode) Huang was higher than genetic variability levels of *E. rutaecarpa* (Juss.) Benth. Our study shown that both SRAP and AFLP molecular markers are in high efficiency in detecting the genetic diversity of *E. rutaecarpa.*

Key words: *Euodia rutaecarpa*, variety amplified fragment length polymorphism (AFLP) markers, sequence-related amplified polymorphism (SRAP) markers, genetic diversity.

INTRODUCTION

The genus *Euodia* also has a synonym of *Evodia*, includes more than 150 species, and it is distributed in Asia, southern Africa, and Australia. There were about 20 species and 5 varieties in China. The dried and nearly ripe fruit of *Euodia rutaecarpa* (Juss.) Benth., *E. rutaecarpa* var. *officinalis* (Dode) Huang named Fructus Evodiae or medicinal Evodia Fruit ("Wuzhuyu" in Chinese) officially listed in the Chinese Pharmacopoeia (Chinese Pharmacopoeia Commission, 2010), is one of used in China for treatment of headache, abdominal pain the most popular and multi-purpose herb traditionally, postpartum hemorrhage, dysentery and amenorrhea (Jia and Hu, 2010). Evodiamine and rutaecarpine, the main alkaloids isolated from these plants, were approved as an effective treatment for IgE-induced allergic diseases such as atopic dermatitis and rhinitis (Shin, et al., 2007). They were also widely studied for anti-tumor activity (Liao et al., 2005; Ueng et al., 2006).*E. rutaecarpa* var. *officinalis* (Dode) Huang is an important variety of *E. rutaecarpa* (Juss.) Benth. They are widely planted in south of the Yangtze River and were genuine medicinal materials to Guizhou and Hunan province. Due to long-term natural

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Table 1. Plant code and locations of 33 samples of *Euodia rutaecarpa* in China. Note: " * " are sign of populations of *Evade rutaecarpa* (Juss.) Benth. Var. *officinalis* (Dode) Huang used for Nei's Original Measures of Genetic Identity and Genetic distance; and "◆"are sign of populations of *Evade rutaecarpa* (Juss.) Benth used for Nei's Original Measures of Genetic Identity and Genetic distance.

Species	Location	Population code	Altitude (m)	Latitude N	Longitude E
Evade rutaecarpa (Juss.) Benth.	Wanzai, Jiangxi	JXB1	110	28°11′49″	114°26′58″
Evade rutaecarpa (Juss.) Benth. var. *officinalis* (Dode) Huang	Wanzai, Jiangxi	JXo2- JXo 4	99-112	28°11′12″-12′14″	114°26′33″-58″
Evade rutaecarpa (Juss.) Benth	Liuyang, Hunan ◆	LYB1-LYB5	105-115	28°17′02″-06″	113°46′22″-29″
Evade rutaecarpa (Juss.) Benth. Var. *officinalis* (Dode) Huang	Liuyang,Hunan *	LYo6-LYo8	105-115	28°17′02″-06″	113°46′22″-29″
Evade rutaecarpa (Juss.) Benth	Xiangxiang, Hunan ◆	XXB1-XXB3	107-113	27°42′06″-12″	112°07′31″-35″
Evade rutaecarpa (Juss.) Benth. Var. *officinalis* (Dode) Huang	Loudi, Hunan *	LDo1-LDo3	160-194	27°42′35″-42″	111°56′30″-36″
Evade rutaecarpa (Juss.) Benth. Var. *officinalis* (Dode) Huang	Xinhuang,Hunan *	XHo1-XHo4	418-422	27°11′24″-40″	108°56′06″-24″
Evade rutaecarpa (Juss.) Benth. var. *officinalis* (Dode) Huang	Tongren, Guizhou *	TRo1-TRo3	650-672	27°57′41″-43″	109°16′40″-43″
Evade rutaecarpa (Juss.) Benth.	Pengshui, Chongqing ◆	PSB1-PSB3	482-490	29°15′57″-16′01″	108°04′41″-05′02″
Evade rutaecarpa (Juss.) Benth.	Youyang,Chongqing ◆	YYB1-YYB3	652-661	28°45′18″	108°49′06″-09″
Evade rutaecarpa (Juss.) Benth. var. *officinalis* (Dode) Huang	Xiushan, Chongqing	XSo1 XSo2	532	28°34′11″	109°03′17″

and artificial choices, obvious differentiation has been occurred in these populations. A survey found significant variation in morphology and growth habit in *E. rutaecarpa*. Therefore, the study of their differences at the molecular level is in imperative for the purpose of authenticating the populations. In Huang's (2008) study, there are some differences of ITS (internal transcribed spacer) sequence among them.

Recently, a new technique called amplified fragment length polymorphism (AFLP) was introduced as a new tool for genetic analysis (Vos et al., 1995), and it is already widely used in genetic studies in plants (Mikes et al., 1996; Van et al., 1995). Sequence-related amplified polymorphism (SRAP) is an efficient genetic marker system, revealing genetic variation in open reading frames among related organisms (Li and Quiros 2001), which has been successfully used in examination of genetic diversities (Riaz et al., 2001; Ferriol et al., 2003) in many plant species (Sun et al., 2006; Alasaad et al., 2008; Song et al., 2010). Nevertheless, to our knowledge, there were no reports using these markers in the field of *E. rutaecarpa* before the present study. The objective of the present study was to investigate the genetic variability among *E. rutaecarpa* (Juss.) Benth and *E. rutaecarpa* var. *officinalis* (Dode) Huang using the novel AFLP and SRAP markers. The comparison between the two marker systems is discussed.

MATERIALS AND METHODS

33 accessions of *E. rutaecarpa* from nine different locations which are the main producing areas of China (Table 1) were gathered with random sampling method, Tender leaves of *E. rutaecarpa* stored in ultra low temperature freezer (U410 Premium, New Brunswick Scientific, New Jersey, USA) at -8°C as samples for DNA extraction. Every sample should be tagged with sample ID, e.g. TRO2: short for Tongren, Guizhou *E. rutaecarpa* var. *officinalis* (Dode) Huang. XXB4 short for Xiangxiang, Hunan *E. rutaecarpa* (Juss.) Benth. The first and second alphabets stand for the origin of *E. rutaecarpa* the third alphabet stands for variant, "O" stands for *E. rutaecarpa* var. *officinalis* (Dode) Huang, "B" stands for *E. rutaecarpa* (Juss.) Benth, the last number stands for the sample order.

DNA extraction

A modified version of the CTAB method was used to extract genomic DNA from the leaf samples. A 1.5 ml tube containing approximately 0.3 g of tender leaf tissue was placed into liquid nitrogen for 30 s and its contents crushed with a small plastic bar. Then, 0.5 ml of 2×CTAB buffer was added to the tubes and incubated at 65°C for 90 min. After incubation, 0.4 ml of chloroform was added and the tubes were centrifuged at 14,000 rpm for 3 min. The supernatant was transferred into a new tube and the DNA precipitated in a 0.6 vol of 2-propanol. The DNA was then washed with 70% ethanol and dissolved in TE buffer and quantified by electrophoresis at 5 V/cm, on a 0.8% agarose gel in 1×TAE buffer (40 mM Tris-acetate and 1 mM EDTA,

Table 2. Polymorphism based on 10 pairs of SRAP primers and AFLP primers. Note: Me: 5' TGA GTC CAA ACC GG 3'; Em: 5' GAC TGC GTA CGA ATT 3'. Note: M: 5' GATGAGTCCTGAGTAA 3'; E: 5' GACTGCGTCCAAATTC 3'.

Marker	No.	Combinations	Total bands	Polymorphic bands	Polymorphism rate (%)
SRAP primers	1	Me-TA+ Em- CAA	12	8	66.7
	2	Me-GC+ Em- CAA	24	12	50.0
	3	Me-AT+ Em- GCA	12	6	50.0
	4	Me-CC+ Em- AAT	28	26	92.9
	5	Me-CC+ Em- AAC	31	29	93.5
	6	Me-CG+ Em- CAC	11	7	63.6
	7	Me-GG+ Em- CAC	14	12	85.7
	8	Me-GG+ Em- CAG	4	3	75.0
	9	Me-GG+ Em- CAT	22	17	77.3
	10	Me-AA+ Em- AAC	30	25	83.3
	Total	10 pairs of primer	188	145	77.1
AFLP primers	1	M-CAG+ E-AAC	69	45	65.2
	2	M-CGA+E-AGG	55	41	74.5
	3	M-CAA+E-AGC	75	49	65.3
	4	M-CAC+E-AAG	40	23	57.5
	5	M-CAT+E-AGG	66	39	59.1
	6	M-CTA+E-ACC	48	31	64.6
	Total	6 pairs of primer	353	228	64.6

pH8.0), the resulting DNA was stored at -20°C.

SRAP analysis

In this assay, 10 pairs of primer (Table 2) that produced scorable polymorphic bands were used to amplify the accessions of *E. rutaecarpa*. Each 25 µl PCR reaction mixture consisted of genomiIn this assay, 10 pairs of primer (Table 2) that produced scorable polymorphic bands were used to amplify the accessions of *E. rutaecarpa*. Each 25 µl PCR reaction mixture consisted of genomic DNA (50 ng/µl) 1 µl, sterile deionized water 18.5 µl; 10×PCR Buffer (contain 20 mM/L Mg^{2+}) 2.5 µl; dNTP (10 mM/L) 0.5 µl; forward Primer (10 pM/µl) 1.0 µl; reverse primer (10 pM/µl) 1.0 µl; Taq DNA Polymerase (2.5 U/µl) 0.5 µl. PCR amplification was performed under the following conditions: 5 min of denaturing at 94°C, 5 cycles of three steps: 1 min of denaturing at 94°C, 40 s of annealing at 35°C and 1 min of elongation at 72°C. In the following 32 cycles, the annealing temperature was increased to 50°C, with a final elongation step of 8 min at 72°C.

SRAP PCR products were separated on 8% denaturing polyacrylamide gel (acrylamide: bisacrylamide = 29:1) and then silver stained. The gel was screened in calibrated densitometer (Bio-RAD, USA).

Amplified fragment length polymorphism (AFLP) analysis

In this assay, 6 pairs of primers (Table 2) that produced scorable polymorphic bands were used to amplify the accessions of *E. rutaecarpa*. Genomic DNA was digested with *Eco*RI and *Mse*I, *Eco*RI and *Mse*I adapters were ligated to the ends of the restriction fragments, The adapter ligated DNA was pre-amplified by primers *E*-A and *M*-C with the following parameters: 20 cycles of 94°C for 30 s, 56°C for 60 s and 72°C for 60 s. Pre-amplified products were diluted 10 folds and used as template for selective amplification reaction using *Eco*RI and *Mse*I primers with three selective nucleotides at 3'-end. This amplification was carried out by programming a touch-down cycle profile as follows: 94°C for 30 s, 66°C (-1°C per cycle) for 50 s and 72°C for 1 min over ten cycles, until reaching the optimal annealing temperature of 56°C. 25 more cycles were performed to complete the second amplification, with a final elongation step of 8 min. The PCR products were separated on 8% denaturing polyacrylamide gel (acrylamide: bisacrylamide = 29: 1) and AFLP bands were stained using silver staining.

Data analysis

Clear and unambiguous DNA bands were manually scored as 1 (for presence) and 0 (for absence) from the images of the gels. Statistical analysis was based on polymorphic AFLP and SRAP markers. The resulting binary data matrix was first analyzed using POPGENE version 1.31 (Yeh et al., 1999). The following genetic diversity parameters including the percentage of polymorphic loci (PPL), Shannon's information index (I) (Lewontin, 1972) and Nei's gene diversity (H) (Nei's, 1973) were obtained at species level. To examine the genetic relationship among populations, Nei's Original measures of genetic identity and genetic distance were generated by POPGENE and a dendrogram was constructed from Nei's genetic distance with the unweighted pair-group method of averages (UPGMA) between the populations selected (Table 1), modified from NEIGHBOR procedure of PHYLIP Version 3.5 (Joseph, 1986).

The resulting binary data matrix of SRAP, AFLP, SRAP + AFLP was also analyzed using NTSYS pc 2.1 (Rohlf, 2000). Genetic similarity matrixs using SM coefficient was calculated. Dendrogram the cophenetic correlation for the dendrogram was constructed with UPGMA method in SHAN program. The representativeness of the dendrogram was evaluated by estimating and comparing it with the similarity matrix in the MXCOMP program, using Mantel's matrix correspondence test (Mantel, 1967). The result of this test is a cophenetic correlation coefficient, indicating how well the dendrogram represents similarity data.

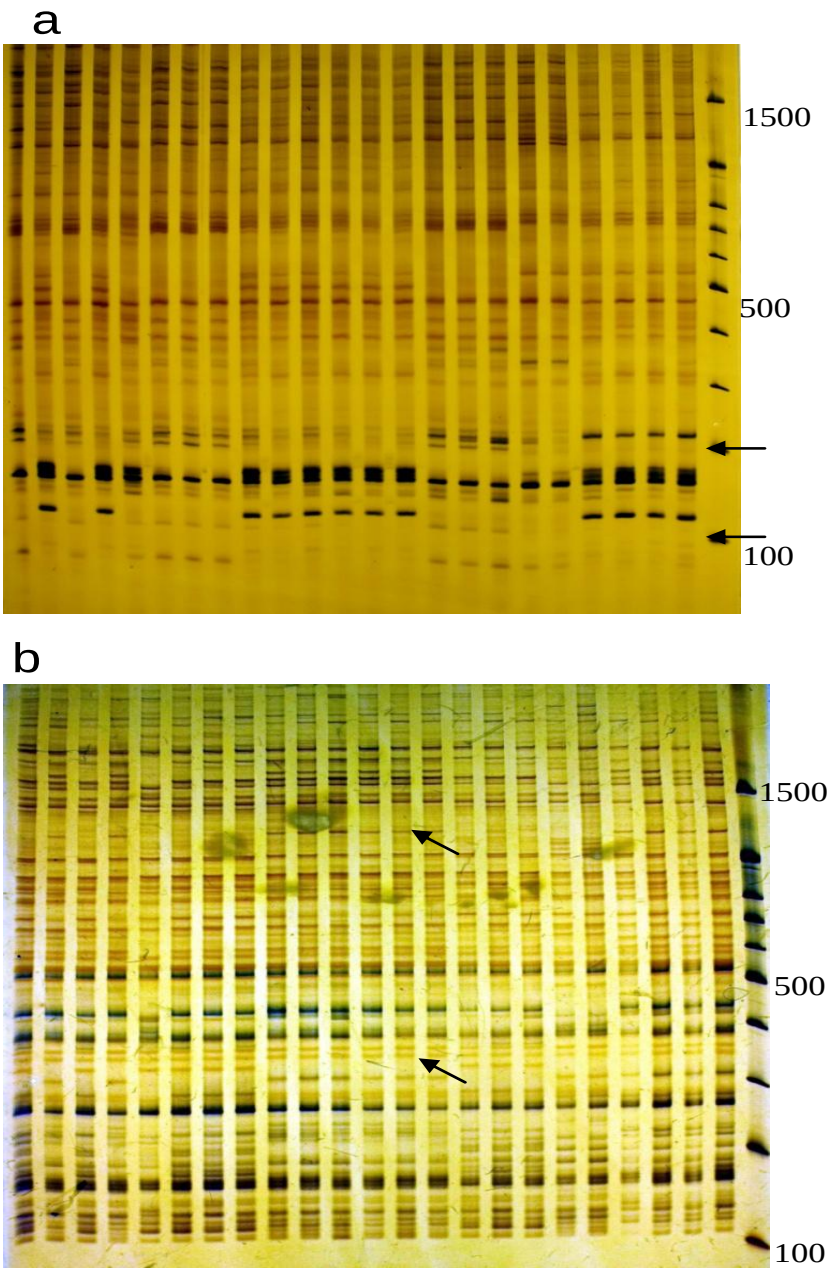

Figure 1. (a) SRAPs amplified by primers 5'-TGAGTCCAAACCGG-CC-3' + 5'-GA CTG CG TACGAATT-AAT-3' in *Euodia* rutaecarpa. (b) AFLP profile showing the genetic polymorphism in *Euodia* rutaecarpa, detected with primer combination M:5'-GATGAGTCCTGAGTAA-CAG-3'+5'–GACTGC GT CCAAAT TC -AAC-3'. Note: samples in the figures from left to right are YYB3 JXB1 JXo2- JXo 4 XXB1- XXB3 LDo1- LDo3 LYo1- LYo3 LYB4- LYB8 XHo1- XHo4 M(100bp ladder).

RESULTS AND DISCUSSION

Genetic parameters of SRAP and AFLP analysis

Ten SRAP primers amplified a total of 188 scorable bands (Table 2 and Figure 1a), and among them, 145 bands were polymorphic, accounting for 77.1%, the SRAP fragments sized from 100 to 1500 bp. The number of bands varied from 4 (Me-GG+ Em- CAG) to 31 (Me-CC+ Em- AAC) with an average of 14.5 polymorphic fragments per primer. The AFLP primer combinations produced 353 scorable markers (Table 2 and Figure 1b), and among them, 228 bands were polymorphic, accounting for 64.6%. The AFLP fragments sized from 40

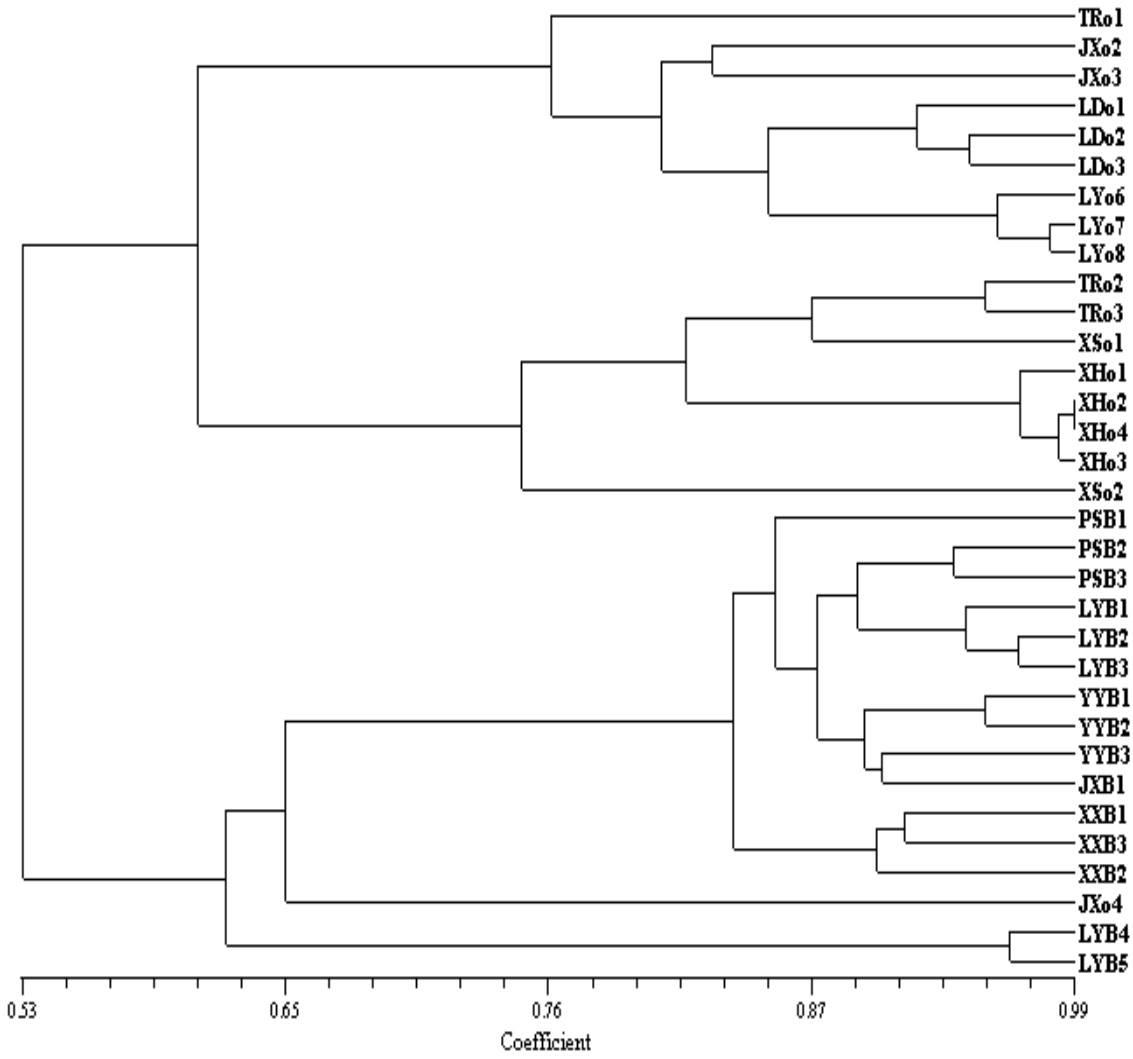

Figure 2. Dendrograms of 33 *E. rutaecarpa* cultivars generated by the SM method based on SRAP+ AFLP data.

to 1500 bp, and the number of bands varied from 40 (M-CAC+E-AAG) to 75 (M-CAA+E-AGC) with an average of 58.8 polymorphic fragments per primer, polymorphic bands per individual ranged from 23 to 49, confirming the higher multiplex ratio obtainable with this technique.

Similarity coefficients and polymorphism among 33 *E. rutaecarpa* accessions

Aiming at obtaining more consistent and balanced results, the 541 bands scored from SRAP and AFLP analysis were pooled together and analyzed as a single binary matrix. Genetic similarity matrix was calculated and a conjoint dendrograms was constructed (Figure 2), high cophenetic correlation coefficient (r = 0.96) was found, the dendrogram was good represent the similarity data. The clustering analysis separated the samples into two groups. One mainly obtains the samples of *E. rutaecarpa* var. *officinalis* (Dode) Huang (except JXo4) another mainly has the samples of *E. rutaecarpa* (Juss.) Benth. On the basis of SRAP and AFLP data, dendrogram were constructed in almost the same way. MXCOMP test shows high cophenetic correlation coefficient (r = 0.94 SRAP) (r = 0.98 AFLP).

In the SRAP and AFLP marker system, two primer pairs (SRAP) 5'-TGA GTC CAA ACC GG-CC-3'+ 5'-GAC TGC GTA CGA ATT-AAT-3' and (AFLP) 5'-GAT GAG TCC TGA GTA A-CAG-3'+ 5'–GAC TGC GTC CAA ATTC–AAC-3' (Figure 1) were shown to distinguish the two varieties with good efficiency.

Genetic diversity of *E. rutaecarpa* revealed by SRAP and AFLP markers

Genetic diversity parameters including the percentage of polymorphic loci (PPL), Nei's gene diversity (H) and

Table 3. Genetic diversity parameters of SRAP and AFLP. Note: NPL: The number of polymorphic loci, PPL: The percentage of polymorphic loci, H: Nei's gene diversity, I: Shannon's Information index.

Species	Sample size	AFLP				SRAP			
		NPL	PPL (%)	H	I	NPL	PPL (%)	H	I
E. rutaecarpa var. officinalis (Dode) Huang	17	153	43.34	0.1427	0.2140	88	46.81	0.1742	0.2573
E. rutaecarpa (Juss.) Benth.	16	123	34.84	0.1103	0.1668	99	52.66	0.1599	0.2431
Total	33	181	51.27	0.1665	0.2518	117	62.23	0.2260	0.3341

Table 4. Nei's Original Measures of Genetic Identity and Genetic distance by SRAP and AFLP. Note: Nei's genetic identity (above diagonal) and genetic distance (below diagonal).

Marker	pop ID	TRO	LDO	LYO	XHO	PSB	YYB	XXB	LYB
SRAP	TRO	****	0.8159	0.7927	0.8535	0.7135	0.7190	0.7433	0.7625
	LDO	0.2035	****	0.8870	0.7847	0.7266	0.7124	0.7697	0.7737
	LYO	0.2323	0.1199	****	0.7445	0.7622	0.7335	0.6798	0.7764
	XHO	0.1585	0.2425	0.2950	****	0.6849	0.7187	0.7303	0.7374
	PSB	0.3376	0.3194	0.2715	0.3785	****	0.9364	0.8813	0.9198
	YYB	0.3299	0.3391	0.3099	0.3304	0.0657	****	0.9082	0.9033
	XXB	0.2966	0.2618	0.3859	0.3143	0.1264	0.0963	****	0.8804
	LYB	0.2711	0.2566	0.2531	0.3046	0.0836	0.1017	0.1274	****
AFLP	TRO	****	0.8918	0.8848	0.9140	0.8234	0.8196	0.8041	0.8513
	LDO	0.1145	****	0.9701	0.8339	0.8168	0.8163	0.8051	0.8571
	LYO	0.1224	0.0304	****	0.8190	0.8073	0.8042	0.8034	0.8517
	XHO	0.0899	0.1817	0.1997	****	0.7753	0.7837	0.7704	0.8200
	PSB	0.1943	0.2024	0.2141	0.2545	****	0.9774	0.9526	0.9459
	YYB	0.1990	0.2029	0.2179	0.2438	0.0228	****	0.9596	0.9393
	XXB	0.2180	0.2168	0.2189	0.2608	0.0485	0.0413	****	0.9345
	LYB	0.1610	0.1541	0.1605	0.1984	0.0556	0.0626	0.0678	****

Shannon's information index (I) at species level are shown in Table 3. At the species level, the percentage of polymorphic loci (PPl) was estimated to be 62.23/51.27%, Nei's gene diversity (H) was 0.2260/0.1665, Shannon's information index (I) was 0.3341/0.2518 based on SRAP and AFLP data, respectively.

George and Joseph (1979) demonstrate that both heterozygosity estimates and genetic distance estimates are far more severely affected by the number of loci sampled than by the number of individuals sampled, and according to Nei's (1978) research, the number of individuals to be used for estimating average heterozygosity can be very small if a large number of loci are studied and the average heterozygosity is low. In our research, 4 populations of *E. rutaecarpa* var*. officinalis* (Dode) Huang (Table 1) and another 4 populations of *E. rutaecarpa* (Juss.) Benth were chosen to analyze the genetic distance and genetic identity.

Genetic identity and genetic distance between 8 populations are listed in Table 4 (SRAP and AFLP). Nei's genetic distance ranged from 0.0657 to 0.3859 based on the SRAP analysis. The largest genetic distance (0.3859) occurred between Liuyang (LYO) and Xiangxiang (XXB) populations and the least (0.0657) between Youyang (YYB) and Pengshui (PSB) populations. The AFLP analysis gave similar results, with the largest genetic difference between Xinhuang (XHO) and Xiangxiang (XXB) populations at the value of 0.2608 and the least (0.0228) between Youyang (YYB) and Pengshui (PSB). The average genetic distances of *E. rutaecarpa* var*. officinalis (Dode)* Huang derived from SRAP and AFLP markers were 0.2086 and 0.1231, while in *E. rutaecarpa* (Juss.) Benth were 0.1002, 0.0498 derived from SRAP and AFLP markers, respectively. Genetic distance was revealed to be higher in *E. rutaecarpa* var*. officinalis (Dode)* Huang than *E. rutaecarpa* (Juss.) Benth by both SRAP and AFLP marker technology.

A dendrogram of eight populations was constructed with the UPGMA algorithm using Nei's genetic distance

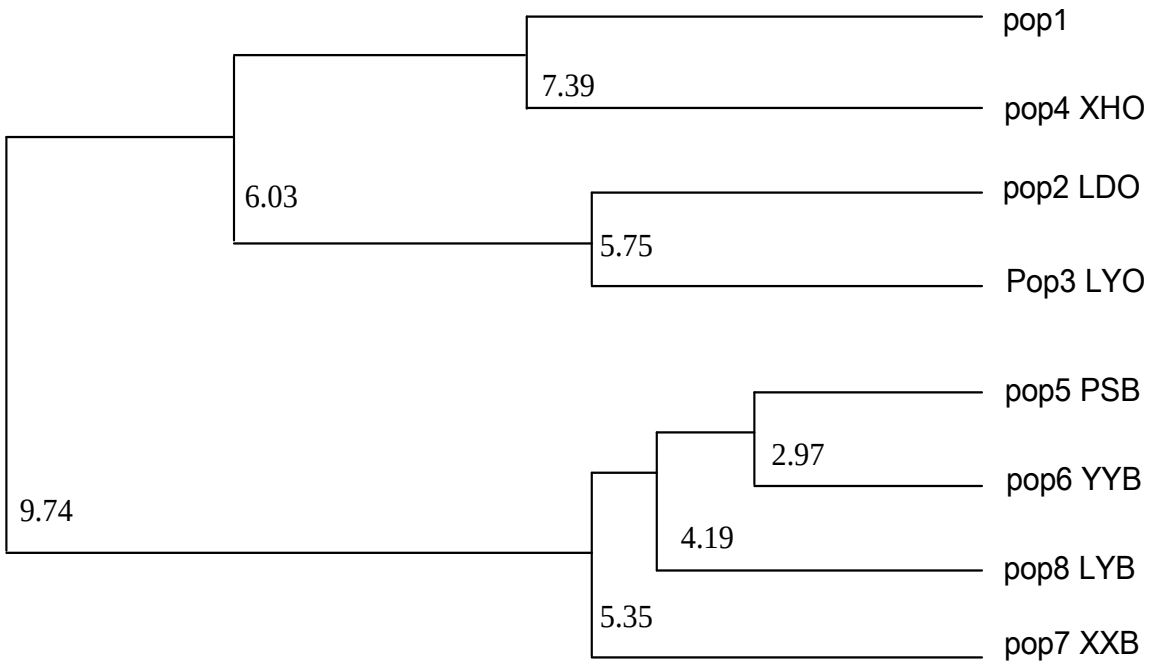

Figure 3. UPGMA dendrogram showing genetic relationship among populations based on Nei's (1978) genetic distance of SRAP + AFLP data matrix. Note: pop1(TRO): Tongren,Guizhou; pop2(LDO): Loudi, Hunan; Pop3(LYO): Liuyang, Hunan; pop4(XHO): Xinhuang, Hunan are populations of *Euodia rutaecarpa var. officinalis* (Dode) Huang. pop5 (PSB): Pengshui, Chongqing; pop6(YYB): Youyang,Chongqing; pop7(XXB): Xiangxiang,Hunan*;* pop8(LYB): Liuyang, Hunan are populations of *Euodia rutaecarpa* (Juss.) Benth.

values (Figure 3). The dendrogram grouped the 8 populations into two main clusters. POP 1, POP 2, POP 3 ,POP 4 of *E. rutaecarpa* var. *officinalis* (Dode) Huang formed a cluster genetically distinct from four other populations of *E. rutaecarpa (Juss.)* Benth.

Efficiency of the two molecular markers

In this work, SRAP and AFLP markers were applied to assess the genetic diversity in two varieties of *E. rutaecarpa*. The percentage of polymorphic loci (PPl = 46.81%), Nei's gene diversity (H = 0.1742) and Shannon's information index (I = 0.2573) at varieties of *E. rutaecarpa*. var*. officinalis* (Dode) Huang generated by SRAP primer were higher than that of AFLP analysis (PPl = 43.34%, H = 0.1427, I = 0.2140). The same trends were found in *E. rutaecarpa* (Juss.) Benth (PPl = 52.66%, H = 0.1599, I = 0.2431 by SRAP to PPl = 34.84%, H = 0.1103, I = 0.1668 by AFLP), and also average genetic distance in the species of *E. rutaecarpa* were high by SRAP (0.3100) than AFLP (0.2073) marker.

Conclusion

It was the first attempt that we used combined results of SRAP and AFLP markers to investigate the genetic structure of *E. rutaecarpa*. For SRAP and AFLP markers, a high reproducibility in dendrogram topologies was obtained. SRAP and AFLP can be used to authentic the genetic differences of *E. rutaecarpa* even different varieties resource. The genetic diversity of *E. rutaecarpa* var. *officinalis (Dode)* Huang is larger than *E. rutaecarpa* (Juss.) Benth.

In this study, two molecular marker techniques: SRAP and AFLP have been compared in order to decide which technique is suitable in genetically characterize of *E. rutaecarpa*. We found that both two marker systems are feasible in identification of *E. rutaecarpa*, especially with the SRAP marker system, which produced some bands, could effectively distinguish the two varieties and revealed large genetic distance and higher genetic parameters of *E. rutaecarpa*.

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