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Genetic Diversity and Population Genetic Structure of *Jatropha curcas* L. Accessions from Different Provenances Revealed by Amplified Fragment-Length Polymorphism and Inter-Simple Sequence Repeat Markers

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Abstract: The genetic diversity and structure of 17 populations of *J. curcas*, including 92 accessions from different provenances (tropical and subtropical), were investigated and effectively evaluated using twelve inter-simple sequence repeats (ISSRs) and seven pairs of florescence-amplified fragment-length polymorphism (AFLP) primers. Genetic diversity, at the overall level among populations of *J. curcas* based on the ISSR markers, showed that the observed number of alleles (Na) was 1.593, the effective number of alleles (Ne) was 1.330, Nei's gene diversity (H) was 0.200, Shannon's information index (I) was 0.303, and the percentage of polymorphic loci was 59.29%, indicating moderate genetic diversity between and within the different populations of *J. curcas*. Based on the genetic diversity analysis of AFLP markers, there were 1.464 (Na) and 1.216 (Ne) alleles, Nei's gene diversity (H) was 0.132, Shannon's information index (I) was 0.204, and the percentage of polymorphic loci was 46.40%. The AMOVA analysis showed that this large variance was due to differences within the populations, with genetic distinctions and limited gene flow among those from varied regions. The 17 populations were clustered into five main groups via UPGMA clustering analysis based on Nei's genetic distance, and the genetic relationships among the populations exhibited no significant correlations with geographical provenances. The genetic variation among Chinese populations of *J. curcas* distributed in dry-hot valley areas was remarkable, and the American germplasm presented with distinct genetic differentiation.

Keywords: *Jatropha curcas*; different provenance; dry-hot valley; molecular markers; genetic diversity; population structure

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1. Introduction

Jatropha curcas L. (Euphorbiaceae) is a perennial small tree or large shrub that is widely distributed across semiarid tropical and subtropical regions of the Americas, Africa, and Asia [1,2]. It is a diploid plant species ($2n = 2x = 22$) [3]. *Jatropha* is a monoecious shrub producing unisexual flowers, with male and female flowers on the same inflorescence [1]. But some studies have reported the occasional presence of hermaphroditic flowers. Previous studies have confirmed that *J. curcas* can reproduce by different mechanisms, namely, natural pollination, self-pollination, and apomixis [4]. In the last few years, *J. curcas* has become popular due to its potential economic value, as its fruit has a relatively high content of seed oil, up to 60%, which can be used as biodiesel [5,6]. At present, *Jatropha curcas* is being recognized as a bioenergy tree.

Other researchers have hypothesized that *J. curcas* is native to Central America and was later cultivated in the tropical and subtropical regions of South America, Asia, and

Africa, where it was grown as a hedge crop [1]. *Jatropha* is believed to have been introduced to China 300 years ago, although no studies have reported an available migration route. *J. curcas* is widespread and wild or semi-natural in China and is mainly distributed in the provinces of Yunnan, Guizhou, Sichuan, Guangdong, Guangxi, Fujian, and Hainan; however, it is mostly found in Yunnan province [7]. *Jatropha* is widely distributed in the dry-hot valley regions of the Jinsha River, Lantsang, Red River, and Nujiang basin and has a favorable ecological effect in soil and water conservation, desertification, greening barren hills, vegetation growth, etc. [7,8]. The geographic origin of *J. curcas* remains controversial. Its current geographical distribution is vague and insufficient for recovering its geographic origin and genetic diversity due to the interference of forces like domestication and potential genotype–environment interactions.

Jatropha grows in semiarid tropical and subtropical climates, does not tolerate frost, and flowers only under specific temperature, light, and phenological conditions. *J. curcas* can be propagated easily from seeds or vegetative cuttings. Studies on the morphological traits of *Jatropha* accessions and genetic diversity are essential for identifying groups with similar genetic backgrounds for conserving, evaluating, and utilizing their genetic resources. At present, research on the genetic diversity of *J. curcas* germplasm resources is still in the initial stages. *J. curcas* accessions representing 13 countries have been elucidated using molecular and biochemical trait analyses, displaying narrow genetic variations among the accessions from different regions of the world and rich diversity among the Mexican genotypes [9]. The agronomic traits were observed and studied in five populations of Jatropha plants collected from China, Indonesia, Suriname, Tanzania, and India, and significant variations were found for all the agronomic traits [10]. A total of 182 *J. curcas* accessions from Asia, Africa, and South and Central America were evaluated at the phenotypic level, and much genetic variation was found in the early growth traits, as indicated by the significant differences between the accessions. The Central American accessions showed the highest phenotypic variation [11]. The phenotypic characteristics of potential planting materials are largely influenced by environmental variables. DNA molecular markers are not influenced by environmental factors and are unbiased and more useful in genetic diversity studies. Inter-simple sequence repeats (ISSRs) have unique advantages over other molecular markers; they do not require any genomic information of the target species, and their analysis consumes a small amount of template DNA and can be rapidly conducted, making it an efficient and rapid method for detecting the genetic diversity of species [9]. The AFLP technique is based on the detection of genomic restriction fragments by PCR amplification and can be used for DNA of any origin or complexity. AFLPs have been recommended over other marker systems for genetic diversity studies due to their efficiency, high quantity of polymorphic information, and reproducibility [10–12]. The molecular markers applied to the genetic diversity analysis of *J. curcas* are mainly AFLPs [13–18], ISSRs [19,20], SSRs [21–23], and RAPD [24–26]. The genetic structures of more than 210 *J. curcas* germplasm resources were studied using ISSR markers, and the results showed that those of Chinese provenance exhibited higher genetic differentiation [19]. AFLP molecular markers were used to study the genetic diversity of 37 domestic germplasms grown in Hainan, and UPGMA clustering analysis showed that the genetic relationships between those of different provenance were not closely associated with their geographical origins [15]. New SSR markers have been developed for Indian *Jatropha* germplasm resources, and the molecular variation analysis showed that the genetic variation within populations was as high as 94% [27].

J. curcas, as an important potential biodiesel crop, has received much attention; however, there are few studies on its genetic diversity and the population's genetic structure of its germplasm resources, which are mainly distributed in Asia. In this present study, the comprehensive genetic diversity and population structure of collected *J. curcas*, including 92 accessions from different provenances (tropical and subtropical regions), were analyzed using ISSR and AFLP markers. These markers can be used to systematically

evaluate and reveal the genetic diversity, population structure, and relationships among *J. curcas* germplasm resources from Asia, Africa, and America for the genetic improvement, breeding, and conservation of this industrial bioenergy tree.

2. Materials and Methods

2.1. Plant Materials and DNA Extraction

Ninety-two accessions of *J. curcas* were collected from 12 countries in Asia, Africa, and America. All these accessions were distributed in the tropics and subtropics. The Asian accessions were highly over-represented in the collection. The Chinese accessions were mainly collected from dry and damp valleys (Table S1). A total of 61 Chinese accessions were collected, of which 59 were natural trees distributed among five provinces in Southwest and Southern China. According to their geographic distribution, 59 Chinese accessions of *J. curcas* were divided into 5 geographic natural populations, namely, Sichuan (SC), Guizhou (GZ), Yunnan (YN), Guangxi (GX), and Hainan (HN). The two cultivated varieties were named BR. A total of 31 exotic accessions were collected by scientific associates, and those from each country were simply grouped into one population due to the lack of accurate geographic distribution information, including 3 from Thailand (TL), 2 from Laos (LA), 5 from Burma (MM), 5 from Vietnam (VN), 4 from Indonesia (IN), 6 from India (ID), 2 from Brazil (BZ), 1 from the United States (US), 1 from Zambia (ZB), 1 from Burkina Faso (BF), and 1 from Mali (ML). The sample number and provenance, such as the longitude, latitude, and altitude, are listed in Table S1. The sample materials were provided by the Institute of Tropical Research, Yunnan Academy of Agricultural Sciences, the Southern China Botanical Garden of the Chinese Academy of Sciences, and the Tropical Biotechnology Research Institute of the Chinese Academy of Tropical Agricultural Sciences. Five representative individuals of *Jatropha* were randomly selected for each accession; fresh leaves were collected and wrapped with marked tin foil and then quickly preserved and frozen in liquid nitrogen. Total DNA was extracted from the leaves following the CTAB method and using the Plant Genomic DNA kit (TIANGEN Biotech, Beijing, China). Purified DNA was visualized using 1% agarose gel electrophoresis and quantified using a NanoDrop 2000c UV (Thermo scientific Inc., Shanghai, China) and then stored at −20 °C for subsequent analyses.

2.2. ISSR-PCR Amplification

Inter-simple sequence repeat marker analysis was conducted using 12 single primers, which were selected from a total of 65 ISSR primers (the UBC primer set no. 9, University of British Columbia) according to the amplification efficiency and reproducibility of the band patterns. The primer sequences are listed in Table 1. ISSR-PCR amplification was carried out in a 25 L PCR reaction mixture containing 1.5 μL of template DNA at a concentration of 50 ng/μL, including 12.5 μL of 2×Taq PCR Master MiX (4 mM $MgCl_2$, 0.4 mM dNTPs of each nucleotide, and 0.05 units/μL Taq DNA polymerase), 1.0 μL of 10 μM primer, and 25 L of ddH_2OL. The amplification reactions were performed with an initial denaturing step at 94 °C for 5 min, followed by 35 cycles of 1 min of denaturing at 94 °C, 1 min of annealing at 50 °C, a 2 min extension at 72 °C, and a final extension step at 72 °C for 10 min on the BIO-RAD S1000 TM Thermal cycler. The amplified products were separated on a 1.5% agarose gel using electrophoresis in 1×TAE buffer, and then the segments were compared with DNA Marker.

Table 1. The sequences of 12 selected ISSR primers.

Primers	Primer Sequences (5′→3′)	Annealing Temperature Tm (°C)
UBC808	AGAGAGAGAGAGAGAGC	52.0
UBC823	TCTCTCTCTCTCTCTCC	52.0
UBC825	ACACACACACACACACT	50.5
UBC834	AGAGAGAGAGAGAGAGYT	53.5
UBC835	AGAGAGAGAGAGAGAGYC	55.5

Table 1. *Cont.*

Primers	Primer Sequences (5′→3′)	Annealing Temperature Tm (°C)
UBC836	AGAGAGAGAGAGAGAGYA	52.5
UBC856	ACACACACACACACACYA	53.0
UBC873	GACAGACAGACAGACA	49.5
UBC884	HBHAGAGAGAGAGAGAG	55.5
UBC888	BDBCACACACACACACA	51.5
UBC889	DBDACACACACACACAC	51.0
UBC891	HVHTGTGTGTGTGTGTG	51.0
Total	12	-

Y = (C,T); B = (non A); D = (non C); V = (non T); H = (non G).

2.3. AFLP-PCR Amplification

The AFLP analysis was carried out using the modified protocol described by Vos et al. [10]. Genomic DNA was digested using the standard restriction enzymes EcoRI and MseI. The DNA oligonucleotides, including the adapters and primers, were synthesized by Thermo Fisher Scientific, Inc.,Shanghai, China. The genomic DNA was digested with the PstI and MseI restriction enzymes for 3 h at 37 °C. Adapters of the known sequences MseI F (5′ GACGATGAGTCCTGAG 3′), MseI R (5′ TACTCAGGA CTCAT 3′), EcoRI F (5′ CTCGTAGACTGCGTACC 3′), and EcoRI R (5′ AATTGGTACGCAGTCTAC 3′) were ligated to 1 µL of restricted DNA using T4 ligase and its buffer. The resultant mixtures were incubated at 37 °C for 6 h, followed by denaturation at 70 °C for 15 min. The restricted ligated DNA samples were analyzed by agarose gel (1.0%) electrophoresis and used for pre-selective amplification. Pre-selective PCR amplification was performed using the adaptors E01 (5′ GACTGCGTACCAATTCA 3′) and M01 (5′ GATGAGTCCTGA GTAAC 3′). A total of 25 µL reaction mixture containing 2 µL of adaptor-ligated DNA was subjected to PCR under the following conditions: 94 °C for 2 min, and then 30 cycles of 94 °C for 30 s, 56 °C for 60 s, and 72 °C for 60 s, with a final extension of 72 °C for 10 min. The pre-selective amplification products were visualized with 1.5% agarose gel electrophoresis and diluted 6 times in TE buffer. Ther selective PCR amplification was performed in a final volume of 25 µL, containing 2 µL of template DNA from the pre-selective PCR, considering seven primer pair combinations (Table 2). The cycling conditions were performed in a two-step PCR DNA thermo-cycler that was programmed as follows: The first stage was composed of 12 cycles with an initial denaturation temperature of 94 °C for 3 min, and each cycle was run at 94 °C for 30 s, and then 65 °C for 30 s, decreasing by 0.7 °C per cycle for the next 11 cycles, followed by 72 °C for 60 s. The second stage was composed of 23 cycles with 94 °C for 30 s, 56 °C for 30 s, and 72 °C for 60 s, followed by a final extension at 72 °C for 5 min. The selective EcoRI primers were labeled with fluorescent dyes at the 5′ end (Alexa Fluor 750). The PCR-amplified products from the selective amplifications were separated and visualized by electrophoresis on 2.0% polyacrylamide gel in an LI-COR 4300 Genetic Analyser. The DNA fingerprints were analyzed using GeneMapper 3.0 software (LI-COR, Inc. Lincoln, NE, USA).

Table 2. The 7 pairs of screened primer combination sequences used for AFLP analysis.

Primer Combination	Selective EcoRI Primers (5′→3′)	Selective MseI Primers (5′→3′)
E-AAG */M-CCC	GACTGCGTACCAATTCAAG	GATGAGTCCTGAGTAACCC
E-ACA */M-CCT	GACTGCGTACCAATTCACA	GATGAGTCCTGAGTAACCT
E-AAC */M-CTC	GACTGCGTACCAATTCAAC	GATGAGTCCTGAGTAACTC
E-AGG */M-CTT	GACTGCGTACCAATTCAGG	GATGAGTCCTGAGTAACTT
E-AAA */M-CAG	GACTGCGTACCAATTCAAA	GATGAGTCCTGAGTAACAG
E-AAA */M-CTT	GACTGCGTACCAATTCAAA	GATGAGTCCTGAGTAACTT
E-AGA */M-CTG	GACTGCGTACCAATTCAGA	GATGAGTCCTGAGTAACTG

* Selective EcoRI primers were labeled with fluorescent dyes at the 5′ end (Alexa Fluor 750).

2.4. Data Analysis

Only those that were reproducible and distinct at a particular locus were considered for data analysis, and fragment polymorphism (%) was determined. The band profiles generated by AFLP and ISSR were scored and converted into binary matrices on the basis of the presence (1) or absence (0) of selected bands. Nine were assigned if amplification did not occur. The PopGene 1.32 software [28] was used to calculate and evaluate the genetic diversity of the population, which included the following aspects: the number (NP) and percentage of polymorphic loci (Pp); the observed (Na) and effective numbers of alleles (Ne); Shannon's information index (I); total genetic diversity (Ht); gene flow (Nm); and Nei's genetic diversity index (H), distance (D), and identity (I) [29,30]. The AMOVA 1.55 software was used to analyze the molecular variance and calculate the variance-squared sum (SS) and genetic differentiation coefficient (φst), which reflected the distribution of genetic variation among the populations [31]. The genetic similarity coefficient was calculated among all the individuals using the Dice method with NTSYS-pc 2.10e software. Based on the genetic similarity coefficient matrix, the SAHN model was used for the UPGMA (the unweighted pair group method with an arithmetic average) clustering analysis and dendrogram construction [32].

3. Results

3.1. Analysis of Polymorphism Using ISSR and AFLP Markers

In this study, 60 GC-rich ISSR primers were screened and assessed. Twelve primers that targeted multiple amplification sites and reproducibly generated clear bands of polymorphic amplification products were selected for genotyping 92 *J. curcas* L. germplasms from 17 populations. These 12 ISSR primers produced 140 DNA fragments of high clarity and stability, and 78 of them exhibited polymorphisms. The amplified primers showed 30%–80% polymorphism, with maximum and average percentages of 80% and 55%, respectively. On average, 11.67 DNA and 6.5 polymorphic fragments were produced by each primer (Table 3). The amplification products produced by the ISSR primers ranged in size from 100 bp to 2000 bp. Figure S1 shows the polymorphism profiles of the DNA fragments amplified by the primer UBC888 across all 92 *J. curcas* germplasms. The polymorphism detection rate was high among the products amplified from *J. curcas* genomic DNA by the ISSR primers, and the detected polymorphisms were significantly different among the various primers.

Table 3. Amplification and polymorphism results of ISSR and AFLP markers.

Primer/Primer Combination	Total Number of Fragments	Number of Polymorphic Fragments	Percent Polymorphism (%)
UBC808	10	8	80.00
UBC823	8	4	50.00
UBC825	12	8	66.67
UBC834	14	9	64.29
UBC835	13	9	69.23
UBC836	12	5	41.67
UBC856	13	6	46.15
UBC873	16	11	68.75
UBC884	10	3	30.00
UBC888	10	4	40.00
UBC889	12	4	33.33
UBC891	10	7	70.00
Total	140	78	-
Average	11.67	6.50	55.01
E-AAG/M-CCC	73	45	61.64
E-ACA/M-CCT	67	41	61.19
E-AAC/M-CTC	102	50	49.02

Table 3. *Cont.*

Primer/Primer Combination	Total Number of Fragments	Number of Polymorphic Fragments	Percent Polymorphism (%)
E-AGG/M-CTT	83	39	46.99
E-AAA/M-CAG	48	16	33.33
E-AAA/M-CTT	88	20	22.73
E-AGA/M-CTG	65	31	47.69
Total	526	242	-
Average	75.14	34.57	46.08

From sixty-four pairs of AFLP primers, seven pairs that generated amplification products with high stability, reproducibility, polymorphism, and resolution were selected to analyze the genomic diversity and relationships among *J. curcas*. These seven primer pairs produced 526 clear bands of DNA products, and 242 of them were polymorphic. The polymorphism rates of the DNA products amplified by each primer pair were 22.73%–61.64%, with maximum and average values of 61.64% and 46.08%, respectively. Each primer pair generated 48–102 amplification and 16–50 polymorphic fragments. On average, each primer set amplified 75.14 DNA and 34.57 polymorphic fragments (Table 3). The DNA fragments amplified by the AFLP primer set E-AGG/M-CTT were relatively short, as shown in Figure S2. The genomic DNA amplification of *J. curcas* by the AFLP marker revealed a high polymorphism detection rate, with substantial differences among the primer pairs.

3.2. Genetic Diversity among J. curcas Populations

The genetic diversity of the ISSR markers in the 17 *J. curcas* populations were analyzed using POPGENE (Table 4), revealing several key findings. The observed number of alleles (N_a) = 1.593 ± 0.493, the number of effective alleles (N_e) = 1.330 ± 0.345, Nei's genetic diversity index (H) = 0.200 ± 0.190, Shannon information index (I) = 0.303 ± 0.275, and the percentage of polymorphic loci (P_p) = 59.29%, indicating moderate genetic diversity between and within the different populations of *J. curcas*. At the population level, genetic diversity varied considerably; the number of polymorphic loci (N_p) ranged from 0 to 70, P_p ranged from 0 to 50%, N_a ranged between 1.000 and 1.500, N_e ranged between 1.000 and 1.356, H ranged from 0 to 0.201, and I ranged from 0 to 0.293. The overall genetic diversity parameters were somewhat consistent across the populations. Among the 17 populations, the Hainan (HN) population had the highest genetic polymorphic information index, i.e., I = 0.293 ± 0.306, and the other parameters, such as N_a (1.500 ± 0.502), N_e (1.356 ± 0.398), H (0.201 ± 0.214), and P_p (50.00%), were all highest among all the populations. These data suggest that the Hainan population has the most genetic variation. The populations from Burkina Faso (BF), Mali (ML), Zambia (ZB), and the United States (US) had the lowest values of all the genetic parameters.

The genetic diversity analysis of the AFLP markers in the 17 *J. curcas* populations (Table 4) revealed several estimates of variability: N_a = 1.464 ± 0.499, N_e = 1.216 ± 0.324, H = 0.132 ± 0.177, I = 0.204 ± 0.256, and P_p = 46.40%. These data indicate a relatively high level of genetic variability at the species level. At the population level, the genetic diversity parameters varied; N_p ranged from 0 to 163, P_p ranged from 0% to 30.87%, N_a ranged between 1.000 and 1.309, N_e ranged between 1.000 and 1.207, H ranged from 0 to 0.113, and I ranged from 0 to 0.168. The genetic diversity parameters had consistent trends among the populations. Among the 17 populations, the population from Hainan (HN) had the highest genetic polymorphic level according to the AFLP genotypes across all the population genetic parameters. These population genetic statistics indicate that the *J. curcas* population from Hainan had the most genetic variation. By contrast, the populations from Burkina Faso (BF), Mali (ML), Zambia (ZB), and the United States (US) exhibited the lowest values across all the genetic parameters.

Table 4. Analysis of genetic diversity for 17 geographical groups of *J. curcas* germplasm based on ISSR and AFLP markers.

Pop	ISSR Markers						Pop	AFLP Markers					
	N_a	N_e	H	I	N_p	P_p (%)		N_a	N_e	H	I	N_p	P_p (%)
SC	1.350	1.238	0.134	0.196	49	35.00	SC	1.142	1.055	0.036	0.057	75	14.20
GZ	1.350	1.212	0.124	0.186	49	35.00	GZ	1.171	1.122	0.068	0.099	90	17.05
YN	1.400	1.214	0.127	0.193	56	40.00	YN	1.309	1.190	0.113	0.168	163	30.87
GX	1.307	1.189	0.109	0.162	43	30.71	GX	1.239	1.171	0.094	0.137	126	23.86
HN	1.500	1.356	0.201	0.293	70	50.00	HN	1.305	1.207	0.117	0.172	161	30.49
BR	1.043	1.030	0.018	0.026	6	4.29	BR	1.008	1.005	0.003	0.005	4	0.76
TL	1.064	1.035	0.022	0.034	9	6.43	TL	1.011	1.006	0.004	0.006	6	1.14
LA	1.057	1.040	0.024	0.035	8	5.71	LA	1.006	1.004	0.002	0.003	3	0.57
MM	1.157	1.118	0.064	0.093	22	15.71	MM	1.042	1.019	0.012	0.018	22	4.17
VN	1.264	1.147	0.089	0.135	37	26.43	VN	1.015	1.005	0.004	0.006	8	1.52
IN	1.207	1.142	0.081	0.119	29	20.71	IN	1.074	1.040	0.025	0.038	39	7.39
ID	1.279	1.192	0.108	0.158	39	27.86	ID	1.125	1.079	0.044	0.066	66	12.50
BZ	1.200	1.141	0.083	0.121	28	20.00	BZ	1.049	1.035	0.020	0.030	26	4.92
US	1.000	1.000	0.000	0.000	0	0.00	US	1.000	1.000	0.000	0.000	0	0
ZB	1.000	1.000	0.000	0.000	0	0.00	ZB	1.000	1.000	0.000	0.000	0	0
BF	1.000	1.000	0.000	0.000	0	0.00	BF	1.000	1.000	0.000	0.000	0	0
ML	1.000	1.000	0.000	0.000	0	0.00	ML	1.000	1.000	0.000	0.000	0	0
Total	1.593	1.330	0.200	0.303	83	59.29	Total	1.464	1.216	0.132	0.204	245	46.40

Notes: Pop, geographical groups; N_a, observed number of alleles; N_e, effective number of alleles; H, Nei's genetic diversity index; I, Shannon's information index; N_p, number of polymorphic loci; P_p, percentage of polymorphic loci.

3.3. Genetic Structure and Differentiation among J. curcas Populations

The genetic differentiation index (F_{ST}) among populations is calculated using allele frequencies under the assumption that the population is in Hardy–Weinberg equilibrium [33,34]. The analysis of molecular variance (AMOVA; $p < 0.0001$) is commonly used to calculate the genetic differentiation coefficients (Φ_{ST}) and analyze the genetic differentiation of a population structure [35]. The genetic differentiation coefficient (Φ_{ST}) among the *J. curcas* populations based on the ISSR marker data was 0.341 (Table 5), indicating that of the total genetic variation in *J. curcas*, 34.12% occurs among the populations, with the remaining 65.88% occurring within each population. Moreover, both the inter- and intra-population variations reached high levels of significance ($p < 0.001$). The genetic variation among the *J. curcas* populations, as revealed by the ISSR markers, exhibits some differentiation among those of various geographic origins, but there was less inter- than intra-population variation, suggesting that the genetic variation in *J. curcas* mainly occurs within populations. The total gene diversity (H_t) at the species level, which is calculated based on Nei's genetic diversity index, is 0.184, and the population genetic gene flow rate (N_m) was 0.304. These data imply that the overall genetic diversity of *J. curcas* is relatively low and that there is limited gene flow among the 17 *J. curcas* populations.

The AFLP marker data were also examined using AMOVA ($p < 0.000$). The genetic differentiation coefficient (Φ_{ST}) among the *J. curcas* populations was 0.441, indicating that 44.09% of the genetic variation was inter-population, while 55.91% of the genetic variation was intra-population (Table 5). Moreover, both the inter- and intra-population variations were highly significant ($p < 0.001$). The genetic variation among the *J. curcas* populations, as revealed by the AFLP markers, exhibited relatively high genetic differentiation among

the populations, but there was still less inter- than intra-population variation, suggesting that the genetic variation in *J. curcas* mainly occurs within populations. The total gene diversity (H_t) at the species level, which was calculated based on Nei's genetic diversity index, was 0.101, and the rate of gene flow (N_m) was 0.231. These data imply that the overall genetic diversity of *J. curcas* is relatively low and that there is limited gene flow among the 17 *J. curcas* populations.

Table 5. Analyses of molecular variance (AMOVA) for 17 geographical groups of *J. curcas* based on statistics of ISSR, AFLP, and ISSR + AFLP markers.

Molecular Markers	Source of Variation	df	Sum of Squares	Variance Components	Percentage of Variation (%)	Φ_{ST}	*p*-Value
ISSR	inter-population	16	571.55	5.081	34.12		
	intra-population	75	735.93	9.812	65.88	0.341	<0.001
	Total	91	1307.48	14.894	100		
AFLP	inter-population	16	1448.26	14.216	44.09		
	intra-population	75	1352.16	18.029	55.91	0.441	<0.001
	Total	91	2800.42	32.244	100		
ISSR + AFLP	inter-population	16	2019.81	19.297	40.94		
	intra-population	75	2088.09	27.841	59.06	0.409	<0.001
	Total	91	4107.90	47.138	100		

Notes: df, degree of freedom; Φ_{ST}, genetic differentiation coefficient; *p*-value was obtained through significance test using 1000 permutations.

The combined AMOVA of the ISSR and AFLP data shows that the genetic differentiation coefficient (Φ_{ST}) of the *J. curcas* populations was 0.409, indicating that 40.94% of the variation occurred among the populations, while 59.06 occurred within each population. Moreover, both the inter- and intra-population variations reached high levels of significance ($p < 0.001$). The genetic variations among the *J. curcas* populations, as revealed by the ISSR and AFLP markers, exhibited significant genetic differentiation among those of various geographic origins, but there was less inter-than intra-population variation. These data suggest that the genetic variation in *J. curcas* mainly occurs within individual populations. The total gene diversity (H_t) at the species level, which is calculated based on Nei's genetic diversity index, was 0.1184, and the population genetic gene flow rate (N_m) was 0.253. These data imply that the overall genetic diversity of *J. curcas* is relatively low and that there is limited gene flow among the 17 *J. curcas* populations.

3.4. Genetic Distances and Clustering among J. curcas Populations

The genetic relationships of the ISSR marker differentiation among the *J. curcas* populations were calculated using Nei's method (Table S2). The value of genetic identity (I) varied between 0.786 and 0.982, with an average of 0.878. The genetic distance (D) varied from 0.019 to 0.241, and the average genetic distance was 0.132. The furthest genetic distance was between the Zambian (ZB) and United States (US) populations, with a genetic distance of 0.241 and, correspondingly, the lowest genetic identity value of 0.786. The shortest genetic distance was between the Thailand (TL) and Breed (BR) populations, with a genetic distance of 0.019 and, correspondingly, the highest genetic identity value of 0.982. Based on Nei's genetic distances inferred from the ISSR markers, a clustering dendrogram was constructed using the UPGMA method (Figure 1). The UPGMA clustering showed that the *J. curcas* populations from 17 different geographic origins can be classified into five groups, namely groups A, B, C, D, and E. In group A, the shortest genetic distance (0.028) lies between the *J. curcas* populations from Yunnan (YN) and Guangxi (GX); thus, these two populations were clustered first, followed by the populations from Sichuan (SC) and Guizhou (GZ). In group C, the shortest genetic distance (0.019) lies between the Thailand (TL) population and the Breeds (BR), and thus these two populations were first clustered in a branch. Next, the populations from Laos (LA) and Indonesia (IN) are clustered together because of their relatively short genetic distance, and this cluster then forms another branch sequentially

with the populations from Myanmar (MM), Vietnam (VN), and India (ID). The Hainan (HN) population alone forms group B, and it forms sister clusters with groups A and C, suggesting that group B is genetically similar to groups A and C; however, the genetic variation is huge within this population. In group D, the populations from Burkina Faso (BF) and Mali (ML) were first clustered together, joined with the United States (US) population. The populations from Zambia (ZB) and Brazil (BZ) are clustered together into group E, suggesting this group is more distantly related to groups A, B, C, and D.

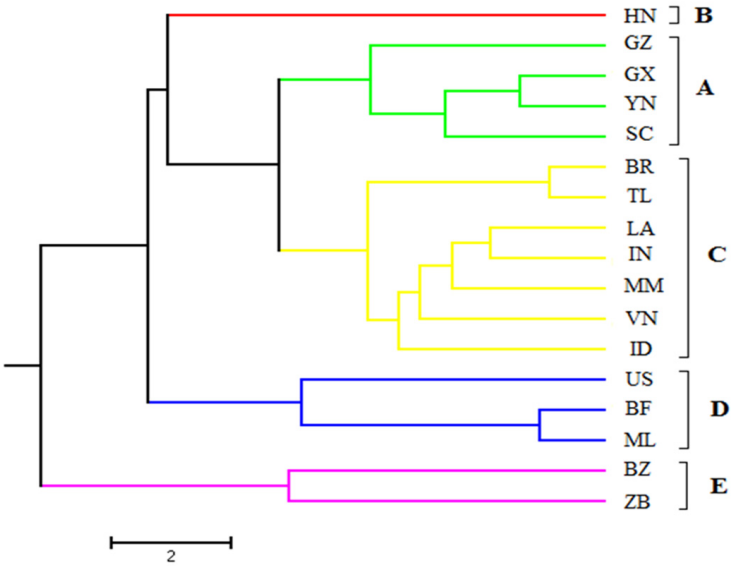

Figure 1. UPGMA dendrogram based on Nei's (1978) unbiased genetic distance among 17 *J. curcas* populations using ISSR markers. The scale represents Nei's similarity coefficient. Bold letters A, B, C, D, and E represent clustering groups; different colored lines correspond to different groups.

The genetic differentiation of the AFLP markers among the *J. curcas* populations was calculated using Nei's method (Table S3). The genetic identity (*I*) varied between 0.847 and 0.996, with an average value of 0.878. The genetic distance (*D*) varied from 0.004 to 0.166, with an average of 0.077. The longest genetic distance was between the Sichuan (SC) and Brazilian (BZ) populations, with a genetic distance of 0.166 and, correspondingly, the lowest genetic identity of 0.847. The shortest genetic distance was between the populations from Myanmar (MM) and Laos (LA), with a genetic distance of 0.004 and, correspondingly, the highest genetic identity of 0.996. Based on Nei's genetic distances inferred from the AFLP markers, a clustering dendrogram was constructed using the UPGMA method (Figure 2). The UPGMA cluster showed that the 17 *J. curcas* populations can be classified into five branches or groups, namely groups A, B, C, D, and E. In group A, the shortest genetic distance (0.030) was between the populations from Yunnan (YN) and Guizhou (GZ); thus, these two populations were clustered first and then sequentially joined with the Guangxi (GX) and Sichuan (SC) populations, indicating that these populations share a close genetic relationship. The Hainan (HN) population alone forms a single branch, i.e., group B, and it is genetically distant from the other Chinese populations. In group C, the populations from Laos (LA) and Myanmar (MM) were first clustered together, owing to the remarkably short genetic distance between them (D = 0.004). Next, the populations from Vietnam (VN), Thailand (TL), Indonesia (IN), India (ID), and the Breeds (BR) sequentially joined to form group C. Group D consists of the Burkina Faso (BF) and Mali (ML) populations, and their genetic distance is consistent with their geographic distance. Group E contains the populations from Brazil (BZ), the United States (US), and Zambia (ZB), suggesting that these three populations share a close genetic relationship.

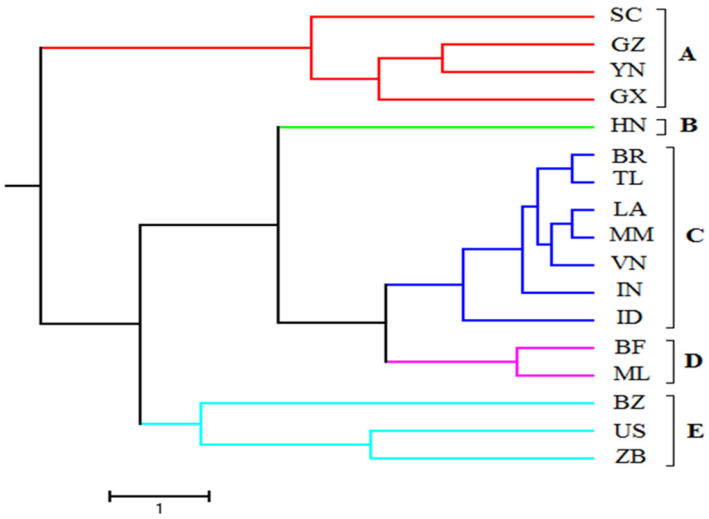

Figure 2. UPGMA dendrogram based on genetic distance among 17 *J. curcas* populations using AFLP markers. The scale represents Nei's similarity coefficient. Bold letters A, B, C, D, and E represent clustering groups; different colored lines correspond to different groups.

The genetic differentiation among the *J. curcas* populations was further analyzed with a combined database of the ISSR and AFLP markers. The inter-population genetic identity (*I*) and genetic distance (*D*) were calculated using Nei's method (Table S4). The genetic identity (*I*) varied between 0.843 and 0.993, with an average value of 0.917. The genetic distance (*D*) varied from 0.007 to 0.171, with an average of 0.088. The highest genetic distance was between the populations from Sichuan (SC) and Brazil (BZ), with a genetic distance of 0.171 and, correspondingly, the smallest genetic identity value of 0.843. The shortest genetic distance was between the Thailand (TL) and Breed (BR) populations, with a genetic distance of 0.007 and, correspondingly, the highest genetic identity value of 0.993. Based on Nei's genetic distances, inferred from the combined ISSR and AFLP marker data, a clustering dendrogram was constructed using the UPGMA method (Figure 3). The UPGMA clustering showed that the *J. curcas* populations from 17 different geographic origins can be classified into five groups, namely, groups A, B, C, D, and E. In group A, the shortest genetic distance (0.030) was between the *J. curcas* populations from Yunnan (YN) and Guangxi (GX), and thus, these two populations were first clustered and then sequentially joined with the populations from Guizhou (GZ) and Sichuan (SC), suggesting that these populations have a relatively close genetic relationship. The Hainan (HN) population alone forms a single branch, i.e., group B, and it shows relatively high levels of genetic variation compared with those of the other Chinese populations. In group C, the populations from Laos (LA) and Myanmar (MM) were first clustered together, owing to their short low genetic distance (0.014), indicating a close relationship between these two populations. The populations from Indonesia (IN), Vietnam (VN), Thailand (TL), India (ID), and the Breeds (BR) joined sequentially, indicating the short genetic distances separating these populations. Group D consists of the populations from Burkina Faso (BF) and Mali (ML), and their genetic distance is consistent with their geographic distance. Group E contains the populations from Brazil (BZ), the United States (US), and Zambia (ZB), suggesting that these three populations share a close genetic relationship.

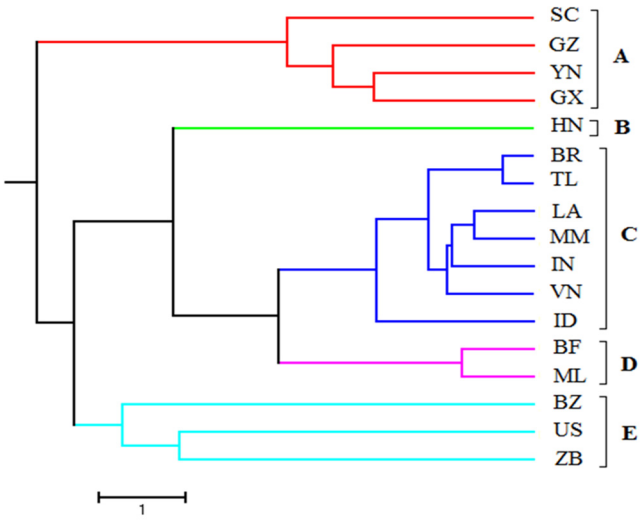

Figure 3. UPGMA dendrogram based on genetic distance among 17 *J. curcas* populations using combined ISSR and AFLP markers. The scale represents Nei's similarity coefficient. Bold letters A, B, C, D, and E represent clustering groups; different colored lines correspond to different groups.

4. Discussion

4.1. Genetic Diversity of J. curcas

In this study, the ISSR and AFLP markers were used to analyze the genetic diversity in *J. curcas* populations from 17 different geographic origins. A combined analysis using all the markers revealed the following overall genetic diversity parameters: PPB = 49.10%, N_a = 1.491, H_e = 0.146, and Φ_{ST} = 0.409. Compared with the mean genetic diversity statistics reported by Hamrick et al., the genetic diversity parameters in the *J. curcas* populations based on a combined marker analysis are all slightly lower, including PPB, N_a, and H_e. These data suggest that the overall genetic diversity of *J. curcas* is slightly below average relative to those of other species, and this is probably related to the quantity and scope of the selected germplasm resources. This present study examined a limited variety of test materials from Central Africa and America, especially Central American (i.e., Mexican) germplasms, which are recognized as the genetic origins of *J. curcas*. This limited sampling may be an important reason for the relatively low overall genetic diversity in this study. In contrast, the genetic differentiation coefficient (Φ_{ST}) was 0.409, which is above the average level, indicating that there is substantial genetic variation and differentiation among the *J. curcas* populations from various geographic origins and that there is high genetic diversity among these populations.

The biological characteristics of a species, such as its life cycle, seed dispersal mechanisms, pollination, mating, reproduction, and mutation rate, have a significant impact on its evolution and genetic variation. Factors that influence the genetic diversity of a species include its reproductive system, geographical distribution, life form, and historical migration [36]. In this present study, the combined analysis of the ISSR and AFLP data revealed an overall estimate of *Ht* of 0.118 at the species level and a P_p estimate of 49.10%, suggesting that the overall diversity of *J. curcas* is average, which suggests our findings are consistent with those of previous studies. The toxicity of *J. curcas* seeds makes them unlikely to be dispersed by animals, especially over long distances. In addition, the genetic diversity level of *J. curcas* is related to its mode of propagation, i.e., asexual propagation from cuttings [18,24,36–39]. Compared with the germplasms from other countries, the Chinese populations of *J. curcas* have a relatively high intra-population genetic variation. The levels of genetic variation are especially high in the Hainan and Yunnan populations. The germplasm resources from Yunnan and Hainan exhibit a relatively high level of genetic variation, probably owing to the complex geographic and climate conditions in these areas. For example, the typical tropical or subtropical dry-hot valley climate in Southwest China is believed to promote and maintain the high genetic diversity of *J. curcas* [40–42]. The high

genetic variation among the *J. curcas* populations is likely attributable to the environmental differences among the geographic regions in which *J. curcas* populations grow, such that beneficial mutations occurring within each population are favored and disproportionately inherited by the offspring [19].

4.2. Genetic Structure of J. curcas Populations

The genetic structure of plant populations is influenced and determined by a number of factors, including geographical distribution, ecological features, reproductive system, evolutionary history, and the seed dispersal mechanism [36,43]. Species that mainly rely on self-pollination exhibit high proportions of genetic variation among populations, with the highest genetic variance occurring among populations. Other studies on the flowering phenology and pollination of *J. curcas* have revealed that staminate and pistillate flowers are separate and differ during flowering. These observations suggest a tendency of infrequent outcrossing and cross-pollination in *J. curcas* [44]. In this study, the AMOVA of the combined ISSR and AFLP marker data shows that the genetic differentiation coefficient (Φ_{ST}) among the *J. curcas* populations is 0.409 (Table 5), which means that the inter-population variation accounts for 40.94% of the total genetic variation, suggesting a relatively strong tendency toward genetic differentiation. In accord, 59.06% of the genetic variation occurs within the populations, indicating that intra-population genetic variation is predominant in *J. curcas*. This finding is consistent with those of the previous studies [16,18,19,45,46]. According to the genetic differentiation coefficient (Φ_{ST}) and considering the typical flowering and pollination characteristics of *J. curcas* and its tendency toward remote outcrossing and cross-pollination, self-pollination may be the dominant mating system in *J. curcas*, with self- and cross-pollination (i.e., outbreeding) co-occurring in this species [44,47,48].

Generally, higher gene flow and more frequent exchange among populations are associated with lower genetic differentiation. In contrast, limited gene flow leads to differentiation among populations and is associated with higher genetic differentiation [49,50]. According to Wright's conceptual framework, the inter-population gene flow when $N_m < 1$ suggests that the geographic distances among populations may be large, such that a limited gene flow is the main reason for genetic differentiation among populations. In contrast, the inter-population gene flow when $N_m \geq 1$ suggests that the geographic distances among populations are short or that there is a high degree of gene flow, causing homogenization and preventing population differentiation [51]. The N_m values from Nei's genetic diversity analyses using the ISSRs, AFLPs, and both markers are 0.3043, 0.2306, and 0.2530, respectively, suggesting that the gene flow among the *J. curcas* populations is relatively low and limited at the species level. Such limited gene flow is not sufficient to resist the differentiation of genetic structures resulting from genetic drift within individual populations. Gene flow in plants mainly relies on the dispersal and movement of genetic material carriers, such as pollen and seeds, the major means of spreading and propagating genetic material [36]. *J. curcas* seeds are heavy and toxic and, therefore, unlikely to be dispersed by animals and spread over long distances; thus, gene flow among populations via seeds is limited. *J. curcas* is widely distributed in tropical and subtropical areas across broad geographic areas, complex terrains, and contrasting ecological environments. The typical dry-hot valley climate of Southwest China and the tropical rainforest climate at low altitudes (i.e., Hainan Island) give rise to habitat fragmentation, which limits and blocks the spread of *J. curcas* pollen. Therefore, we speculate that reproductive isolation has been caused by limitations to insect-mediated pollination and that geographic isolation blocks gene flow, resulting in further genetic differentiation among *J. curcas* populations [16,45,52,53].

4.3. Genetic Relationships among J. curcas Populations

Geographically isolated populations accumulate genetic differences as they adapt to different environments. In China, wild or semi-wild *J. curcas* is mainly distributed in the tropical, subtropical, and dry-hot valley areas of Southwest and Southern China, and it is classified into various ecological types and geographical provenances according to

geography, botanical morphology, and population ecology [7]. In this present study, Nei's genetic distances were inferred from each ISSR and AFLP marker and a combination of the two using the UPGMA clustering method, which clustered the 17 *J. curcas* populations into five groups (A, B, C, D, and E). The clustering maps demonstrate shared genetic variations in the relationships among the populations; the overall clustering relationships are consistent across the three maps. In this present study, combined clustering analysis using both the ISSR and AFLP markers appears to be the strongest. A comparison of the geographical distributions and clustering patterns reveals that group A comprises four Chinese populations, including those from Sichuan (SC), Guizhou (GZ), Yunnan (YN), and Guangxi (GX). These data suggest that the Chinese populations exhibit a close genetic relationship and that they probably share recent ancestry. The Hainan (HN) populations do not cluster with the other Chinese populations but cluster independently as group B, suggesting that they are genetically distant from the other Chinese populations. As the Hainan (HN) populations exhibit significant genetic variations and evolutionary patterns, we speculate that they may have an independent origin. Group C represents a mixed group of Asian populations, including populations from India (ID) and the Breeds (BR), as well as the populations from Myanmar (MM), Vietnam (VN), Thailand (TL), Laos (LA), and Indonesia (IN) in Southeast Asia. This analysis indicates that these populations share a close genetic relationship, suggesting that the populations from India and Southeast Asia may share ancestry. Group D is an African group consisting of the populations from Burkina Faso (BF) and Mali (ML). Group E is an American group consisting of the populations from Brazil (BZ), the United States (US), and Zambia (ZB), implying that the Zambian *J. curcas* population might have been introduced from America. The overall clustering relationships reveal that the *J. curcas* populations from various origins are somehow associated with their geographic distribution. However, the genetic relationships among the populations do not exhibit a significant association with their geographic distribution. Additionally, the American population forms an independent cluster, implying that their genetic differentiation and variation levels are relatively high [16,19,23].

4.4. Comparative Analysis between ISSR and AFLP Markers

The ISSR and AFLP markers are the dominant markers used for analyzing genetic variance in genomic DNA. Other studies have shown that the tests using the dominant RAPD, AFLP, and ISSR markers produce similar and comparable results [54,55]. Thus far, studies examining the genetic diversity of *J. curcas* germplasms from different countries using both the ISSR and AFLP markers have been very rare. In this present study, several genetic diversity estimates of *J. curcas* that were calculated from the AFLP and ISSR markers varied, and the percentage of ISSR-marked polymorphic loci (59.29%) was higher than that of the AFLP-marked polymorphic loci (46.40%). This is probably owing to the different detection mechanisms of these two marker types, i.e., they contain different test loci. The ISSR markers amplify fragments of simple repeat sequences with high mutation rates, and the anchored ISSR-PCR can detect polymorphisms occurring at multiple sites in the genome. In contrast, AFLP markers correspond to specific restriction fragments, and the numbers of total and polymorphic bands amplified by the AFLP markers are far greater than those amplified by the ISSR markers, suggesting that the AFLP markers offer higher-resolution data for genetic analyses [34,56].

5. Conclusions

The present study results demonstrate that the ISSR and AFLP markers were both able to assess genetic diversity among various *J. curcas* populations with high efficiency and stability. The genetic variation in *J. curcas* mainly occurs within populations. There was significant genetic differentiation among the populations, and the level of inter-population gene flow was relatively low. The UPGMA clustering analyses based on Nei's genetic distances, as inferred from the ISSR and AFLP markers, show that these two types of markers provide consistent assessments of the genetic structure and clustering relation-

ships of the 17 *J. curcas* populations into five groups. Clustering is correlated with the geographic distribution of the populations, but the genetic relationships among the populations exhibited no significant correlation with their geographic distribution. In population clustering, the genetic variation among the Chinese populations of *J. curcas* distributed in dry-hot valley areas was remarkable, and the American germplasm formed a separate cluster, presenting high levels of genetic differentiation. In this present study, the materials of African and American provenances were insufficient. The comprehensive and efficient material collection is of great significance for studying the global genetic diversity of *J. curcas*, promoting genetic improvement and variety selection for cultivation.

The Mantel correlation analysis of the genetic distances, inferred from the ISSR and AFLP markers, revealed a significant correlation, suggesting that both types of markers are stable and reliable for analyzing genetic diversity among populations. However, correlation analysis examining the ISSR, AFLP, and combined marker data revealed a stronger correlation between the AFLP and combined data. Moreover, the AFLP-based cluster map was similar to the clustering structure based on both marker types, suggesting that AFLP markers are more reliable tools for assessing genetic diversity and conducting clustering analyses of *J. curcas* germplasms.

Supplementary Materials: The following supporting information can be downloaded at https://www.mdpi.com/article/10.3390/f15091575/s1. Table S1: *Jatropha curcas* accessions used in ISSR and AFLP molecular marker analyses and their place of origin. Table S2: Nei's genetic distance (below diagonal) and genetic identity (above diagonal) between selected genotypes based on ISSR markers. Table S3: Nei's genetic distance (below diagonal) and genetic identity (above diagonal) between selected genotypes based on AFLP markers. Table S4. Nei's genetic distance (below diagonal) and genetic identity (above diagonal) between selected genotypes based on combined statistics of ISSR and AFLP markers.

Author Contributions: Conceptualization, G.G., L.T. and Y.X.; methodology, G.G.; software, G.G.; validation, G.G.; formal analysis, L.T.; data curation, G.G. and Y.X.; investigation, L.T.; resources, Y.X.; funding acquisition, L.T.; writing—original draft preparation, G.G.; writing—review and editing, Y.X. All authors have read and agreed to the published version of the manuscript.

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Data Availability Statement: The datasets used and analyzed during the current study are available from the corresponding author upon reasonable request.

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Conflicts of Interest: The authors declare no conflicts of interest.

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