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Full Length Research Paper

Genetic diversity and germplasm resource research on tung tree (*Vernicia fordii*) cultivars, investigated by inter-simple sequence repeats

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Tung tree is an important woody oil-rich plant in the world. In order to determine the genetic diversity, germplasm resource and breeding method on tung tree, inter-simple sequence repeats (ISSR) was used to investigate the cultivars in China. Among the total 110 bands amplified with 12 primers, 90 were polymorphic. The mean genetic similarity coefficient (*Gs*), the mean Nei's gene diversity (*h*), and the mean Shannon's information index (*I*) of tung cultivars were 0.8273, 0.1770 and 0.2453, respectively. Both UPGMA cluster and PCA showed clear genetic relationship among the 64 tung cultivars. The multilocus marker system also yielded useful strategies for germplasm collection and conservation of tung.

Key words: Tung tree, genetic diversity, ISSR, germplasm resource.

INTRODUCTION

China wood oil tree also known as tung tree or tung oil tree, which has been planted and used for over a thousand years, is a native woody oil plant in subtropical area in China. Tung oil, extracted from tung seeds, contains 80% (w/w) α-eleostearic acid, which is an unusual trienoic fatty acid (18:3$\Delta^{9cis,\ 11trans,\ 13trans}$) that imparts useful drying quality to the oil (Sonntag et al., 1979). As the best drying oil, tung oil possesses the excellent characteristics such as insulation, acid and alkali resistance, as well as anticorrosion properties. It has gradually been applied to manufacturing modern paint, high-quality printing ink, plasticizer, medicine and chemical reagents, etc. Meanwhile, the oil-rich character makes tung tree considered as the raw material for biodiesel to solve the energy crisis. Therefore, the strategies for germplasm resource and breeding are basal and significant.

Since 1980s, molecular markers have successfully overcome the problems associated with morphological or isozyme markers. In 1980, the first molecular or DNA marker Restriction Fragment Length Polymorphisms (RFLP) based on southern blotting enhanced the application of marker technology (Bostein et al., 1980). Since that time, the development of marker system based on the polymerase chain reaction (PCR) has grown to include Random Amplified Polymorphism DNA (RAPD) (William et al., 1990), microsatellite or Simple Sequence Repeat (SSR) (Ali et al., 1986), and Amplified Fragment Length Polymorphism (AFLP) (Karp et al., 1997). Since 1994, a new molecular marker based on SSR, called inter-simple sequence repeats (ISSR) has been available (Zietkiewicz et al., 1994). ISSR uses anchored or non-anchored SSR primers to amplify DNA sequences between two inverted SSRs made up the same sequence. Such amplification does not require the previous knowledge of the genome sequence and leads to high polymorphous patterns. Furthermore, ISSR can also detect more diversity than RAPD because of the longer length of the primers (Bornet and Branchard, 2001). ISSR has been proved 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).

So far, a few tung genes about fatty acid synthesis have been cloned (Dyer et al., 2002; Hwang et al., 2004; Shoc-

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

No.	Cultivar name	Origin	No.	Cultivar name	Origin
1	Jiangchengxu 79-9	Zhejiang	33	Shuangjiang 3	Yunnan
2	Jiangchengxu 9-10	Zhejiang	34	Xianju 1	Zhejiang
3	Long Zhe 3 9-8	Zhejiang	35	Jiancan 5	Zhejiang
4	Long Zhe 3 9-11	Zhejiang	36	Jiancan 4	Zhejiang
5	Shuangjiang 3	Yunnan	37	Jiancan 3	Zhejiang
6	Long 9-16	Zhejiang	38	Jiancan 2	Zhejiang
7	Long 9-15	Zhejiang	39	Jiancan 1	Zhejiang
8	Long 9-13	Zhejiang	40	Shuangjiang 2	Yunnan
9	Henglu 7	Jiangxi	41	Shaungjiang 4	Yunnan
10	Henglu 15	Jiangxi	42	Sichuan Xiaomi	Sichuan
11	Henglu 21	Jiangxi	43	Sichuan Dami	Sichuan
12	Henglu 12	Jiangxi	44	Sichuan Wanlong	Sichuan
13	Henglu 20	Jiangxi	45	Sichuan Shibing	Sichuan
14	Henglu 23	Jiangxi	46	Sichuan Wan'gan 1	Sichuan
15	Henglu 17	Jiangxi	47	Sichuan Wan'gan 3	Sichuan
16	Henglu 22	Jiangxi	48	Wanlong 1	Sichuan
17	Henglu 14	Jiangxi	49	Sichuan Wan'gan 2	Sichuan
18	Henglu 19	Jiangxi	50	Yunnan Yiliang1	Yunnan
19	Chenjiaxu 9-24	Zhejiang	51	Henan Guzhuaqing	Henan
20	Chenjiaxu 9-27	Zhejiang	52	Yelizhi 79-1-28	Zhejiang
21	Chenjiaxu 9-18	Zhejiang	53	Hena Wuzhua	Henan
22	Chenjiaxu 9-20	Zhejiang	54	Hunan 72-30	Hunan
23	Chenjiaxu 9-22	Zhejiang	55	Hunan 72-159	Hunan
24	Chenjiaxu 9-23	Zhejiang	56	Hunan 72-213	Hunan
25	Mi 10	Zhejiang	57	Hunan 74-1	Hunan
26	Chenjiaxu shibing	Zhejiang	58	Changxing 210-1	Zhejiang
27	Taishun 5	Zhejiang	59	Changxing 31-1	Zhejiang
28	Pujiang 67	Zhejiang	60	Changxing 68-3	Zhejiang
29	Taishun xiaomi	Zhejiang	61	Changxing 68-4	Zhejiang
30	Pujiang 66	Zhejiang	62	Changxing 187-5	Zhejiang
31	Jinhua 2	Zhejiang	63	Changxing 31-6	Zhejiang
32	Shuangjiang 1	Yunnan	64	Gongcheng Duinian	Guangxi

key et al., 2005; Shockey, et al. 2006; Henderson et al., 2007), however, few attempts have been made to characterize the genetic diversity of tung cultivars through molecular markers. Therefore, in the present study, the ISSR technology was used to investigate the genetic diversity of 64 tung cultivars in China to provide the information for establishing appropriate breeding and germplasm collection and conservation strategies.

MATERIALS AND METHODS

Plant materials

64 tung cultivars, described in Table 1, were used. Young leaves were obtained from the National Gene Pool of Tung Tree in Dongfanghong Forest Farm, Zhejiang Province, China.

DNA extraction and PCR amplification

Genomic DNA was extracted from fresh leaflets following the CTAB protocol (Doyle and Doyle, 1987). 50 ISSR primers from the University of British, Columbia were initially screened. 12 primers were selected for further analysis. Reaction was carried out in a total volume of 20 µL containing 20 ng template DNA, 1.0 U Taq DNA polymerase, 2.0 mmol/L Mg^{2+}, 0.20 mmol/L dNTP, 0.4 µmol/L primer, 1 × PCR buffer. The PCR amplifications were performed in a 2720 Thermal Cycler under the following cycle profile: 5 min at 94°C ; followed by 45 cycles of 45 s at 94°C , 1 min at annealing temperature (T_a, depend on primers used, Table 2), and 1.5 min at 72°C; and 8 min final elongation.

Electrophoresis and data analysis

PCR products were analyzed by electrophoresis using 1.5% (w/v) agarose gels buffered with 0.5 × TBE (45 mmol/L Tris-borate, pH

Table 2. ISSR primer sequences used for analysis of tung, with primer annealing temperature, number of bands amplified, and number of polymorphic bands amplified.

Primer	Sequence	Annealing temperature (°C)	No. of bands scored	No. of polymorphic bands
808	$(AG)_8C$	57.0	8	4
810	$(GA)_8T$	51.0	8	5
811	$(GA)_8C$	57.5	11	6
823	$(TC)_8C$	56.5	7	5
834	$(AG)_8YT$	57.0	10	8
835	$(AG)_8YC$	56.0	6	5
844	$(CT)_8RC$	59.0	9	8
848	$(CA)_8RG$	59.0	16	16
868	$(GAA)_6$	47.0	7	6
873	$(GACA)_4$	51.5	9	9
876	$(GATA)_4$	41.0	8	8
881	$GGGT(GGGGT)_2G$	59.0	11	10
Total			110	90

R = Purine, Y = pyrimidine.

8.0, 1 mmol/L EDTA). Gels were stained with ethidium bromide and visualized under UV light. Band size was estimated from a 200 bp DNA ladder. ISSR bands were used to assign loci for each primer and scored as present (1) and absent (0). The band presence / absence data matrix was analyzed using POPGENE 1.31 (Yeh et al., 1999) to estimate the Nei's gene diversity (Nei, 1973), Shannon's information index (Lewontin, 1972), genetic similarity and genetic distance. A dendrogram generated with Nei's genetic distance (Nei, 1972) and the UPGMA (unweighted pair group method arithmetic averages) method and the principle coordinates analysis (PCA) were plotted using the software NTSYS pc2.10.

RESULTS

ISSR diversity

Of the 50 ISSR primers screened, 12 produced clear and repeatable fragments and were selected for further analysis (Table 2). These primers consistently amplified a total of 110 bands, of which 90 (81.82%) were polymorphic. The bands amplified by each primer differed in number and intensity, with fragment size raging from 100 to 2000 bp. The bands per primer produced varied from 6 to 16, with a mean of 9.17. ISSR sequences corresponded either to high or low intensity bands from fingerprint patterns (Figure 1).

Genetic diversity and differentiation

Using the data from all PCR amplified bands shown by 12 ISSR primers, the genetic similarity matrix among all sources used in present work was obtained by multivariate analysis using Nei's coefficient. Similarity coefficients ranged from 0.6636 to 0.8818 with an average of 0.8273. The highest genetic similarity coefficient (0.8818) was

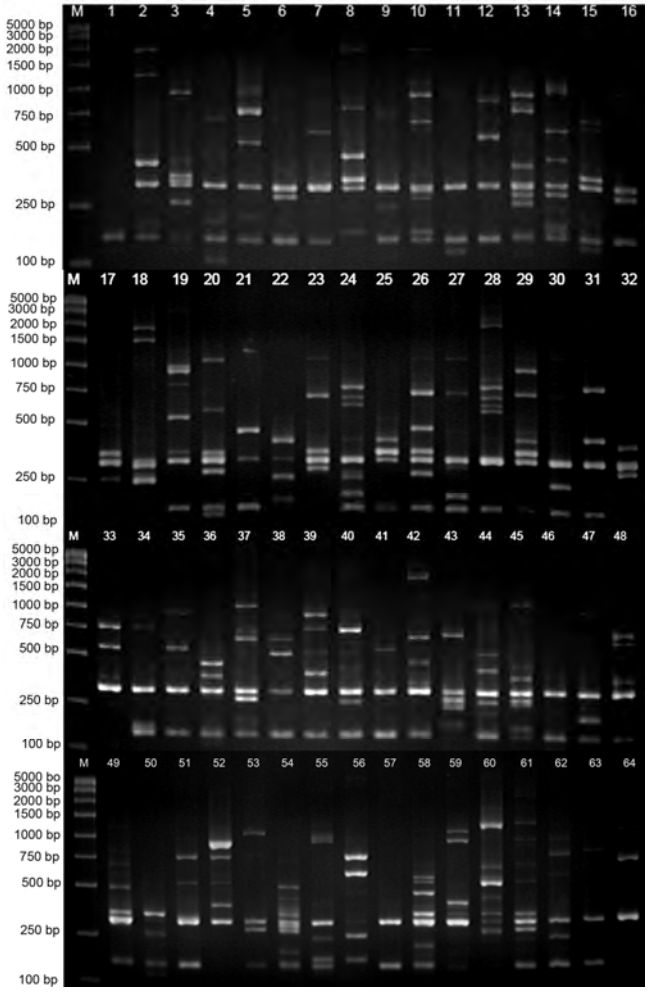

Figure 1. ISSR Fingerprints of 64 tung cultivars amplified with $(CA)_8RG$. M is the 200 bp DNA ladder.

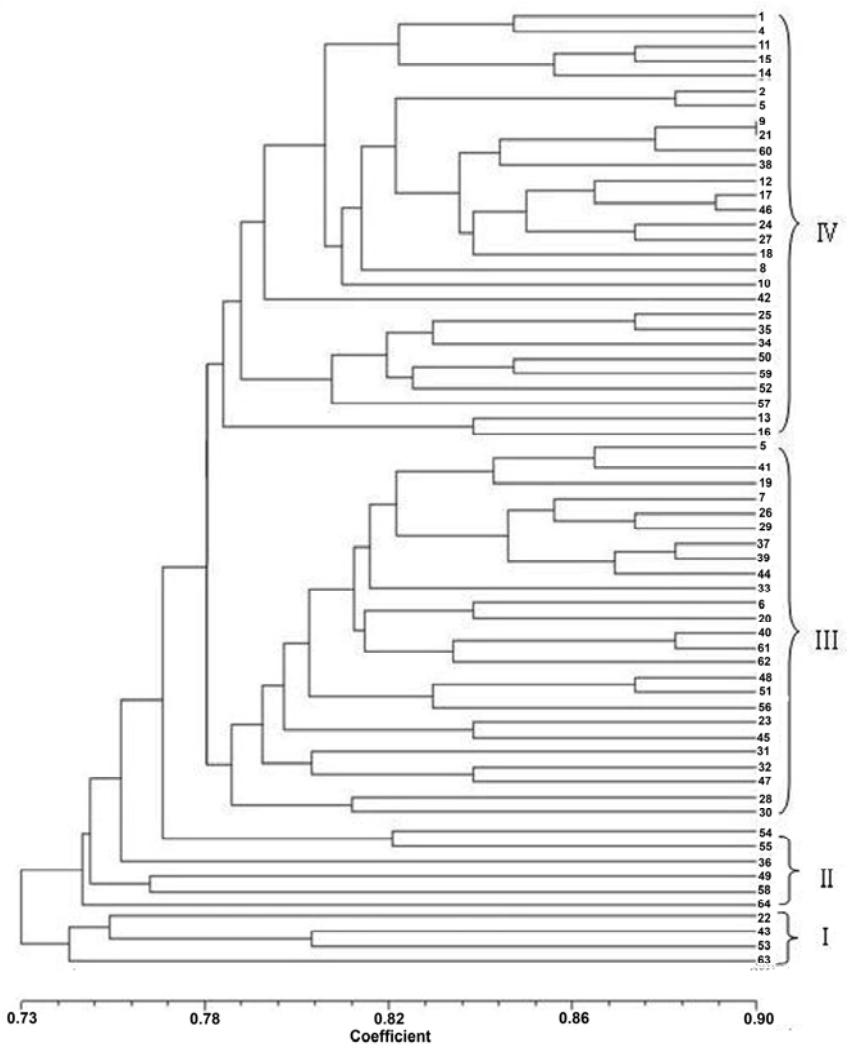

Figure 2. Dendrogram (UPGMA method) of 64 tung cultivars based on genetic distance.

found between Henglu 7 and Chenjiaxu 9 - 18, indicating that they are closely related. The lowest genetic similarity coefficient (0.6636) was found between Hunan 72 - 159 from Hunan Province and Chanxing 31 - 6 from Zhejiang Province, indicating that that they are remote in relationship. The mean Nei's genetic diversity and Shannon's information index among 64 tung cultivars were 0.1770 and 0.2453, respectively. On the basis of the genetic distance, a dendrogram was generated with UPGMA clustering analysis (Figure 2). UPGMA cluster analysis divided the 64 cultivars into 4 clusters and showed clear genetic relationships among them. Most cultivars from the same place are mainly in the same cluster, but with exceptions. Most of the members from Zhejiang were in cluster, but Taishun 5 and Xianju 1 were in cluster, and Chenjiaxu 9 - 20 is in cluster. Meanwhile, cultivars with geographical difference may be in the same cluster. To check the reliability of the results above, the principle coordinates analysis (PCA) was also used. The same genetic relationships among the cultivars were observed.

DISCUSSION

Identification and analysis of the genetic diversity of tung cultivars are traditionally based on the morphological characters. It takes years for ligneous plants. Due to environmental influence, phenotypic traits in many cases fail to serve as unambiguous markers for diversity analysis (Wang and Tanksley, 1989). Evolution and identification of germplasm using ISSR are playing a significant role in studies of genetics and breeding. In this paper, 12 primers were used for fingerprinting and genetic diversity of tung cultivars. Among the primers used, the 848 primer had the highest polymorphism. Each cultivar can be identified from the unique band patterns amplified with 848, indicating that ISSR was a very effective tool in the

identification of tung cultivars.

Principle coordinate analysis (PCA) is one of the multiple approaches to grouping, based on the similarity coefficients or variance-covariance among the traits of the entries (Akond et al., 2007). Since this is the first study on tung diversity using molecular markers, no comparison is possible. However, the coherence of the dendrogram and the PCA strongly support the reliability of the marker system.

The present data revealed relatively higher polymorphism (81.82%) but lower genetic similarity coefficient (from 0.6636 to 0.8818), indicating that the genetic diversity of tung is low at the level of the total 64 cultivars, but some individuals are remote from others. The data also suggest that cultivars cultivated in the same place now may have different genetic backgrounds. Namely they were selected from places far away and have different ancestors, and cultivars with different location can also have the same origin. All of these are the evidence that, because of long cultivation history, human factors exert influence on the inconsistency between genetic base and current situations of cultivars. This is different from earlier studies on genetic diversity at population level of some other plants, which showed spatial patterns corresponding to geographic locations (Wang et al., 2004; Zhang et al., 2006). Although the tung varieties were bred in different circumstances, they have some common pedigrees. But internal factors do not determine everything, and external situation such as eco-condition increases cultivation adaptation. At the molecular level, this may be gene mutation or other chromosome changes. Those multiple factors made the genetic background rather complex.

Furthermore, the information obtained from the present study could be of practical use for mapping the tung genomes as well as classical breeding. For instance, according to heterosis theories, the two cultivars with the lowest genetic similarity coefficient (Hunan 72 - 159 and Chanxing 31 - 6) may generate progenies with fine traits, and can be chosen for hybrid test. The informative primers identified in our studies will be useful in genetic analysis of tung accessions in germplasm holdings. The putative cultivar-specific bands can be used for genotype characterization and grouping germplasm accessions. To help overcome some of the problems usually associated with a tree crop improvement program, the study provide a basis for tung breeders to make informed choices on selection of parental material based on genetic diversity. Dangi et al. (2004), Jeung et al. (2005) and Escandón et al. (2007) had used more than one marker system for genetic diversity research, and that could overcome shortages of using a marker only. In order to get more accurate results, SSR, AFLP and RAPD methods can be applied to further studies.

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