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Full Length Research Paper

Genetic diversity and association of ISSR markers with the eleostearic content in tung tree (*Vernicia fordii*)

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Tung tree is a useful woody oil plant in the world. In this study, both the genetic diversity and biochemical traits were analyzed in order to improve the breeding methods on tung tree. The mean genetic similarity coefficient (*Gs*), the mean Nei's gene diversity (*h*) and the mean Shannon's information index (*I*) of tung tree were 0.7821, 0.2192 and 0.3424, respectively. In dendrogram based on UPGMA, 30 cultivars were divided into three groups, A, B and C, which displayed a descending trend on eleostearic content. A conclusion was drawn that ISSR makers associated with the biochemical trait, can be used in marker-associated breeding programs for tung crop improvement targeted at high eleostearic content variety.

Key words: Tung tree, genetic diversity, fatty acid.

INTRODUCTION

Tung tree or tung oil tree (*Vernicia fordii* Hemsl.) is a species in Euphorbiaceae, native to southern China, Burma and northern Vietnam. Tung oil, which is derived from seed kernels of the tung tree, is commonly used in formulations of inks, dyes, coatings and resins because of its unique ability to dry to a clear, hard protective barrier (Sonntag, 1979). Meanwhile, Chang and Wan, (1947) and Crossley et al. (1962) have proved tung oil to be a raw material for biodiesel. Tung oil is water, acid and alkali resistant and this property makes it an excellent candidate for outdoor finishes, due to an unusual fatty acid α-eleostearic (18:3$\Delta^{9cis,\ 11trans,\ 13trans}$), which accounts for approximately 80% of the total fatty acids in the oil. And the content of this trienoic fatty acid is the most important factor affecting the quality of tung oil. Therefore, it is significant to collect data on fatty acid composition of tung oil and germplasm resource of tung tree.

The availability of a variety of DNA markers, such as restriction fragment length polymorphism (RFLP), amplified fragment length polymorphism (AFLP), random amplified polymorphic DNA (RAPD), simple sequence repeat (SSR) and inter-simple sequence repeat (ISSR) has enabled researchers to investigate genetic diversity among various species across natural populations. ISSR, for instance, has several advantages for assessing genetic diversity (Gupta et al., 1994). ISSR analyses are more specific than RAPD analyses, due to the longer SSR-based primers with higher primer annealing temperature, which enable higher-stringency and greater band reproducibility amplifications (Wolfe et al., 1998a). ISSR has been reported to be useful to the study of genetic diversity (Luan et al., 2006; Cortesi et al., 2005), genetic mapping and gene mapping (Sankar and Moore, 2001), germplasm identification (Blair et al., 1999) and fingerprinting construction (Davierwala et al., 2001; Rao et al., 2006).

Most of the former researches on tung tree with molecular methods focused on nucleotide and peptide about fatty acid synthesis (Dyer et al., 2002a; Shockey et al., 2006). Previously, we applied ISSR to characterizing the genetic diversity of 64 tung cultivars (Li et al., 2008). However, there have been no reports on the association of the main economic characters with DNA markers on tung tree. The aim of this work was to gain more information on relationship between genetic diversity and the main economic characters of 30 tung cultivars in China.

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Table 1. Names and source of tung cultivars analyzed in this study.

No.	Cultivar names	Origins	No.	Cultivar names	Origins
1	Long 9 - 16	Zhejiang	16	Sichuan Xiaomi	Sichuan
2	Long 9 - 15	Zhejiang	17	Sichuan Wanlong	Sichuan
3	Long 9 - 13	Zhejiang	18	Sichuan Wan'gan 1	Sichuan
4	Henglu 7	Jiangxi	19	Sichuan Wan'gan 3	Sichuan
5	Henglu 15	Jiangxi	20	Wanlong 1	Sichuan
6	Henglu 23	Jiangxi	21	Henan Guzhuaqing	Henan
7	Henglu 14	Jiangxi	22	Yelizhi 79-1-28	Zhejiang
8	Henglu 19	Jiangxi	23	Hunan 72-30	Hunan
9	Chenjiaxu 9 - 24	Zhejiang	24	Hunan 72-159	Hunan
10	Chenjiaxu 9 - 27	Zhejiang	25	Hunan 72-213	Hunan
11	Chenjiaxu 9 - 20	Zhejiang	26	Hunan 74-1	Hunan
12	Taishun Xiaomi	Zhejiang	27	Changxing 210-1	Zhejiang
13	Shuangjiang 1	Yunnan	28	Changxing 31-1	Zhejiang
14	Jiancan 5	Zhejiang	29	Changxing 187-5	Zhejiang
15	Jiancan 3	Zhejiang	30	Gongcheng Duinian	Guangxi

Table 2. List of primer sequences used for ISSR for 30 tung cultivars.

Primer	Sequence (5'→3')	Tm (%)	Ta (%)	Bands scored	Polymorphic bands
UBC808	AGA GAG AGA GAG AGA GC	54.59	57.0	8	4
UBC810	GAG AGA GAG AGA GAG AT	52.18	51.0	8	5
UBC811	GAG AGA GAG AGA GAG AC	54.59	57.5	11	6
UBC823	TCT CTC TCT CTC TCT CC	54.59	56.5	7	5
UBC834	AGA GAG AGA GAG AGA GYT	53.88	57.0	10	8
UBC835	AGA GAG AGA GAG AGA GYC	56.16	56.0	6	5
UBC844	CTC TCT CTC TCT CTC TRC	54.16	59.0	9	8
UBC848	CAC ACA CAC ACA CAC ARG	56.16	59.0	16	15
UBC868	GAA GAA GAA GAA GAA GAA	48.19	47.0	7	6
UBC873	GAC AGA CAG ACA GAC A	51.55	51.5	9	8
UBC876	GAT AGA TAG ATA GAT A	41.30	41.0	8	8
UBC881	GGG TGG GGT GGG GTG	61.77	59.0	11	9
Total				110	87

R = Purine, Y = Pyrimidine.

MATERIALS AND METHODS

Plant materials

Thirty tung cultivars used in the present study and listed in Table 1, were obtained from the National Gene Pool of Tung Tree in the Red East Forest Farm, Zhejiang Province, China. These tung trees in the gene pool were collected from 6 provinces in China in natural environment.

DNA extraction and ISSR-amplification

Genomic DNA was extracted from fresh young leaves using a modification of the 2 × CTAB method of Doyle and Doyle (1987). Nuclear DNA was then PCR-amplified using ISSR primers obtained from the University of British Columbia. Following an initial screen of 100 primers, 12 were selected for further analysis.

The specific annealing temperature (Ta) was determined for each primer; primer sequences and Ta are shown in Table 2. PCRs were performed as described by Li et al. (2008). PCR products were analyzed using an agarose (1.5% w/v) electrophoresis gel stained with ethidium bromide.

Fatty acid extraction

Tung oil was extracted from kernels according to Soxhlet's procedure (Soxhlet, 1879). For analysis of fatty acid content, methyl esters of extracted oil were prepared using potassium methoxide (Christie et al., 1984). Then fatty acids were converted into fatty acid methyl esters (FAMEs).

Gas chromatography (GC) with flame ionization detection (FID) was also performed as described (Dyer et al., 2002b). FAMEs were

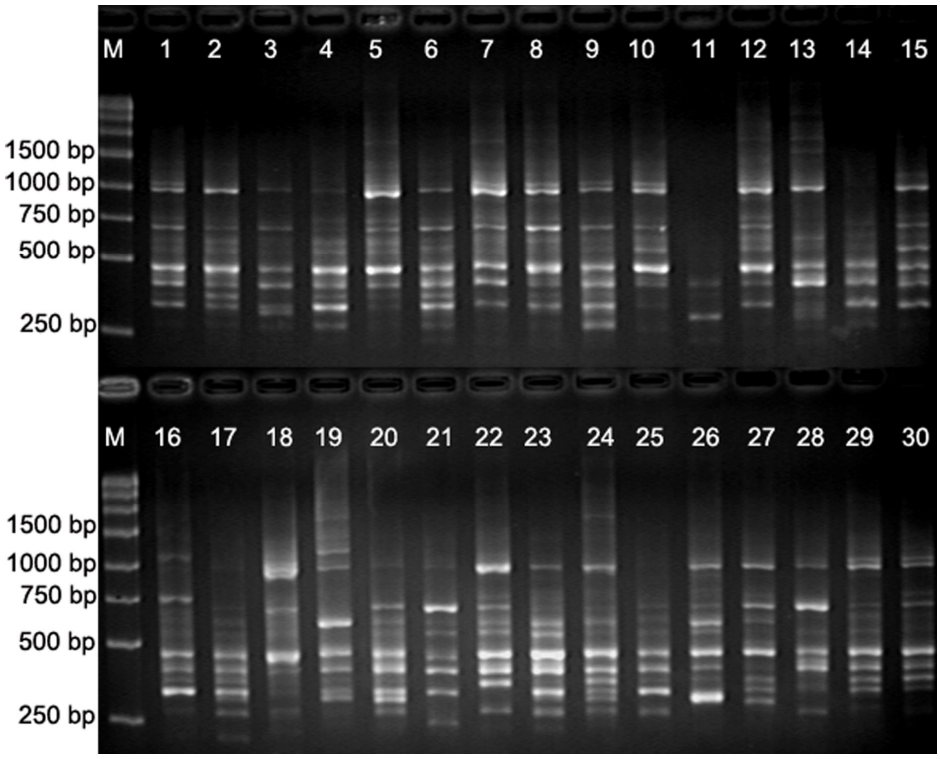

Figure 1. ISSR fingerprint patterns of the 30 tung cultivars, amplified with primer UBC873. M is the DNA marker.

prepared using sodium methoxide in methanol and methyl heptadecanoate was used as an internal standard. FAMEs were analyzed on an Agilent 6890 gas chromatograph and identified by comparison of relative retention times with FAME standards. Percentages of each FAME were calculated based on total FAME area counts. The operating conditions for GC/FID analysis were set as follows: injector, 195℃; FID, 195℃; split ratio, 10:1; flow rate of air, 20 ml/min. No hold at initial column temperature of 150℃, program rate was 5℃ /min and final temperature was 195℃ and held 18 min at final temperature.

Data analysis

To compare ISSR amplification patterns, bands were scored as 1 (present) or 0 (absent) binary characters. The software program POPGENE v. 1.31 (Yeh et al., 1999) was used to obtain the genetic diversity parameters, such as Nei's gene diversity (Nei, 1973), Shannon's information index (Lewontin, 1972), genetic similarity and genetic distance. A dendrogram with Nei's genetic distance (Nei, 1972) and the UPGMA (unweighted pair group method arithmetic averages) method were generated using the software NTSYS pc2.10.

Microsoft Office Excel was applied for comparing the differences among 30 tung cultivars on the content of different fatty acids.

RESULTS

Genetic diversity

In preliminary studies, the repeatability of bands was examined by repeating the ISSR process. It proved that patterns of ISSR were highly reproducible. From prescreening assays using 100 ISSR primers, 12 primers generating bright amplification products and polymorphisms, were used in further analysis (Table 2). A total of 110 reliable fragments were obtained. The number of fragments per primer ranged from 6 to 16 with the average of 9.17. Among them, 87 bands were polymorphic with a ratio of 79.82%. The number of polymorphic bands per primer was 7.25. The results of PCR amplification are given in Figure 1.

Genetic structuring was evident due to the detection of specific bands in each sample examined. Genetic similarity coefficients (*Gs*) of 30 tung cultivars ranged from 0.6257 to 0.8113 with an average of 0.7821. The mean Nei's genetic diversity (*h*) and Shannon's information index (*I*) among 30 tung cultivars were 0.2192 and 0.3424. On the basis of genetic distance, a dendrogram was generated with UPGMA clustering analysis and the total 30 cultivars were divided into 3 groups (Figure 2).

Fatty acid content analysis

There were great differences among the 30 tung cultivars on the main economic traits. By gas chromatography, there were 6 kinds of fatty acids in tung oil: α-eleostearic acid (18:3$\Delta^{9cis, 11trans, 13trans}$), linoleic acid (18: 2$\Delta^{9cis, 12cis}$),

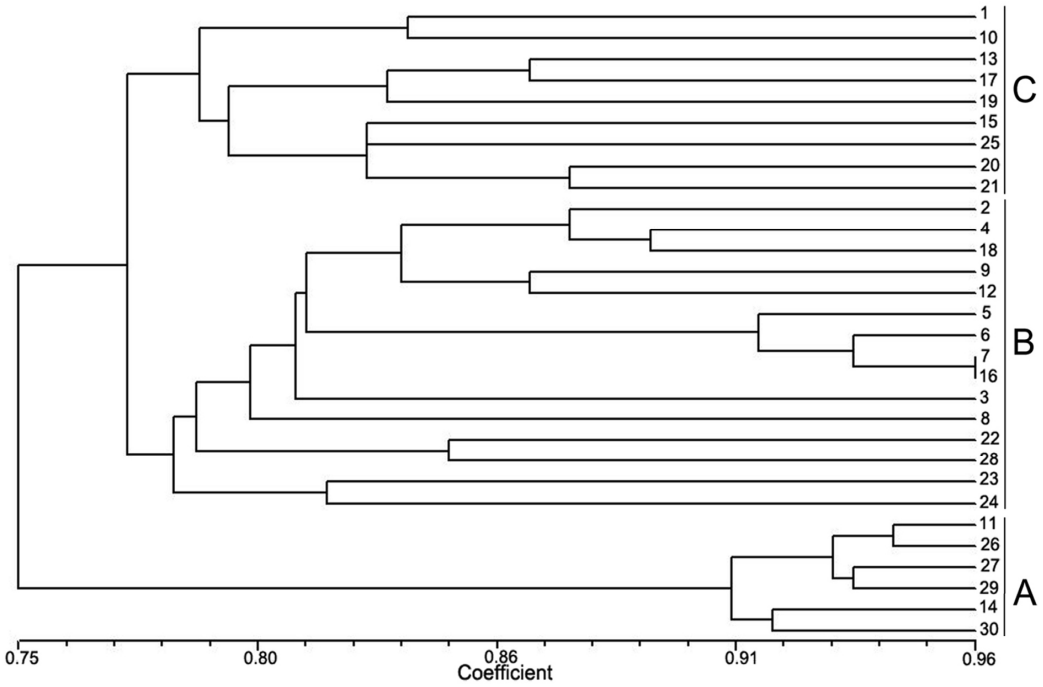

Figure 2. Dendrogram of 30 tung cultivars according to UPGMA cluster analysis.

oleic acid (18: 1Δ^{9cis}), palmitic acid (16: 0), stearic acid (18: 0), gondic acid (20:1Δ^{11cis}) a few individuals had (Table 3). Of the fatty acids, α-eleostearic was of the highest content, from 68.12 to 78.58%, with an average of 74.25%; content of linoleic and oleic acid was much lower, 8.74-11.69% and 7.12 -13.50%, averaged at 10.07 and 9.63%, respectively; tung kernels had the lowest content of 2 saturated fatty acids, palmitic (2.42-3.49%) and stearic acid (2.08-3.47%), with the mean of 2.87 and 2.72%. Cultivars with high content of polyunsaturated fatty acids, usually contained low content of saturated and monounsaturated fatty acids.

According to the comparison between dendrogram and the fatty acid content, there was a descending trend on eleostearic content from group A, B to C. In group A, the eleostearic content ranged from 75.89% (Gongcheng Duinian, NO. 30) to 78.58% (Changxing 187-5, NO. 29). In group B, Henglu 23 (NO. 6) had the highest content of eleostearic (75.71%) and eleostearic content of Hunan 72 -159 (NO.24) was the lowest (73.74%). In group C, nearly all of the members had the lowest eleostearic content of the 3 groups except Henan Guzhuaqing (NO. 21), whose eleostearic content was at the moderate level in group B (Figure 3).

DISCUSSION

Although much work based on morphological traits has been carried out in tung tree for cultivar identification and breeding, the results is still ambiguous, for phenotypic characters are susceptible to developmental stage and environment in many cases (Wang and Tanksley, 1989). ISSR markers can overcome those disadvantages to a certain extent. ISSRs are arbitrary multilocus markers produced by PCR amplification with a single anchored microsatellite primer. They are advantageous because no genomic information is required for their use. This provides a convenient and rapid assessment of the differences in genetic composition of closely related individuals at the DNA level and has been employed in a large number of plant species for characterization and assessment of genetic diversity because of their speed and ease in handling (Wolfe and Liston, 1998b).

The results of the present study using ISSR markers revealed low level of genetic similarity (0.6257 to 0.8113) but high polymorphism (79.82%) within 30 individuals based on the statistical data. The UPGMA analysis based on genetic distance measures clustered the 30 cultivars into 3 major groups. Nevertheless, the clustering did not show a consistency between genetic similarity and geographic location of the cultivations. Genetic diversity in a species is affected by a number of evolutionary factors including mating system, gene flow and seed dispersal, geographic range as well as natural selection (Hamrick and Godt, 1989). Of these factors, the mating system appears to influence the levels of genetic diversity within cultivations greatly for such a woody crop which has been cultivated for more than a thousand years.

In 2008, an attempt was made to find associations between ISSR markers and some biochemical traits in mulberry maker-assisted breeding. Stepwise multiple

Table 3. Concentration of each fatty acid in tung oil (%).

No.	Eleostearic	Linoleic	Oleic	Palmitic	Stearic	Gondoic
1	72.48	10.10	11.03	2.89	3.17	0.33
2	73.51	10.01	8.21	3.04	2.30	-
3	74.91	10.15	8.51	2.78	2.87	0.79
4	74.13	9.94	9.74	2.97	2.80	0.42
5	75.19	9.68	10.05	2.67	2.08	0.34
6	75.71	9.49	9.08	2.80	2.66	0.26
7	75.12	9.82	9.22	2.74	2.69	0.41
8	74.78	9.56	9.50	2.80	2.97	0.39
9	75.11	9.37	9.93	2.70	2.56	0.34
10	68.31	11.41	13.50	2.88	3.11	0.74
11	76.93	10.25	7.31	2.45	2.61	0.44
12	74.28	10.57	9.39	3.24	2.52	-
13	74.94	9.71	8.87	3.03	2.61	0.84
14	76.13	9.50	8.07	2.72	3.09	0.45
15	71.16	10.61	11.92	3.80	2.84	0.38
16	74.48	9.77	9.49	2.99	2.82	0.44
17	73.18	10.50	10.91	2.67	2.44	0.29
18	74.92	10.11	9.36	2.63	2.51	0.40
19	73.82	10.06	9.84	2.91	2.74	0.64
20	72.10	10.52	11.37	3.06	2.96	-
21	75.00	9.94	9.24	2.77	2.75	0.30
22	73.63	9.82	9.46	3.01	3.24	0.83
23	68.12	11.66	13.03	3.49	3.47	0.25
24	73.74	9.85	10.31	2.94	2.60	0.56
25	71.99	11.31	10.95	2.97	2.79	-
26	76.41	10.71	7.56	2.61	2.70	-
27	78.08	9.50	7.12	2.62	2.33	0.34
28	74.78	9.67	9.50	2.87	2.70	0.48
29	78.58	8.74	7.29	2.66	2.31	0.42
30	75.89	9.73	9.22	2.42	2.42	0.33
Mean	74.25	10.07	9.63	2.87	2.72	0.46

regression analysis identified four ISSR markers associated with protein content with highly positive correlation. Therefore, DNA markers for proteins seem promising and can be used for mulberry breeding program (Kar et al., 2008). Fatty acid composition is an important index for tung oil quality evaluation. In this study, by comparing the cultivars graded for the fatty acid content with the groups in the dendrogram, we have shown that ISSR markers revealed accordance between genetic diversity and eleostearic content among separated samples, namely there was a descending trend from group A, B to C on eleostearic content (Figure 3). In dendrogram based on DNA diversity, individuals in the same group meant their genetic relationship was closely related. Furthermore, clustering depending on eleostearic content showed similar phenomenon. It is concluded that the ISSR makers, associated with the biochemical trait, can be used in marker-associated breeding programs for tung crop improvement targeted at high eleostearic content variety.

Fatty acid concentration of tung oil affects the applicability. Tung oil for traditional industrial usage with superior quality usually has high content of unsaturated fatty acids, especially α-eleostearic, which imparts useful drying quality to the oil (Sonntag et al., 1979). Hence, oil of Changxing 187-5 (containing 78.58% eleostearic acid) can be used to manufacture modern paint, printing ink, etc. On the other hand, tung oil for biodiesel producing needs superior raw materials containing low content of poly-unsaturated fatty acids and high content of mono-unsaturated fatty acids (Harrington, 1986). Thus, cultivars (Hunan 72-30, Chenjiaxu 9-27, etc) with such a trait can be planted as the candidates to supply oil for biodiesel.

In this study, it was found that tung tree cultivars of high content of eleostearic usually yield low content of oleic and linoleic acid. For instance, Hunan 72-30 contained the lowest concentration of eleostearic acid but the

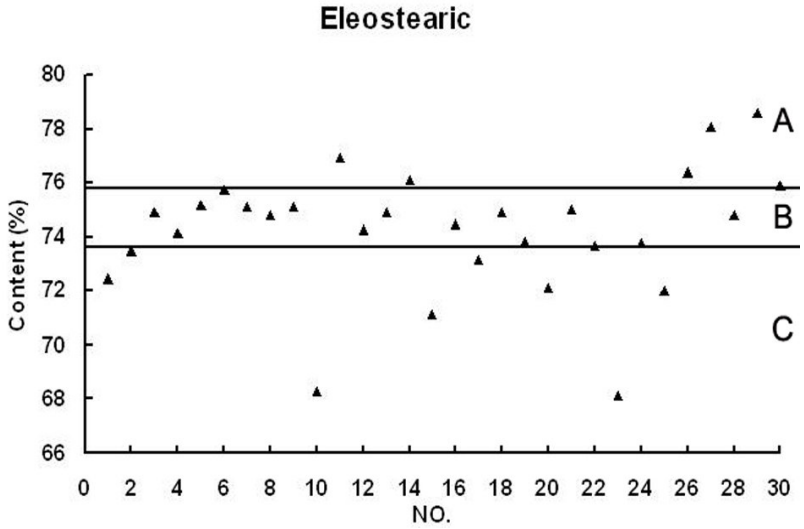

Figure 3. Eleostearic content of 30 tung cultivars, indicating the difference among groups A, B and C.

highest content of oleic, stearic and palmitic acid of the 30 individuals. It might be caused by the difference of fatty acid desaturases (FADs, e.g. FAD2 and FADX) bioactivity of different cultivars. In the biosynthetic pathway of eleostearic acid, it was revealed that FAD2 converted oleic acid into linoleic acid and that FADX converted linoleic acid into α-eleostearic acid (Dyer et al., 2002a). Therefore, it will have a promising prospect to utilize molecular technologies to regulate the gene expression of enzymes about the fatty acid synthesis in tung tree, to enhance or reduce eleostearic concentration for different usage. In our laboratory, both the construction of antisense expression vector of FADX gene and tobacco, tung transformation have been successively completed (Wang et al., 2007).

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