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Article

Genetic Diversity and Population Structure of *Toona Ciliata* Roem. Based on Sequence-Related Amplified Polymorphism (SRAP) Markers

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Abstract: Sequence-related amplified polymorphism (SRAP) markers were used to investigate the genetic diversity among 30 populations of *Toona ciliata* Roem. sampled from the species' distribution area in China. To analyze the polymorphism in the SRAP profiles, 1505 primer pairs were screened and 24 selected. A total of 656 SRAP bands ranging from 100 to 1500 bp were acquired, of these 505 bands (77%) were polymorphic. The polymorphism information content (PIC) values ranged from 0.32 to 0.45, with an average of 0.41. An analysis of molecular variance (AMOVA) indicated that the most significant variation was attributable to differences among the populations and that variation within the populations was

small. STRUCTURE analysis divided the 30 populations into two parts. The unweighted pair group method of arithmetic averages (UPGMA) clustering and principal coordinates analysis (PCoA) showed that the 30 populations could be classified into four types. The results demonstrate a clear geographical trend for *T. ciliata* in China and provide a theoretical basis for future breeding and conservation strategy of *T. ciliata.*

Keywords: *Toona ciliata* Roem.; SRAP markers; PIC; genetic diversity

1. Introduction

Toona ciliata Roem. (family *Meliaceae*) is a deciduous or semi-deciduous tree. It is one of the precious timber species in China and listed as a level II national key protected wild plant in the *China Plant Red Data Book* [1]. The flowers are small and hermaphroditic, with the pollen spread primarily by wind. The seeds are light with wings, dispersed mainly by wind also. Propagation by seed is most common. *T. ciliata* is mostly distributed in hilly and mountainous areas, and its vertical distribution range is extensive [2]. Commonly known as Chinese mahogany, the tree grows with straight trunk and produces red wood with beautiful grains [3]. Low natural regeneration and over-exploitation have resulted in the continual decline of *T. ciliata* leading to it being classified as an endangered species. It is also listed on *China's reference list of cultivated rare species* [4]. Given the huge developmental potential of this species, it has become the focus of a development and utilization program targeting the planting of fast-growing timber species in southern China [5–7]. In addition, the China's State Forestry Administration strictly controls the cutting of *T. ciliata* in order to protect and manage well. [8]. An in-depth study of the species, focusing on understanding its traits to improve breeding and cultivation, will be of great significance and value in forestry production and industrial development [9]. As a rare and endangered species, analyses of the genetic diversity and structure in *T. ciliata* are particularly important for species conservation, exploration of genetic resources, and development of breeding programs.

Molecular markers are useful and effective for assessing genetic diversity and population structure of plant species. Molecular markers that can be directly estimated from DNA are the most widely used type of genetic marker in forestry studies for several reasons. They can be obtained from plants of any growth stage and are not influenced by environmental or seasonal factors [10,11]. Of the various molecular marker techniques available, sequence-related amplified polymorphism (SRAP) is a novel third-generation technique. The SRAP protocol is a polymerase chain reaction (PCR) based marker system designed to amplify open reading frames (ORFs) [12,13]. The protocol is simple, efficient, and has a high production rate. It has been used successfully in genetic diversity analysis and construction of genetic maps of many plant species [14–23].

Due to the sporadic natural distribution of *T. ciliata*, its genetic diversity has not been investigated systematically; this has seriously hampered further study on the development and utilization of this species. In this study, SRAP was used for the first time to assess the genetic diversity of 30 populations of *T. ciliata* in China to define the level of genetic diversity and the relationships among different populations. The data further provide a theoretical and experimental basis for future breeding, evaluation, management, and conservation of the species.

2. Materials and Methods

2.1. Plant Material

Samples from 30 populations of *T. ciliata* were collected from distribution range in 11 provinces of in China. Information regarding the plant materials is provided in Table 1. The number of parent trees representing each population was 30; a few had less due to small population size. The distance between mother trees was at least 50 m in order to reduce the probability of sampling trees that were closely related. Young, healthy leaves were collected, dried with silica gel, then stored at −80 °C until DNA extraction.

Table 1. Location of 30 populations of *T. ciliata* used in this study.

No.	Code	Province	Latitude (°′ N)	Longitude (°′ E)	Sample Number
1	YF	Guangdong	22°46′	111°34′	15
2	LC	Guangdong	25°07′	113°20′	20
3	JX	Anhui	30°41′	118°24′	30
4	HS	Anhui	30°16′	118°08′	6
5	CH	Guizhou	24°59′	105°48′	30
6	GL	Guizhou	25°52′	105°37	30
7	WM	Guizhou	25°10′	106°05′	30
8	LD	Guizhou	25°25′	106°44′	30
9	XY	Guizhou	25°06′	104°54′	30
10	PP	Yunnan	25°04′	99°06′	30
11	PE	Yunnan	22°46′	100°58′	30
12	PW	Yunnan	22°23′	101°04′	30
13	YR	Yunnan	25°01′	101°32′	30
14	GS	Jiangxi	27°19′	115°26′	30
15	JLS	Jiangxi	24°54′	114°47′	30
16	JGS	Jiangxi	26°44′	114°17′	30
17	WYS	Jiangxi	28°18′	117°42′	30
18	MTS	Jiangxi	27°41′	117°03′	30
19	HPS	Hunan	29°01′	111°41′	30
20	CB	Hunan	27°14′	111°28′	30
21	XN	Hunan	28°18′	109°44′	32
22	TL	Guangxi	24°17′	106°13′	30
23	LL	Guangxi	24°46′	105°20′	30
24	XL	Guangxi	24°29′	105°05′	30
25	XJ	Zhejiang	28°45′	119°92′	30
26	SC	Zhejiang	28°59′	119°25′	30
27	NP	Fujian	26°38′	118°10′	30
28	SP	Hubei	30°17′	109°28′	30
29	HD	Sichuan	27°23′	102°09′	30
30	DC	Sichuan	26°40′	102°32′	30

2.2. DNA Extraction

About 100 mg dried leaves were used for DNA extraction using EZNA® High-Performance DNA Mini Ki (Omega Bio-Tek Inc., Norcross, GA, USA) and the quality of DNA was tested by 0.8% (w/v) agarose gel electrophoresis. DNA concentrations were measured with an ultraviolet spectrophotometer, adjusted to 50 ng μL^{-1}, and stored at −20 °C for PCR amplification.

2.3. SRAP Analysis

The SRAP technique followed a combination of two primers with arbitrary sequences: a forward primer of 17 bases and a reverse primer of 18 bases. Pairs of primers with AT- or GC-rich cores were used to amplify intragenic fragments for polymorphism detection. Both the forward and reverse primers contained three elements: a 5' 10–11-base filler of different but no specific sequence, a CCGG sequence in the forward primer and AATT in the reverse primer, and a 3' three-nucleotide selective sequence. Variation in these three selective nucleotides generated a set of primers sharing the same core sequence [13].

Eight individual plant materials from different populations were used for the initial screening. In a subsequent test of material from 16 individuals, 24 different combinations of primers, including 14 forward and 15 reverse primers, were employed. The SRAP primer sequences are listed in Table 2. For SRAP analysis, PCR amplification was carried out in a total volume of 25 μL, containing 1 μL 50 ng μL^{-1} template DNA, 2.5 μL 10 × PCR buffer (100 mM Tris-HCl, pH 8.3, 500 mM KCl), 2 μL 2.5 mM each deoxy-ribonucleoside triphosphate (dNTP), 0.75 μL 10 mM each primer, 2.5 μL 25 mM $MgCl_2$, and 2 U *Taq* DNA polymerase (TaKaRa). PCR amplification began with a 5-min denaturation at 94 °C followed by five cycles of three steps (1 min of denaturing at 94 °C, 1 min of annealing at 35 °C, and 1 min of elongation at 72 °C). The next 35 cycles used the same three steps, but with an annealing temperature of 50 °C, and were followed by a final elongation step of 10 min at 72 °C. PCR products were separated on 6% denatured polyacrylamide gels and detected by silver staining. Gels were photographed or dried for the following analysis.

Table 2. Primer sequences used in SRAP analysis of *T. ciliata.*

Primer	Primer Combination Sequences	
Me17Em21	F:5'TGA GTA CAA ACC GG TAG 3'	R:5'GAC TGC GTA CGA ATT TCG 3'
Me19Em13	F:5'TGA GTA CAA ACC GG TTG 3'	R:5'GAC TGC GTA CGA ATT CTA 3'
Me19Em15	F:5'TGA GTA CAA ACC GG TTG 3'	R:5'GAC TGC GTA CGA ATT CTT 3'
Me19Em27	F:5'TGA GTA CAA ACC GG TTG 3'	R:5'GAC TGC GTA CGA ATT CCA 3'
Me22Em21	F:5'TGA GTA CAA ACC GG GTC 3'	R:5'GAC TGC GTA CGA ATT CCA 3'
Me22Em27	F:5'TGA GTA CAA ACC GG GTC 3'	R:5'GAC TGC GTA CGA ATT CCA 3'
Me23Em22	F:5'TGA GTA CAA ACC GG AGT 3'	R:5'GAC TGC GTA CGA ATT GTC 3'
Me23Em31	F:5'TGA GTA CAA ACC GG AGT 3'	R:5'GAC TGC GTA CGA ATT CTCA 3'
Me27Em28	F:5'TGA GTA CAA ACC GG GAT 3'	R:5'GAC TGC GTA CGA ATT TGA 3'
Me27Em7	F:5'AGC GAG CAA GCC GG GAT 3'	R:5'GAC TGC GTA CGA ATT GAG 3'
Me28Em18	F:5'GAC CAG TAA ACC GG TGG 3'	R:5'GAC TGC GTA CGAATT AGC 3'
Me31Em24	F:5'TGA GTA CAA ACC GG ATG 3'	R:5'GAC TGC GTA CGA ATT CAG 3'
Me32Em17	F:5'GAG CGT CGA ACC GG GAA 3'	R:5'GAC TGC GTA CGAATTCAG 3'
Me32Em26	F:5'GTA CAT AGA ACC GG GAA 3'	R:5'GAC TGC GTA CGA ATT CAG 3'

Table 2. *Cont.*

Primer	Primer Combination Sequences	
Me34Em13	F:5'GTA CAT AGA ACC GG TAT 3'	R:5'GAC TGC GTA CGAATT CAG 3'
Me35Em13	F:5'GTA CAT AGA ACC GG ATG 3'	R:5'GAC TGC GTA CGAATT CAG 3'
Me35Em14	F:5'TAC GAC GAA TCC GG ATG 3'	R:5'GAC TGC GTA CGAATT CAG 3'
Me35Em32	F:5'CAC AGT CAT GCC GG ATG 3'	R:5'GAC TGC GTA CGA ATT ATT 3'
Me38Em21	F:5'CAC AGT CAT GCC GG AGT 3'	R:5'GAC TGC GTA CGAATT ATT 3'
Me38Em24	F:5'ATC AGT CGG ACC GG AGT 3'	R:5'GAC TGC GTA CGAATT ATT 3'
Me39Em32	F:5'GTA CAT AGA ACC GG ACT 3'	R:5'GAC TGC GTA CGAATT ATT 3'
Me40Em13	F:5'TAC GAC GAA TCC GG ACT 3'	R:5'GAC TGC GTA CGA ATT CTA 3'
Me40Em22	F:5'CAC AGT CAT GCC GG ACT 3'	R:5'GAC TGC GTA CGAATT GTC 3'
Me43Em19	F:5'CAC AGT CAT GCC GG ATT 3'	R:5'GAC TGC GTA CGAATT ACG 3'

2.4. Data Analyses

Amplified fragments with the same gel mobility were scored as 1 for presence or 0 for absence to generate a binary data matrix. Differences in band strength were ignored. POPGENE1.32 was used to estimate genetic diversity parameters [24–26], including Shannon's information index (*I*), Nei's gene diversity (*H*), effective number of alleles (*Ne*), observed number of alleles (*Na*), genetic differentiation (*Gst*), and Nei's genetic distance (1978). Pair-wise Nei's genetic distance estimates were subjected to a cluster analysis by the UPGMA (unweighted pair group method with arithmetic average) method [27]. The degree of genetic relatedness among populations was assessed by principal coordinates analysis (PCoA). Analysis of molecular variance (AMOVA) based on the allele frequency data was performed to explore the genetic differentiation among and within populations [28] using GenAIEx 6.5. STRUCTURE 2.3 was used to analyze the genetic structure [29] using a model based on Bayesian method and genotype data to assign individuals to different clustering. ΔK was calculated through the second order change rate of L (K) between adjacent K value [30].

The polymorphism information content (PIC) was used to evaluate the distinguishing ability of SRAP markers in the assessment of genetic diversity with different primer combinations:

$$\mathrm{PIC}i = 2fi\,(1 - fi) \quad (1)$$

where PICi is the PIC of marker i, fi is the frequency of present marker fragments, and 1-fi is the frequency of absent marker fragments. The PIC value of each primer combination was the average of the bands [25].

3. Results

3.1. SRAP Fragments Amplified Polymorphism Analysis

Twenty-four primer pairs of 1505 primer combinations tested yielded clear, high-stability polymorphic bands. Table 3 shows the total number of bands, the number of polymorphic bands, the percentage of polymorphic bands (PPB) and PIC obtained from the SRAP primer combinations. A total of 656 bands were amplified, of which 505 (77%) were polymorphic. The number of bands of each primer combination ranged from 19 to 36, with an average of 27. The number of polymorphic fragments ranged from

14 to 29, with an average of 21. Each primer combination amplified multiple fragments and polymorphism bands. Polymorphism was highest for the Me39Em32 primer combination (94.74%), while Me35Em32 had the lowest polymorphism (51.85%).

Table 3. Total number of bands, polymorphic bands, polymorphism and polymorphic information content (PIC) obtained from 24 SRAP primer combinations.

Primer Combinations	Total No. of Bands	Polymorphic Bands	Polymorphism (%)	PIC
Me17Em21	27	21	77.78	0.38
Me19Em13	25	21	84.00	0.42
Me19Em15	26	23	88.46	0.42
Me19Em27	25	21	84.00	0.41
Me22Em21	26	21	80.77	0.41
Me22Em27	28	20	71.43	0.41
Me23Em22	30	26	86.67	0.44
Me23Em31	28	21	75.00	0.41
Me27Em28	25	21	84.00	0.39
Me27Em7	25	21	84.00	0.44
Me28Em18	36	27	75.00	0.40
Me31Em24	36	29	80.56	0.43
Me32Em17	28	20	71.43	0.43
Me32Em26	32	28	87.50	0.42
Me34Em13	25	19	76.00	0.39
Me35Em13	27	15	55.56	0.42
Me35Em14	26	15	57.69	0.40
Me35Em32	27	14	51.85	0.45
Me38Em21	24	19	79.17	0.39
Me38Em24	29	21	72.41	0.42
Me39Em32	19	18	94.74	0.43
Me40Em13	28	23	82.14	0.42
Me40Em22	21	15	71.43	0.33
Me43Em19	33	26	78.79	0.43
Total	656	505	1850.36	
Mean	27.33	21.04	77.10	0.41

PIC revealed the discriminatory power of the various primer combinations. The highest PIC value, 0.45, was obtained for Me35Em32 combination, followed by 0.44 for Me23Em22 and Me27Em7, and 0.43 for Me31Em24, Me32Em17, Me39Em32 and Me43Em19. The primer combinations with the higher PIC values could be used to develop a rapid detection method [31–33]. The primer combination Me17Em27 had the lowest PIC value of 0.32.

3.2. Genetic Relationship Analysis Based on SRAP

The PPB of *T. ciliata* was 77.10%. The average value of Nei's gene diversity index (*H)* was 0.3775. Shannon's information index (*I*) ranged from 0.1748 to 0.4482. The average number of alleles (*Na*) and effective number of alleles (*Ne*) were 1.7710 and 1.6587, respectively. According to POPGENE analysis,

the total genetic diversity (*Ht*) was 0.3876, the genetic diversity within populations (*Hs*) was 0.1237, the coefficient of genetic differentiation (*Gst*) was 0.6809, and the estimate of gene flow (*Nm*) was 0.2343.

The dendrogram of genetic relationships based on the UPGMA clustering method is shown in Figure 1. The 30 *T. ciliata* populations could be classified into four major clusters. The result was consistent with biplot of PCoA analysis shown in Figure 2 (the contribution percentage of the first and second principal components was 52.76% and 60.12%, respectively). The first group consisted of the 14 populations from Hubei, Hunan, Jiangxi, Fujian, Zhejiang and Anhui provinces. The second group had only one accession, LC, which originated from Guangdong Province. The 13 populations from Guizhou (excluding GL), Sichuan and Yunnan were grouped in the third cluster. Group four was composed of populations from YF (Guangdong) and GL (Guizhou). The genetic distances among the four clusters are shown in Table 4.

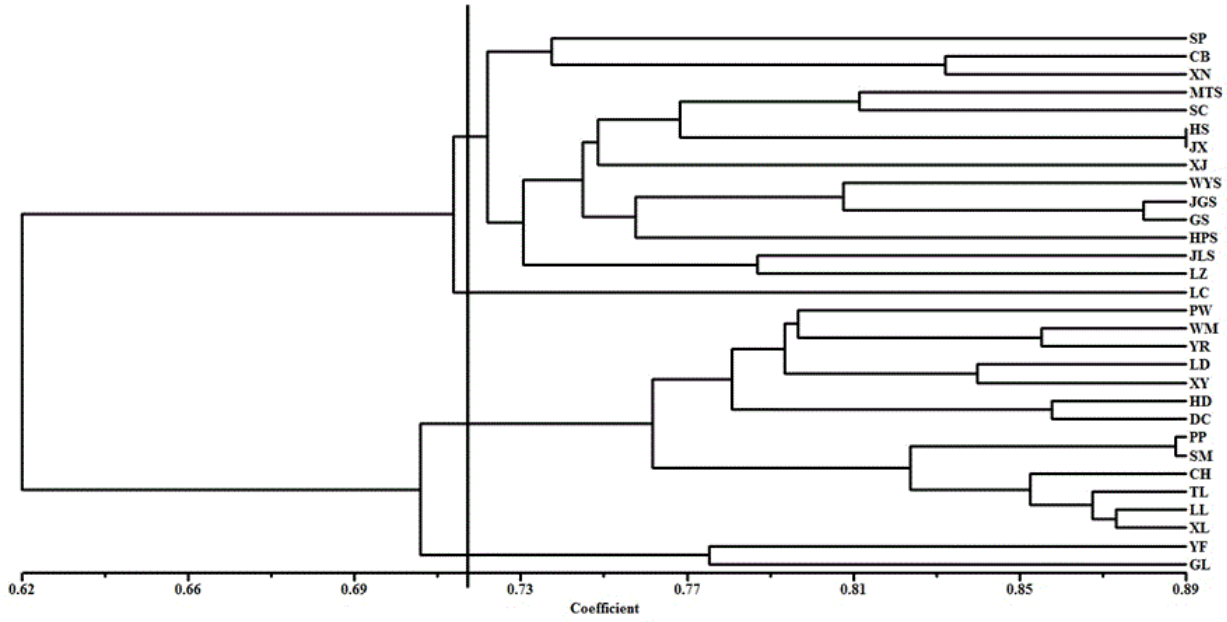

Figure 1. Phylogenetic dendrogram based on the genetic distance by the UPGMA cluster in the 30 populations of *T. ciliata*.

3.3. Genetic Structure Analysis

Results of the model STRUCTURE is showed in Figure 3. The 30 *T. ciliata* populations were divided into two groups. Group I included the populations from central and eastern China. The populations of southwest and south China formed Group II (Figure 4). NP, JLS and LC were in a state of transition.

3.4. AMOVA Analysis

AMOVA showed that 79.24% of the total variation resided among populations, while the proportion of total variation within populations was 20.76%. (Table 5). These findings are consistent with those from the genetic diversity analysis in this study.

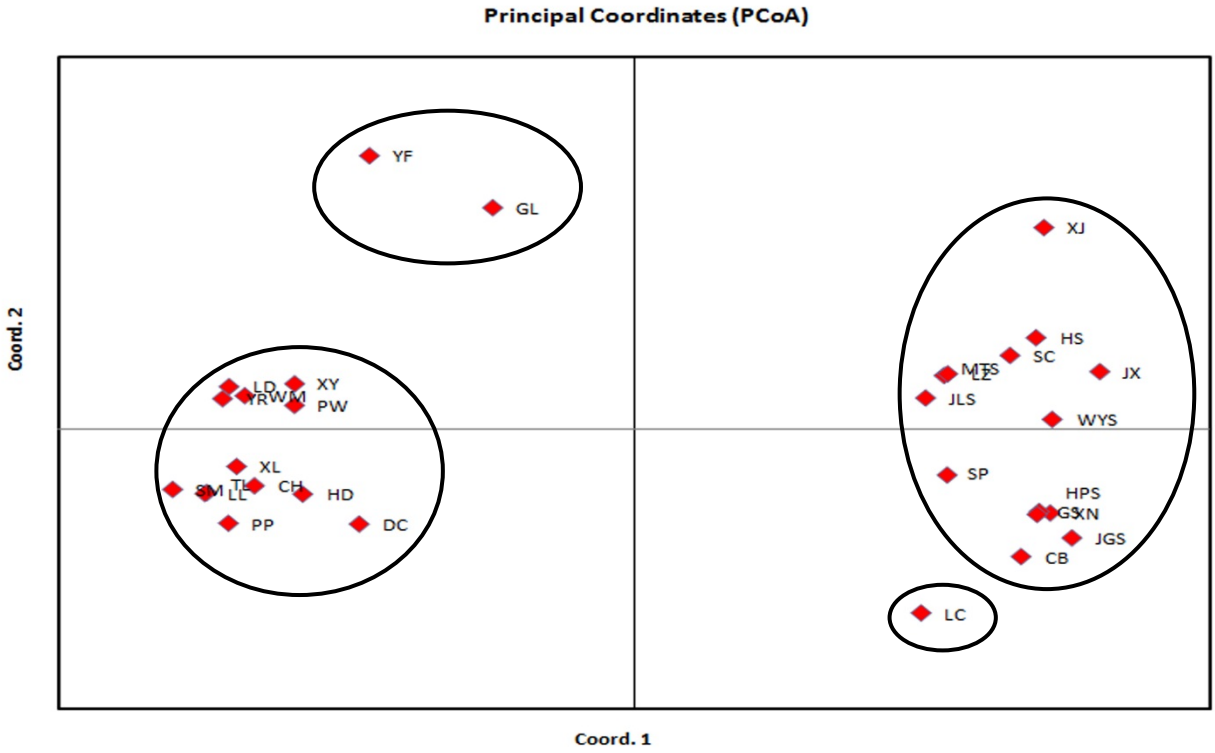

Figure 2. Biplot of the principle coordinates analysis of 30 *T. ciliata* populations. Codes of populations follow those given in Table 1.

Table 4. The genetic distances among the four clusters of *T. ciliata.*

	1	2	3	4
1	****			
2	0.3347	****		
3	0.2321	0.5383	****	
4	0.3038	0.6408	0.2070	****

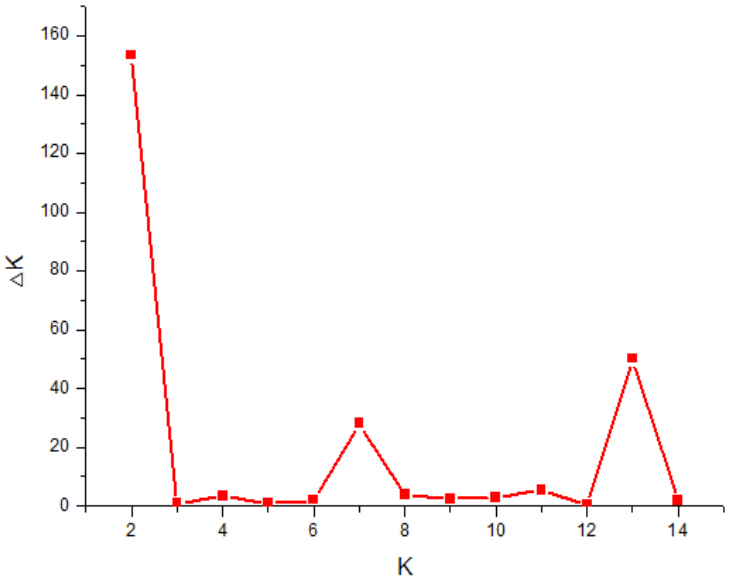

Figure 3. Relations between *K* and *ΔK*.

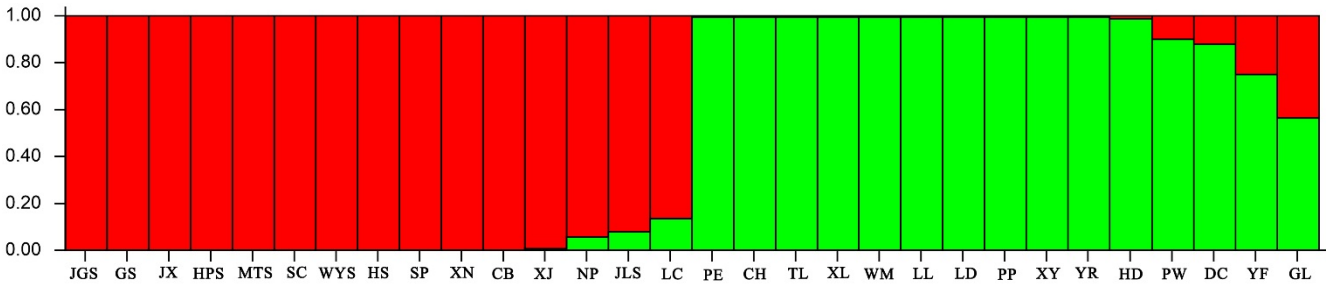

Figure 4. Clustering results of 30 *T. ciliata* populations using STRUCTURE.

Table 5. Analysis of molecular variance (AMOVA) of 30 *T. ciliata* populations.

Source	df	Sum of Squares	Mean Squares	Variation Component	Percentage of Variance (%)	*Φst*	*P*
Among Pops	29	40,273.140	1388.729	81.979	79.24%	0.792	0.001
Within Pops	475	10,197.632	21.469	21.469	20.76%		
Total	504	50,470.772		103.448	100%		

4. Discussion

To the best of our knowledge, this study was the first time to use the SRAP molecular marker to evaluate the genetic diversity of *T. ciliata.* Results show that the novel SRAP marker was suitable for distinguishing *T. ciliata.*

Polymorphic loci are amplified DNA fragments from sites with a frequency of less than 0.99 [34]. The percentage of polymorphic bands (PPB) is an important indicator of the level of genetic variation in a species and an important parameter for measuring genetic diversity. In general, if the PPB of population is high, the population has strong ability to adapt to the environment. On the contrary, a population that has low PPB is unlikely to adapt to the environment and may be eliminated in the long-term evolution [34]. The PPB was 77.10% in this study, indicating a certain degree of genetic variation among populations. For the PIC, when the PIC > 0.5, 0.25 < PIC < 0.5, or the value < 0.25, loci polymorphism could be classified as high, medium, or low, respectively [31–33]. In this study, Me35Em32 was the most informative primer combination for genetic diversity studies among *T. ciliata.* Twenty-four SRAP primer combinations had an average value of 0.41, indicating that the SRAP markers could be used to identify polymorphic loci for assessment of the genetic variation among *T. ciliata.* Shannon's information index (*I*) indicates the level of genetic diversity; a higher value indicates greater genetic diversity [35]. The *I* values for the 30 populations in the study ranged from 0.1748 to 0.4482, indicating various levels of genetic diversity. All of Nei's gene diversity index (*H)*, Shannon's information index (*I*) and AMOVA result all showed that the most significant variation was attributed to differences among the populations, which revealed that population selection is important for the breeding of *T. ciliata.*

Through various studies on perennial woody plants, it is believed that species with longer lives have a broad distribution area spread by animal, possesses abundant genetic diversity [36–38]. The higher genetic diversity among populations of *T. ciliata* and the greater genetic differentiation among populations are closely correlated with their biological characteristics. As a perennial broad-leaf species, *T. ciliata* has a long life

history. In addition, the main strategy of pollen spread is anemophily, and it carries out sexual propagation by virtue of the seeds [39], all of which lay the foundation for preserving the genetic diversity of *T. ciliata* during adaption to the living environment. Simultaneously, larger differences exist with regards to the natural distribution of *T. ciliata*, which has a broad natural distribution range [40]. Through long-term natural selection, *T. ciliata* has established an ability to survive through strong adaptation to the local environments. Its varying flowering period is an important expression of adaptive capacity. Influenced by air temperature, rain and sunshine, the populations close to southwest of China flower from March to April. The flowering period in other regions generally occurs from May to June. The habitat fragmentation that is influenced by constant changes and deterioration of the environment has probably caused the isolation of the species and intensified the genetic differentiation among populations. Additionally, *T. ciliata* has a long generation, which implies the low frequency of gene exchange.

A combination of mutation, genetic drift, and natural selection promotes both the differentiation of population genetics and the formation of genetic structure. However, gene flow weakens the genetic structure of a population [38,41]. A gene flow (*Nm*) value <1 indicates a greater degree of isolation between populations; thus gene flow is insufficient to overcome the differentiation caused by genetic drift [35]. In the current study, the *Nm* value of *T. ciliata* was 0.2343, revealing that gene flow among populations occurred and the extent of differentiation was moderate. The spread of *T. ciliata* pollen is limited, and seed dispersal spread is restrictive, so geographic isolation hindered gene exchange among populations, resulting in a lower and gene flow.

According to the genetic diversity (based on coefficient of 0.72), the 30 populations could be divided into four types that are essentially identical to those determined by PCoA analysis. LC population is from furthest north of Guangdong and formed its own cluster (Cluster II) separately. Its geographical isolation is due to the complex environment with mountain and hills and it is located close to Hunan and Jiangxi provinces. The genetic distance between LC and Cluster I was less than that between LC and Cluster III (Table 4), suggesting that LC is more closely related to the populations from central and eastern China. Cluster IV included two populations from YF (Guangdong) and GL (Guizhou). The YF population was later found to have been introduced from Nanyang (now known as Southeast Asia) in the process of seed investigation. After clustering based on SRAP, YF grouped only with GL (Guizhou). These findings suggest the conclusion that the populations of YF and GL populations may be from the same original source, and the genes they carry may be different from those carried by other populations, which should be further researched in the future. Genetic diversity and population structure data based on SRAP are a theoretical basis for more in-depth study and research on *T. ciliata* breeding. Additionally, in the process of breeding selection, growth indices, such as tree height, diameter and wood quality should be included to determine breeding materials in the same groups.

At present, the distribution of *T. ciliata* is scattered in China, and its natural regeneration is weak. Additionally, excessive development of the land and man-made interference increases geographic and genetic isolation, further hindering regeneration. To protect its economic value and guarantee sustainable development of its biodiversity, the protection and management should be strengthened. The conservation generally includes *in situ* (on-farm or wild) and *ex situ* (gene or field bank) approaches. The achieving complementary *in situ* and *ex situ* holdings is great important for perennial species [42]. As for the broad-leaf species, which has a longer life history, extant natural forests of *T. ciliata* exist in the form of ancient trees. Consequently, to protect the species, it is necessary to maintain the original

living environment and alleviate the intensification of habitat fragmentation. Natural protection areas have been established in some natural distribution areas, providing local protection (*in situ*) for *T. ciliata.* In this study, the populations of Jiangxi were collected from natural protection areas, revealing the excellent effects of protection and management. While carrying out protection of the original habitat, we should take corresponding measures to cultivate and collect seeds for directive breeding (*ex situ*). Moreover, we should reinforce the technology of artificial reproduction. We will collect all current genetic resources and expand the range of collection as far as possible, especially those populations which have lower genetic diversity. Furthermore, we will carry out extensive research, combining pest control, biological studies, and breeding in various ways to develop and expand the directions for both protection and application.

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Author Contributions

Pei Li designed and conducted the experiments, analyzed the data, and wrote the manuscript. Pei Li, Xin Zhan and Xiaomei Deng carried out the population collection. Quemin Qing, Wenting Qu, Juncheng Li, Junjie Zhang and Boyong Liao for DNA extraction and data analysis. Mingqian Liu and Ruiqi Pian for techbical assistance. Kunxi Ouyang and Xiaoyang Chen for their valuable comments to improve the initial manuscript. All authors read and approved the final manuscript.

Conflicts of Interest

The authors declare no conflict of interest.

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