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Population Structure and Genetic Relationships of *Melia* Taxa in China Assayed with Sequence-Related Amplified Polymorphism (SRAP) Markers

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Abstract: The uncertainty about whether, in China, the genus *Melia* (Meliaceae) consists of one species (*M. azedarach* Linnaeus) or two species (*M. azedarach* and *M. toosendan* Siebold & Zuccarini) remains to be clarified. Although the two putative species are morphologically distinguishable, genetic evidence supporting their taxonomic separation is lacking. Here, we investigated the genetic diversity and population structure of 31 *Melia* populations across the natural distribution range of the genus in China. We used sequence-related amplified polymorphism (SRAP) markers and obtained 257 clearly defined bands amplified by 20 primers from 461 individuals. The polymorphic loci (*P*) varied from 35.17% to 76.55%, with an overall mean of 58.24%. Nei's gene diversity (*H*) ranged from 0.13 to 0.31, with an overall mean of 0.20. Shannon's information index (*I*) ranged from 0.18 to 0.45, with an average of 0.30. The genetic diversity of the total population (H_t) and within populations (H_s) was 0.37 $\pm$ 0.01 and 0.20 $\pm$ 0.01, respectively. Population differentiation was substantial (*Gst* = 0.45), and gene flow was low. Of the total variation, 31.41% was explained by differences among putative species, 19.17% among populations within putative species, and 49.42% within populations. Our results support the division of genus *Melia* into two species, which is consistent with the classification based on the morphological differentiation.

Keywords: *Melia azedarach*; *M. toosendan*; Meliaceae; SRAP; genetic diversity; genetic relationship

1. Introduction

The genus *Melia* belongs to the order Rutales and family Meliaceae. Fossil evidence indicates that *Melia* could have evolved in Indochina during the Middle–Lower Miocene [1–3]. *Melia* is widely distributed in China and has a considerable economic value with respect to the development of botanical pesticides, timber, bioremediation in urban industrial districts, and a combination of forestry and agricultural uses [4–11]. However, classification of the species in *Melia* is still in dispute in the literature. Whether the genus *Melia* (Meliaceae) consists of one species (*M. azedarach*) or two species (*M. azedarach* and *M. toosendan*) in China is under debate. According to Flora Reipublicae Popularis

Sinicae [12], both species can be morphologically distinguished. *M. azedarach* has 5–6 ovaries, small fruits of not more than 2 cm length, lobules with obtuse teeth, and an inflorescence length that is often similar to the leaf length. *M. toosendan* has 6–8 ovaries, has large fruit of not more than 3 cm, is lobular around almost the entire margin, has no obvious obtuse teeth, and has an inflorescence length of an approximately half leaf size [12]. Despite these differences, only *M. azedarach* was included in the Flora of China [13]. In a study of the phenological delineation of the *Melia* distribution area in China, all collected *Melia* plants were classified as *M. azedarach* [14]. Zhang reported that toosendanin contents in fruits of *M. toosendan* from China were higher than those of *M. azedarach* [15]. Li compared the high-performance liquid chromatography (HPLC) fingerprints of *M. azedarach* and *M. toosendan* stones and reported differences in the numbers of characteristic peaks, peak values (relative retention time), and peak areas among samples [16]. In a public letter to the editor of Toxicology, Wiart noted that *M. toosendan* did not exist in China and was not listed in the Flora of China, 2008 [17,18]. Therefore, a more comprehensive examination, using molecular working alongside the existing classification based on the morphological traits, is needed.

Apart from the uncertainty in species delineation, studies on population structure and genetic diversity in *Melia* in China have been limited due to the small sizes of local seedlots and the availability of only a few primers for DNA amplification in the species [19,20]. This could also limit the exploitation of *Melia* in genetics and breeding programs, as population structure and genetic diversity provide essential background information for assessing the preliminary provenance. *M. azedarach* is disseminated, and has become naturalized in several tropical and subtropical areas. Because of its widespread cultivation and adaptation to diverse habitats, its original distribution is to be determined [13]. *M. azedarach* L. is found at northern latitudes between 18° and 40° and at altitudes below 2100 m in China. It is typically distributed in mixed evergreen, broad-leaved, and deciduous forests and in sparse forests, field margins, and along roadsides [13]. Its geographic range extends from Baoding (Hebei), Yuncheng (Shanxi), and Longnan (Gansu) in the north to Ya county (Hainan) in the south, and from Taiwan and Chinese coastal provinces in the east to Chengdu (Sichuan) and Baoshan (Yunnan) in the west. Thus, it is native to about one-third of the land area of China [14]. *M. azedarach* is monoclinous, and the first flowers occur 2–3 years after germination. Pollination is realized via both animal agents and wind [21–23]. Seed dispersal is mediated by animals (e.g., birds) or by gravity [24]. Such reproductive ecology suggests that gene flow among natural populations may be limited. It is hypothesized that population differentiation in *Melia* will be expected to be much greater than that in conifer and oak tree species, where gene flow is primarily mediated by wind pollination.

In our genetic analysis, sequence-related amplified polymorphism (SRAP) was used to select markers because SRAP analysis is a relatively simple and highly reproducible DNA-based method. The method is used in linkage mapping and gene tagging in plants [25]. SRAP markers are PCR-based markers, with primers 17 or 18 nucleotides in length that are used to amplify open reading frames (the coding regions in genomes). It can disclose numerous co-dominant markers with a large number of polymorphic loci and allows easy isolation of bands for sequencing. These features could yield a pattern of genetic diversity and phylogenetic relationships among populations derived from mostly functional coding regions; these would differ from other molecular markers in which both coding and non-coding variations are mixed.

To clarify the taxonomic uncertainty and the population structure in *Melia*, we investigated populations covering the natural range of this genus in China. Thirty-one populations were sampled, including the putative species of both *M. azedarach* and *M. toosendan*. Analysis of population genetic diversity and differentiation from the coding regions (SRAP) was used to determine whether the two morphologically distinguished taxa exhibited significant population genetic divergence. The degree of population genetic diversity within each taxon was also assessed.

2. Materials and Methods

2.1. Plant Materials and DNA Extraction

Using the latitude and longitude grid sampling method, we collected seeds from 31 wild populations of *Melia* in 17 provinces in China. The seedlots were evenly located across the natural range of *Melia* in China; the population distributions are shown in Table 1. Figure 1 shows the geographic locations of the sampled populations. Within each population, sample trees were separated by at least 100 m to reduce the probability of collecting seeds derived from crosses between closely related individuals. Seeds were collected from 15 trees in each population (GS, HANI, HEN, and YN3 populations: 14 tree seeds). Seeds collected from 461 parent trees in total were coded with family numbers and were planted in 2014 at the nursery of South China Agricultural University (23.0905000 N, 113.2106000 E). One healthy plant (no diseases or insect pests) was selected randomly from each family of seedlings, and the selected 461 progeny seedlings were numbered according to their respective families. When the selected seedlings reached 40 cm in height, young leaves were collected from each plant and stored separately at −80 °C until DNA extraction.

Table 1. Summary of the 31 *Melia* seed sources sampled in this study.

No.	Provenance Code	Provenance	Latitude (°′ N)	Longitude (°′ E)	Altitude (m a.s.l.)
M. toosendan					
1	GS	Gansu Longnan	33°24′	104°55′	1106
2	GZ1	Guizhou Xingyi	25°03′	104°37′	1407
3	GZ2	Guizhou Ceheng	24°57′	105°41′	1117
4	GZ4	Guizhou Zunyi	27°43′	106°55′	1168
5	SC1	Sichuan Chengdu	30°34′	104°3′	495
6	SC2	Sichuan Dazhou	31°12′	107°28′	593
7	YN1	Yunnan Mengla	21°48′	101°15′	1010
8	YN3	Yunnan Chuxiong	25°02′	101°31′	2173
M. azedarach					
9	AH	Anhui Chuzhou	32°18′	118°19′	15
10	FJ	Fujian Yong'an	25°49′	117°06′	255
11	GD1	Guangdong Kaiping	22°25′	112°43′	7
12	GD2	Guangdong Renhua	25°19′	113°55′	99
13	GX1	Guangxi Guilin	25°16′	110°17′	166
14	GX2	Guangxi Qinzhou	21°58′	108°39′	17
15	GX3	Guangxi Du'an	23°55′	108°6′	373
16	GZ3	Guizhou Liping	26°13′	109°08′	618
17	HAN1	Hainan Wuzhishan	18°47′	109°29′	280
18	HAN2	Hainan Tunchang	19°24′	110°07′	160
19	HEB	Hebei Baoding	38°52′	115°27′	22
20	HUB	Hubei Jingmen	31°02′	112°11′	98
21	HEN	Henan Xuchang	34°02′	113°51′	71
22	HUN1	Hunan Dong'an	26°22′	111°14′	205
23	HUN2	Hunan Yanling	26°27′	113°40′	200
24	HUN3	Hunan Liuyang	28°09′	113°38′	124
25	JX1	Jiangxi Yudu	25°59′	115°25′	132
26	JX2	Jiangxi Ruichang	29°40′	115°40′	18
27	SD1	Shandong Jinan	36°39′	117°07′	122
28	SD2	Shandong Tai'an	36°13′	117°06′	641
29	SX	Shanxi Weinan	34°29′	109°30′	351
30	YN2	Yunnan Malipo	23°06′	104°40′	1180
31	ZJ	Zhejiang Ling'an	30°13′	119°43′	47

Note that the provenance samples were grouped, based on morphological differences, according to the classification of two putative species described in the Flora Republicae Popularis Sinicae [12].

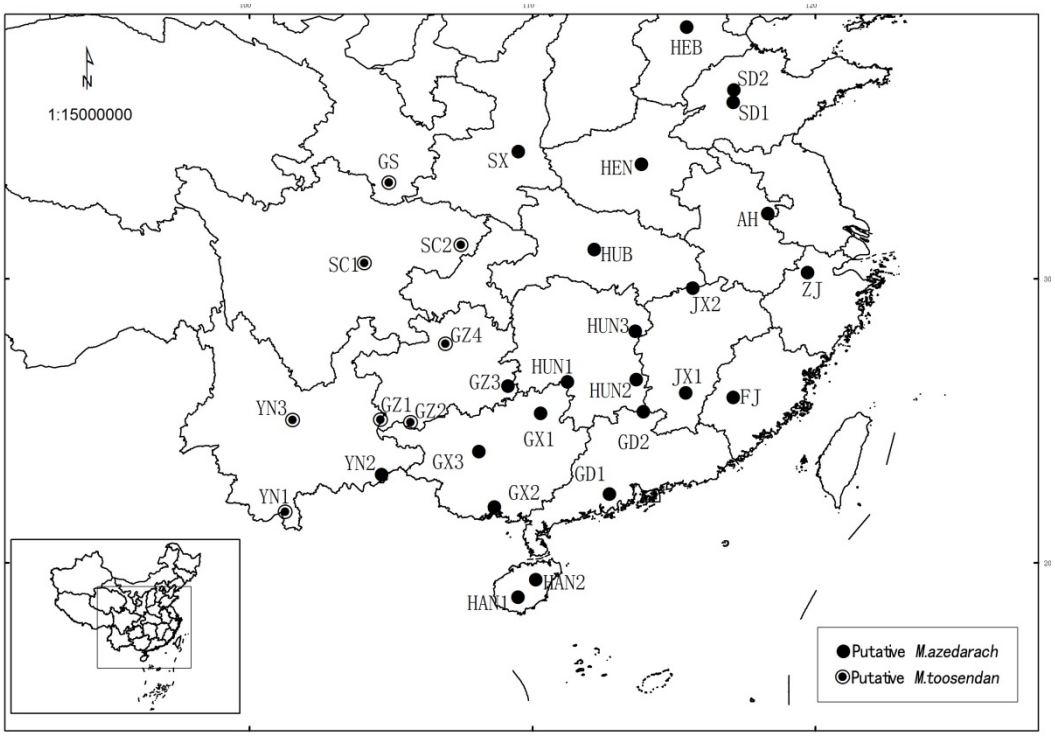

Figure 1. Geographical locations of the sampled populations covering the taxa of both *M. azedarach* and *M. toosendan*. Codes for the populations are given in Table 1.

DNA was extracted from 150 mg of leaves using the E.Z.N.A. high-performance DNA mini kit (Omega Bio-tek, Norcross, GA, USA) and separated by electrophoresis in a 1.0% agarose gel. DNA concentration was measured using a NanoDrop 1000 spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA), adjusted to 50 ng/μL, and stored at −20 °C until PCR amplification.

2.2. SRAP Analysis

SRAP analysis was performed as described by Li and Quiros [25]. All reagents and buffers were supplied by Takara Bio (Otsu, Japan). Each PCR was prepared in a 25-μL reaction mixture containing 50 ng genomic DNA, 200 μM dNTPs, 2.75 mM $MgCl_2$, 0.4 μM of each primer, 2.5 μL PCR buffer, 0.75 U *Taq* DNA polymerase, and sterile double-distilled water. PCR was conducted using the following cycle profile in an Eastwin thermal cycler (EDC-810, Suzhou, China): initial denaturation at 94 °C for 5 min, followed by five cycles of denaturation for 1 min, annealing at 35 °C for 1 min, and elongation at 72 °C for 1 min, and then 35 cycles of denaturation for 1 min and annealing at 50 °C for 1 min, ending with an elongation step at 72 °C for 5 min. Samples were then stored in a refrigerator at 4 °C until use.

The ability of the 783 SRAP primer combinations (27 forward and 29 reverse primers, Table 2) to amplify eight individual plant materials from different populations was assessed. In a subsequent test of material from 16 individuals, 20 SRAP primer combinations, including 12 forward and 13 reverse primers that identified consistently reproducible polymorphisms with clearly defined bands, were used to analyze all samples. PCR products were resolved in a 6% polyacrylamide gel at 12.5 $V \cdot cm^{-1}$ for 1.5 h, and stained with silver nitrate ($AgNO_3$) [26]. Reliable and clearly distinguishable amplified bands of 100–1500 bp were scored as either 1 (present) or 0 (absent), and a SRAP data matrix was constructed.

Table 2. Primers used for sequence-related amplified polymorphism (SRAP).

Forward Primers		Reverse Primers	
Name	Sequence (5′–3′)	Name	Sequence (5′–3′)
Me1	TGAGTCCAAACCGGATA	Em1	GACTGCGTACGAATTAAT
Me2	TGAGTCCAAACCGGAGC	Em2	GACTGCGTACGAATTTGC
Me3	TGAGTCCAAACCGGAAT	Em3	GACTGCGTACGAATTGAC
Me4	TGAGTCCAAACCGGACC	Em4	GACTGCGTACGAATTTGA
Me5	TGAGTCCAAACCGGAAG	Em5	GACTGCGTACGAATTAAC
Me6	TGAGTCCAAACCGGTAA	Em6	GACTGCGTACGAATTGCA
Me7	TGAGTCCAAACCGGTCC	Em7	GACTGCGTACGAATTGAG
Me8	TGAGTCCAAACCGGTGC	Em8	GACTGCGTACGAATTGCC
Me9	TGAGTCCAAACCGGACA	Em9	GACTGCGTACGAATTTCA
Me10	TGAGTCCAAACCGGACG	Em10	GACTGCGTACGAATTCAA
Me11	TGAGTCCAAACCGGACT	Em11	GACTGCGTACGAATTGCA
Me12	TGAGTCCAAACCGGAGG	Em12	GACTGCGTACGAATTCAT
Me13	TGAGTCCAAACCGGAAA	Em13	GACTGCGTACGAATTCTA
Me14	TGAGTCCAAACCGGAAC	Em14	GACTGCGTACGAATTCTC
Me15	TGAGTCCAAACCGGAGA	Em15	GACTGCGTACGAATTCTT
Me17	TGAGTCCAAACCGGTAG	Em16	GACTGCGTACGAATTGAT
Me18	TGAGTCCAAACCGGCAT	Em17	GACTGCGTACGAATTATG
Me19	TGAGTCCAAACCGGTTG	Em18	GACTGCGTACGAATTAGC
Me20	TGAGTCCAAACCGGTGT	Em19	GACTGCGTACGAATTACG
Me21	TGAGTCCAAACCGGTCA	Em20	GACTGCGTACGAATTTAG
Me22	TGAGTCCAAACCGGGCA	Em21	GACTGCGTACGAATTTCG
Me23	TGAGTCCAAACCGGATG	Em22	GACTGCGTACGAATTGTC
Me24	TGAGTCCAAACCGGGAT	Em23	GACTGCGTACGAATTGGT
Me25	TGAGTCCAAACCGGGCT	Em24	GACTGCGTACGAATTCAG
Me26	TTCAGGGTGGCCGGATG	Em25	GACTGCGTACGAATTCTG
Me27	TGGGGACAACCCGGCTT	Em26	GACTGCGTACGAATTCGG
Me28	TGAGTCCAAACCGGATC	Em27	GACTGCGTACGAATTCCA
		Em28	GACTGCGTACGAATTCGA
		Em29	GACTGCGTACGAATTATT

2.3. Data Analysis

POPGENE version 1.32 was used to analyze the genetic datasets [27]. Genetic diversity parameters included the total genetic diversity (H_t), heterozygosity within population (H_s), the proportion of polymorphic loci (P), Nei's genetic diversity index (H), and Shannon's information index (I) [28]. The percentage of polymorphic bands (PPB) was calculated as PPB = $(K/N) \times 100\%$, where K is the number of polymorphic bands and N is the total number of amplified bands. Population genetic differentiation (Gst) was estimated [29], and gene flow was assessed under Wright's island model of population structure [30].

The genetic relationships and genetic structure among 31 populations were examined using different analytical approaches. Analysis of molecular variance (AMOVA) was performed using Genalex 6.5 [31] to estimate the partitioning of genetic variance between the two putative species, among populations within each putative species, and within populations. Nei's genetic distances were used to perform a cluster analysis using the neighbor-joining method with 50,000 bootstraps replications. A dendrogram was constructed from the genetic distance [32] using the POPTREE2 software [33]. A Bayesian-based structure analysis was also carried out using STRUCTURE [34]. Population structure was evaluated for a range of values of K from 1 (testing for panmixis) to 14, and the results were interpreted following the approaches suggested by Pritchard *et al*. [35] and Evanno *et al*. [36]. Multivariate principal coordinate analysis (PCoA) was applied to evaluate genetic relationships among populations using Genalex 6.5 software (Oxford University Press, New York, NY, USA) [31].

To test the effects of geographical distance, we used Mantel's tests to determine whether the population genetic distance is correlated with geographic distance (km) [31,37].

3. Results

3.1. Screening SRAP Primers

Of 461 individuals representing 31 *Melia* populations, 257 clearly defined bands were amplified using 20 combinations of 12 forward and 13 reverse primers. Of these bands, 145 (58.24%) were polymorphic. The total number of bands ranged from 4 to 26, with an average of 12.85. The number of polymorphic bands ranged from 2 to 14, with an average of 7.25 (Table 3).

Table 3. Polymorphism data based on genetic analyses performed using 20 SRAP primer combinations.

Primer Combination	Total Number of Bands	Polymorphic Bands (*n*)	PPB (%)
Me1/Em9	17	14	82.35
Me1/Em17	13	3	23.08
Me2/Em12	12	2	16.67
Me2/Em13	10	9	90.00
Me4/Em5	15	6	40.00
Me5/Em10	14	9	64.29
Me6/Em4	19	13	68.42
Me6/Em5	11	5	45.45
Me6/Em10	11	5	45.45
Me6/Em29	4	2	50.00
Me11/Em29	8	8	100.00
Me17/Em29	26	10	38.46
Me19/Em5	7	5	71.43
Me19/Em7	8	3	37.50
Me20/Em7	17	13	76.47
Me24/Em14	9	8	88.89
Me27/Em4	14	8	57.14
Me27/Em18	16	4	25.00
Me28/Em15	11	10	90.91
Me28/Em19	15	8	53.33
Total	257	145	
Mean	12.85	7.25	58.24

3.2. Genetic Diversity Analysis

Estimates of genetic diversity are summarized in Table 4. The percentage of polymorphic loci (*P*) varied from 35.17% to 76.55%, with an overall mean of 58.24%. Nei's gene diversity (*H*) ranged from 0.13 to 0.31, with an overall mean of 0.20. The total genetic diversity (H_t) was 0.37 $\pm$ 0.01. Shannon's information index (*I*) ranged from 0.18 to 0.45, with an average of 0.30. Genetic diversity within populations (H_s) was 0.20 $\pm$ 0.01.

The population genetic diversity varied among provenances. The GZ2 population, originating from Ceheng (Guizhou), had the highest genetic diversity, followed by the populations from Dazhou (Sichuang), Liping (Guizhou), Yanling (Hunan), Longnan (Gansu), and Mengla (Yunnan). The Tunchang population from Hainan had the lowest genetic diversity, followed by Baoding (Hebei), Tai'an (Shandong), and Ling'an (Zhejiang).

Table 4. Genetic diversity in 31 *Melia* populations.

No.	Code	Percentage of Polymorphic Loci (*P*)	Nei's Gene Diversity (*H*)	Shannon's Information Index (*I*)
M. azedarach				
1	GS	67.59	0.25 ± 0.20	0.38 ± 0.28
2	GZ1	58.62	0.19 ± 0.20	0.28 ± 0.28
3	GZ2	76.55	0.31 ± 0.20	0.45 ± 0.27
4	GZ4	61.38	0.21 ± 0.21	0.32 ± 0.29
5	SC1	50.34	0.17 ± 0.19	0.25 ± 0.28
6	SC2	71.72	0.27 ± 0.20	0.40 ± 0.28
7	YN1	70.34	0.25 ± 0.20	0.38 ± 0.28
8	YN3	55.86	0.18 ± 0.19	0.28 ± 0.28
M. toosendan (all)		64.05	0.23 ± 0.20	0.34 ± 0.28
M. azedarach				
9	AH	64.83	0.24 ± 0.20	0.35 ± 0.29
10	FJ	52.41	0.17 ± 0.19	0.25 ± 0.27
11	GD1	56.55	0.17 ± 0.19	0.27 ± 0.27
12	GD2	55.17	0.18 ± 0.20	0.28 ± 0.28
13	GX1	62.76	0.22 ± 0.20	0.33 ± 0.29
14	GX2	54.48	0.18 ± 0.20	0.27 ± 0.29
15	GX3	53.10	0.18 ± 0.20	0.27 ± 0.29
16	GZ3	75.17	0.27 ± 0.20	0.41 ± 0.27
17	HAN1	35.17	0.19 ± 0.17	0.18 ± 0.26
18	HAN2	40.00	0.13 ± 0.18	0.20 ± 0.27
19	HEB	48.28	0.15 ± 0.19	0.23 ± 0.28
20	HUB	57.24	0.21 ± 0.21	0.30 ± 0.29
21	HEN	50.34	0.17 ± 0.20	0.26 ± 0.28
22	HUN1	68.97	0.25 ± 0.20	0.37 ± 0.28
23	HUN2	73.79	0.26 ± 0.20	0.39 ± 0.28
24	HUN3	67.59	0.25 ± 0.21	0.37 ± 0.29
25	JX1	56.55	0.19 ± 0.19	0.29 ± 0.28
26	JX2	54.48	0.18 ± 0.19	0.27 ± 0.28
27	SD1	48.97	0.17 ± 0.20	0.26 ± 0.29
28	SD2	45.52	0.15 ± 0.19	0.23 ± 0.27
29	SX	46.90	0.16 ± 0.20	0.24 ± 0.29
30	YN2	69.66	0.25 ± 0.20	0.37 ± 0.28
31	ZJ	55.17	0.15 ± 0.18	0.23 ± 0.26
M. azedarach (all)		56.22	0.19 ± 0.20	0.29 ± 0.28
Whole population		58.24	0.20 ± 0.20	0.30 ± 0.28

3.3. Population Structure

Population differentiation in terms of Gst was 0.45, and the average number of migrants per generation was 0.60. Figure 2 shows the results from STRUCTURE, indicating that two groups of 31 populations formed two distinct groups with the largest population differentiation. Group I included the eight populations from western China, whereas Group II consisted of the populations from southeast and south China. A considerable proportion of individuals was seen to introgress from one putative species to the other (Figure 3).

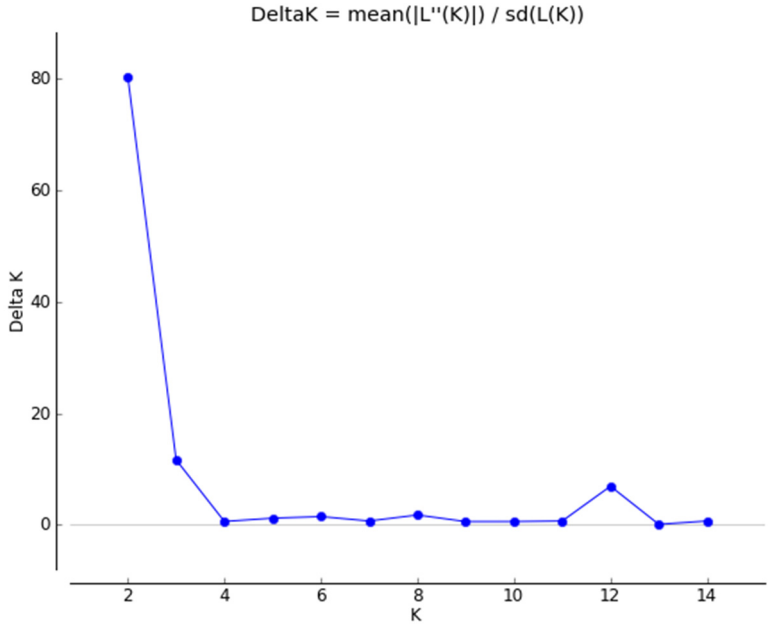

Figure 2. Relationship between *K* and Delta *K*. Delta *K* is an indicator of the optimal number of population groups. The number of groups with the maximum Delta *K* was optimal. Delta *K* was calculated according to Evanno *et al*. (2005) [36].

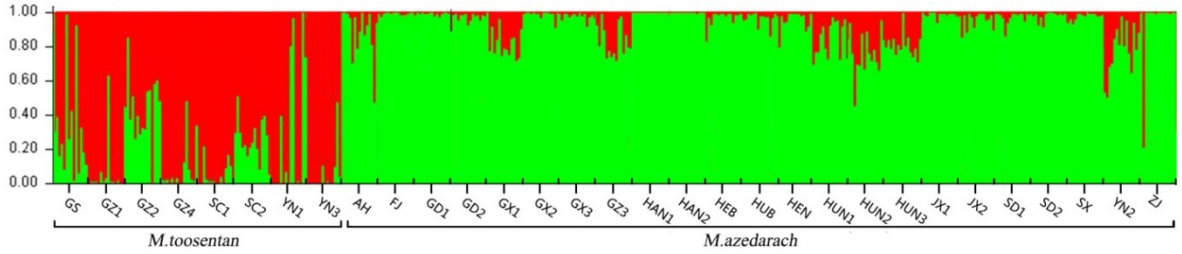

Figure 3. Clustering analysis of 31 *Melia* populations with STRUCTURE.

AMOVA (Table 5) indicated that 31.41% of the total variation corresponded to the variation between putative species (p-value < 0.001), 19.17% corresponded to variation among populations within putative species (Φ_{st} = 0.28, p-value < 0.001), and 49.42% corresponded to variation within populations (p-value < 0.001).

Table 5. Analysis of molecular variance (AMOVA) of 31 *Melia* populations.

Source	Degrees of Freedom	Sum of Squares	Variation Component	Percentage of Variation (%)	*p*
Among putative species	1	1799.23	9.66	31.41	<0.001
Among populations Within putative species	29	2985.25	5.90	19.17	<0.001
Within populations	430	6537.29	15.20	49.42	<0.001
Total	460	11321.76	30.76	100.00	

3.4. Genetic Relationships

A dendrogram, based on Nei's genetic distances and generated using the neighbor-joining clustering method (Figure 4), indicated the presence of two major groups among the 31 studied populations with a 100% support. Group I consisted of eight populations, GZ1 (Xingyi), GZ2 (Ceheng),

GZ4 (Zunyi), GS (Longnan), SC1 (Chengdu), SC2 (Dazhou), YN1 (Mengla), and YN3 (Chuxiong), all of which were from western China. Based on their large fruits and stones, these seedlots were regarded as *M. toosendan* (Table 1). Group II comprised the remaining 23 sources from Guangdong (2), Guangxi (3), Guizhou (1), Hainan (2), Jiangxi (2), Hunan (3), Anhui (1), Hebei (1), Hubei (1), Henan (1), Shandong (2), Shanxi (1), Fujian (1), Zhejiang (1), and Yunnan (1), mainly from eastern and northern China. They were considered to be *M. azedarach* based on their small fruits and stones.

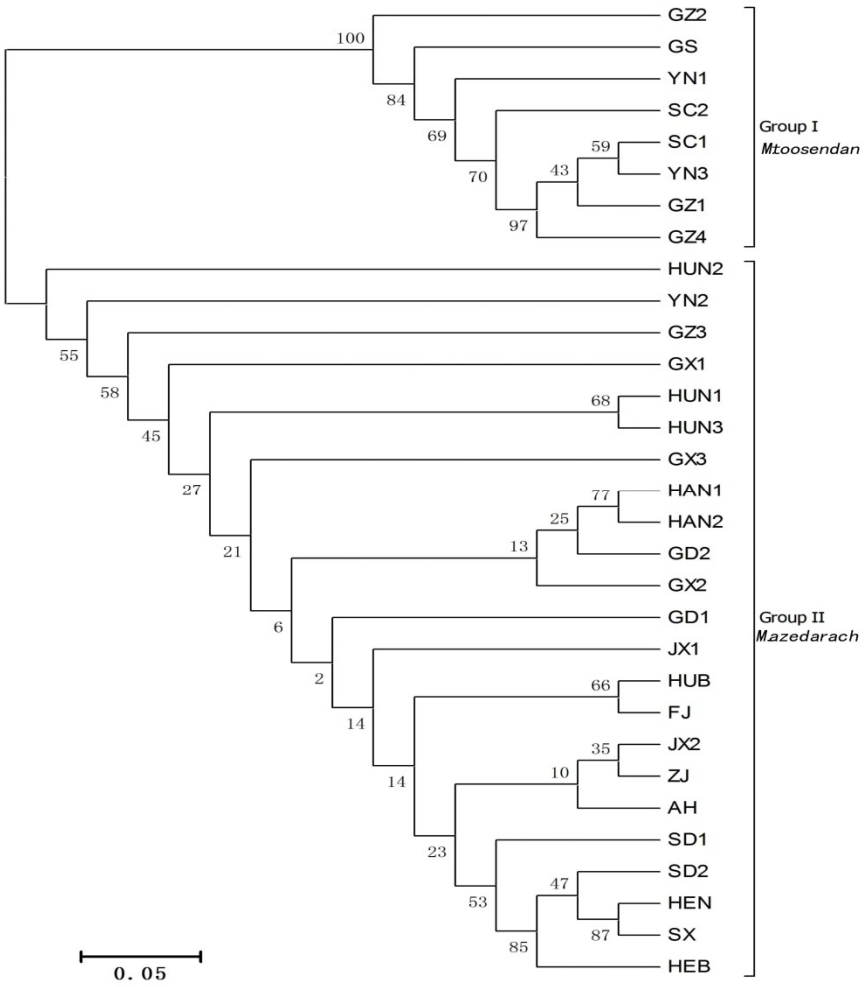

Figure 4. Neighbor-joining dendrogram of the 31 *Melia* provenances.

PCoA analysis revealed that the first two axes in the analysis accounted for 31.83% and 19.22% of the total variation (*i.e.*, 51.05% in total). The biplot with PCoA 1 and PCoA 2 clearly showed two groups in the 31 *Melia* populations, which agreed with the neighbor-joining cluster analysis. Individuals from the same seedlots tended to align closely, and geographically close provenances tended to cluster together (Figure 5).

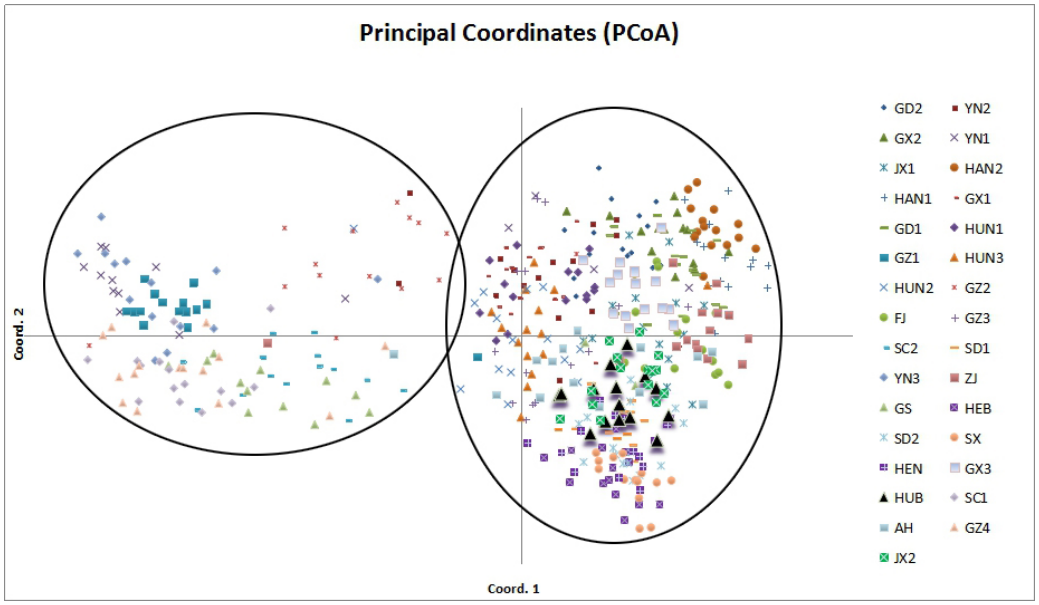

Figure 5. Biplots of PCoA1 and PCoA2 within 31 *Melia* provenances.

3.5. Mantel Test

For the 31 populations, a Mantel test indicated a significant correlation between genetic distance and geographic distance (r = 0.256, p-value $\leqslant$ 0.003 from 1000 permutations; Figure 6). Significant correlation indicated that geographical distance could increase population genetic distance, although this pattern was weak (r-square ~6.6%). However, no significant correlations existed between genetic distance and geographic distances within each putative species (r = −0.123, p-value $\leqslant$ 0.290 within *M. toosendan*; r = 0.001, p-value $\leqslant$ 0.436 within *M. azedarach*).

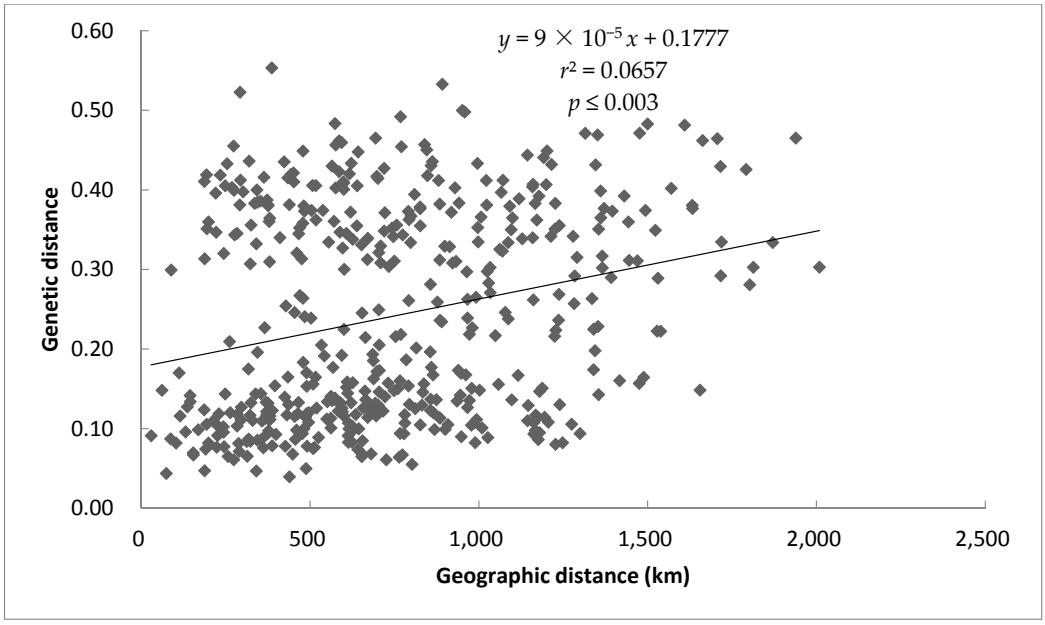

Figure 6. Correlation between the geographic distance (x-axis) and Nei's genetic distance (y-axis).

4. Discussion

This investigation represents the first study using SRAP as a molecular marker to evaluate genetic variation among and within *Melia* populations. The total genetic diversity (H_t = 0.37 ± 0.01) and percentage of polymorphic loci (P = 58.24%) indicated an intermediate level of genetic diversity in *Melia*. Populations from Ceheng and Liping (Guizhou) and Dazhou (Sichuan) had high genetic diversity (H = 0.20 and I = 0.30). The Nei's and Shannon's diversity within the putative *M. toosendan* populations were 0.23 and 0.34, respectively, and were higher than those of the putative *M. azedarach* populations, which were 0.19 and 0.29, respectively.

AMOVA further revealed that 31.41% of the variation was explained by differences between the two putative species, which was greater than population differentiation within each putative species (19.17%). These results were consistent with STRUCTURE analyses, which suggested that the two morphological groups were highly differentiated, with underlying clusters corresponding to the origins of the seedlots. This analysis also indicated that differentiation occurred mainly in populations from Yunnan, Guizhou, and Sichuan provinces. Various different genetic analysis methods (AMOVA, neighbor-joining cluster analysis, and PCoA grouping) indicated a consistent grouping pattern among the 31 populations. The eight populations in Group I (from Yunnan, Guizhou, Sichuan, and Gansu) were closely related to *M. toosendan* and were characterized by larger fruits and stones. The remaining 23 populations in Group II comprised southern, eastern, and northern seedlots, were associated with *M. azedarach* and were characterized by smaller fruits. These two distinct groups coincided with the two putative species described in the Flora Reipublicae Popularis Sinicae [12].

In this study, Groups I and II were putatively *M. toosendan* and *M. azedarach*, respectively. These groupings confirmed the morphological differences in the size and form of fruits and stones (Figure 7). The observation of fruit and seed characteristics showed that *Melia* populations from western China clustered together, and the stones and seeds of those seed lots differed significantly from those of other seedlots [38]. These results are consistent with the morphological differentiation reported by Chen *et al*. [39] and Hou *et al*. [40], and also match with the geographic distribution proposed for the two putative species in China [14,38]. Our genetic evidence supported the recognition of two taxa, *M. toosendan* and *M. azedarach*, in the genus *Melia* in China.

Genetic analyses have suggested the occurrence of a substantial population structure. The Mantel test indicated the presence of geographical distance effects on population genetic distance across the natural distribution range of the genus *Melia* in China. The number of migrants per generation per locus was less than 1, indicating a small extent of gene exchange between populations. This extent of population differentiation in the genus *Melia* was much greater than that in most conifers (F_{st} = 0.008–0.063) [41] and some broad-leaved tree species (F_{st} = 0.041–0.206) [42,43]. Population differentiation was also greater in *Melia* than in other outcrossing (F_{st} = 0.22), perennial (F_{st} = 0.19), and wind-pollinated (F_{st} = 0.13) plants [44,45]. These differences may arise primarily from their distinct dispersal properties and reproductive ecology.

In comparison with other species in the same family (Meliaceae), *Melia* had a degree of population differentiation comparable to those in *Swietania macrophylla* King, *Toona ciliate* Roemer, and *Chukrasia* [46–48], suggesting a similar reproductive ecology among different genera in Meliaceae. Furthermore, analogous to the genus *Melia*, the genus *Chukrasia* had two morphologically distinct groups of populations. This implies evolutionary convergence in population structure under biotic and abiotic environmental conditions.

The main reasons for a low gene flow between populations could be related to several factors. First, gene flow in genus *Melia* relies on gravity and seed dispersal by birds. Such birds include *Pycnonotus sinensis sinensis* Gmelin, *Cyanopica cyana swinhoei* Pallas, *Turdus naumanni eunomus* Temminck, *Turdus naumanni naumanni* Temminck, *Turdus pallidus pallidus* Gmelin, and *Sturnus cineraceus* Temminck. Of these species, *T. n. naumanni* can swallow more than 20 seeds per day during the autumn and winter in southern regions of Jiangsu, and they generally do not carry the seeds over long distances [24]. Gravity-mediated dispersal of non-ingested seeds results in much lower genetic diversity. Furthermore,

seeds dispersed in this way can be washed to the bottom of valleys by streams. If seeds encounter suitable humidity and warm earth, they will germinate from their thick epicarp after the pulp is eaten, usually near water, not far from the seed trees. The second main reason is related to the low levels of inter-population gene flow, which may also be explained by the pollination ecology of *Melia* trees. In general, any geographic distribution cannot extend beyond the limits of the distribution of its pollinators. The main pollinators of *Melia* are insects, such as bees and ants [21,23], which tend to be confined to a particular location; this results in decreased gene flow between populations.

Figure 7. Morphological differences in *Melia* fruits and stones: (**A**)–(**C**) for putative *M. toosendan*; (**D**)–(**H**) for putative *M. azedarach*. The fruits and stones in (**A**)–(**H**) were collected from the same seed trees in different populations located in: (**A**) Chuxiong; (**B**) Zunyi; (**C**) Mengla; (**D**) Xuchang; (**E**) Tunchang; (**F**) Renhua; (**G**) Tai'an; and (**H**) Yanling.

Analysis of the genetic structure of 31 *Melia* populations (Figure 5) revealed partial population genetic admixture, such as in populations GZ3, GX1, YN2, and the populations from Hunan province. In the *M. toosendan* gene pool, GZ2 and SCS also contained a proportion of *M. azedarach* genes. Although YN1, YN3, and YN2 were located in the same province, the YN2 population belonged to another gene pool. This was a case of differentiation in *Melia* within the same region. Genetic admixture implied that natural hybridization may have occurred between the two groups. These natural hybridization groups may form a barrier to gene flow or to germplasm introgression, as occurred in natural eucalyptus and pine tree species groups where germplasm introgression occurred between subspecies [49–52]. Our study provided preliminary experimental results to classify the genus *Melia*. Further study using chloroplast and mitochondrial DNA markers could provide additional genetic evidence on the classification of the genus *Melia*. Alternatively, artificial-pollination control testing and flowering biology observation could be used to test for interspecific hybridization between the two putative species or to ascertain whether the hybrids have a very low fitness compared with the parental fitness.

5. Conclusions

Melia populations exhibited substantial population differentiation, suggesting a low level of gene flow among populations. Genetic evidence indicated that the entire natural range of populations could be classified into two groups, which was consistent with the taxonomic classification based on the morphological characteristics of *M. toosendan* and *M. azedarach.* Our study supports the division of the genus *Melia* into two species in China, namely *M. toosendan* and *M. azedarach*. Additionally, this study also demonstrated that SRAP molecular markers were effective for characterizing population genetic diversity and the genetic relationships of *Melia* taxa and suggests that they could be useful for investigating the population genetic diversity of other broad-leaved tree species.

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Conflicts of Interest: The authors declare that they have no conflict of interest.

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