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Research Article

Keywords: Job's tears, *Coix lacryma-jobi* L., ISSR markers, genetic diversity, population structure

Posted Date: February 19th, 2021

DOI: <https://doi.org/10.21203/rs.3.rs-148352/v1>

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Genetic Diversity and Population Structure of Job's Tears(*Coix lacryma-jobi* L.) Germplasm Based on ISSR Marker

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ABSTRACT

Background: Job's tears (*Coix lacryma-jobi* L.) is a minor cereal and an important food item in some parts of Asia. It has also been used in the traditional Chinese medicine for relieving various ailments, therefore, it plays an important role in our lives. Lack of excellent new varieties hinders the development of coix as a sustainable crop, and it is urgent to provide new cultivars with excellent trait in Chinese Coix industry.

Results: ISSR markers were used to assess the genetic diversity and population structure of 8 populations of Job's tears in China. A genotyping analysis that utilized ten ISSR primer pairs resulted in the production of 116 bands, of which 98 were polymorphic. The Guizhou population (PPB = 81.90%, $h = 0.3113$, $I = 0.4589$) was the most genetically diverse, while the lowest was observed in the Hebei population (PPB = 46.55%, $h = 0.1842$, $I = 0.2701$). Genetic differentiation analyses including GST and AMOVA illustrated that genetic variation was most prevalent within populations while only minor variations were observed among populations. Genetic distance coefficients ranged from 0.0095 to 0.0948 for the 8 populations; the genetic relationship between the Guizhou and Chongqing populations was the closest, while the most distant genetic relationship occurred between the Hubei and Hunan populations. The results of an UPGMA cluster analysis that investigated genetic diversity among the populations were consistent with the genetic distance results. The results of a

STRUCTURE analysis suggested that 94 Job's tears accessions could be grouped into two subpopulations. Moreover, according to a cluster analysis based on the UPGMA for individuals of Job's tears, accessions were divided into two major clusters. The results of the Bayesian cluster and UPGMA cluster analyses were largely consistent despite minor differences. There was no significant correlation between genetic distance and geographic distance ($r = 0.055$, $p = 0.782$).

Conclusions: Our study was undertaken to systematically analyze genetic diversity and population structure in 94 Job's tears accessions using ISSR markers. And this study provides us with valuable information pertaining to germplasm collection, genetic improvement, and systematic utilization of Job's tears.

INTRODUCTION

Job's tears (*Coix lachryma-jobi* L.) belongs to the *Coix* genus, the Andropogoneae tribe, and the Gramineae family^[1]. Job's tears, also known as coixseed, tear grass, hatu mugi, and adlay, is a tall grain-bearing tropical plant^[2]. This crop is predominantly planted in Southeast Asian countries including China, Vietnam, Laos, Japan, and Korea^[3]. Job's tears seeds are sweet to taste and are widely used in both culinary and medicinal practices; they are considered an ideal health food and effective medicine. Indeed, the seeds have been shown to reduce fever, invigorate the spleen, while also exhibiting diuretic, anti-cancer, hypolipidemic, hypoglycemic, antioxidant, anti-inflammatory, and anti-allergic properties^[4-6]. Job's tears seeds are nutritious and are commonly processed into products including Job's tears seed tea, Job's tears seed powder, Job's tears seed biscuits, Job's tears seed alcoholic beverages and vinegar^[7-8]. Until now, research conducted on Job's tears has predominantly focused on agronomic characteristics, inherent chemical constituents and associated pharmacological functions. There is currently a lack of international peer-reviewed articles pertaining to breeding programs associated with Job's tears. Evaluation of genetic diversity and genetic relationships in the germplasm can provide useful information for breeding programs. Morphological or phenotypic descriptors have traditionally been used to distinguish one accession from another. Although this type of agronomical characterization provides useful information, these investigations are subject to environmental influences; they are also time-consuming and must be assessed during a fixed vegetative phase of the crop life cycle^[9].

Molecular markers have significant advantages over morphological and isozyme markers because they are not influenced by growth and environmental conditions and can be applied during any growth phase. Compared with strategies that analyze other molecular markers, analyses investigating inter-simple sequence repeats (ISSR) are attractive methods because they do not require DNA sequence information. These methods facilitate the detection of highly variable sequences; they are also reproducible and cost effective. ISSR analysis has been widely used to study the genetic diversity of various plants^[10]. Several statistical methods including POPGENE analysis, Analysis of molecular variance (AMOVA), Bayesian cluster analysis and Molecular Evolutionary Genetics Analysis (MEGA) have been developed to analyze population structure, genetic diversity and differentiation of the germplasm. However, up until now there has been no comprehensive analysis of the genetic diversity and population structure of Job's tears in China. Therefore, a comprehensive analysis of the genetic diversity of Job's tears is required to facilitate the effective utilization of associated accessions during breeding. This current study was undertaken to systematically analyze genetic diversity and population structure in 94 Job's tears accessions using ISSR markers. The purpose of this study was to assess genetic diversity among accessions of Job's tears that were collected from different regions of China and to evaluate the population structure of these accessions.

MATERIAL AND METHODS

Plant Materials

A total of 94 Job's tears accessions were included in this study (Table 1). Forty-seven cultivated accessions and 57 wild accessions were sampled from different geographical regions from seven different provinces (Guizhou (36), Guangxi (5), Sichuan (7), Chongqing (7), Hunan (7), Yunnan (19), Hubei (4), and Qianxinan Institute of Agricultural Sciences (9)) of China. All of the Job's tears accessions were selected between 2013 and 2014.

Thirty seeds per Job's tears accession were planted in the seedling cups. Seedlings with 3–4 leaves were transplanted to the field at the Germplasm Resources Garden of Guizhou Academy of Agricultural Sciences, Guizhou, China in April 2015. The study was performed using Completely Randomized Design (CRD) and 3 replicates per accession; each replicate consisted of 10 plants with one plant per nest. The row spacing was 60 cm and plant spacing

was 40 cm.

Experimental research and field studies on plants (either cultivated or wild) complied with relevant institutional, national, and international guidelines and legislation. And the permissions or licenses to collect plants described in a manuscript were obtained. All wild plants described in a manuscript are deposited in Institute of Subtropical Crops Research, Guizhou Academy of Agricultural Sciences.

DNA extraction

Ninety-four Job's tears accessions (Table 1) were studied in this experiment. Leaves from 15-day-old seedlings were randomly selected for each accession. These leaf samples were transported to the laboratory in a 4°C ice box and finally stored in a freezer at -20°C prior to DNA extraction. Approximately 75–80 mg of DNA was extracted into Eppendorf tubes following a modified version of a protocol outlined by Murray and Thompson^[11]. Leaf tissues were directly grounded in 500 µL of CTAB (cetyltrimethyl ammonium bromide) extraction buffer containing 2% CTAB, 2 M NaCl, 2% PVP, 20 mM EDTA pH 8.2, 100 mM Tris-HCl pH 8.0, and 1% β-mercaptoethanol (this was performed in Eppendorf tubes). The extracted DNA was quantified on a Microplate Spectrophotometer (Biotec[®] Instruments, Inc.) and stored at -20°C. Approximately 60 ng of DNA were used in amplification reactions^[12].

ISSR Primers

A total of 100 ISSR primers were screened in this study. The primers were synthesized by Shanghai Sangon Biological Engineering Technology & Service Co., Ltd. (China) according to primer sets published by the University of British Columbia (UBC, Canada). Three representative samples from the 94 accessions were initially screened. Ten prime pairs that resulted in the generation of repeatable and polymorphic results with clear bands were used for PCR amplification (Table 2).

PCR amplification

The ISSR reaction mixture (total volume = 25 µL) contained 2.5 µL of 10 × buffer (0.1 M of Tris-HCl, pH 8.3, 0.5 M KCl), 1 µL of DNA template (50 ng), 2 µL of each primer (10 µM concentration), 0.5 µL of Taq DNA polymerase (2.5 U/µL), 1 µL of MgCl₂ (2 mM), 2 µL of dNTPs (2.5 µM) and 16 µL of ddH₂O. The amplifications were performed in an Eppendorf Mastercycler Gradient PCR machine (Eppendorf, Germany) using the following program

conditions: an initial denaturation step of 5 min at 94°C; 35 cycles of 94°C for 30 s, 46–60°C (depending on primer pair chosen) for 30 s, 72°C for 30 s; and a final extension at 72°C for 5 min. The amplification products were electrophoresed at 100 V for 45 min on a horizontal gel apparatus (Bio-Rad, SA) using a 1.5% agarose gel in 1 × TAE (pH 8.0). The gels were stained with 0.8 µg/mL ethidium bromide for approximately 30 min, and then photographed under UV light using the UVP-GDS8000 Gel Documentation System (UVP, USA)^[6].

Data analysis

The discernible and reproducible DNA bands ranging from 250 to 2000 bp were scored 1 for presence and 0 for absence. These bands were used to construct the binary data matrix for statistical analysis. The number of polymorphic bands (NPB), the percentage of polymorphic bands (PPB), the observed number of alleles (Na), Shannon's Information index (I), effective number of alleles (Ne), Nei's gene diversity (h), genetic differentiation, genetic identity and genetic distance were calculated using PopGene 1.32^[13]. AMOVA was used to analyze genetic diversity using ARLEQUIN version 3.01^[14]. Based on the Nei's genetic distance, cluster analysis was performed to generate a dendrogram; the dendrogram exhibited the genetic relationships among populations and was assimilated using the un-weighted pair-group method with arithmetic mean (UPGMA) using the MEGA 4 v. 4.1 software ^[15].

The population structure was also estimated using a Bayesian assignment test as implemented using STRUCTURE v. 2.2 software. K (number of populations) values were set from 1 to 16, 6 repetitions. The posterior probabilities were estimated using the Markov Chain Monte Carlo (MCMC) method. Ninety-four Job's tear individuals were clustered using the admixture model program and the genetic clustering number (K) was inferred; burn-in was 100000 and run-length was 1000000. The value of ΔK was calculated from the values of $\ln P(D)$ corresponding to different values of K^[16], and the possible genetic structures were analyzed. Accessions were assigned to a subpopulation if the probability of membership was greater than 70%^[17]. If membership was $\leq 70\%$, the accessions were assigned to the mixed subpopulation.

The genetic distance matrix was calculated using the NTSYS-pc version 2.1 software package^[18]. The UPGMA^[19] tree was constructed based on the genetic distance matrix using MEGA 4.1 software^[15].

The geographical distance was obtained by the actual latitude and longitude of each population, and the genetic distance was calculated by POPGENE 3.2 software. The Mantel correlation test^[20] in the TFPGA software^[21] was used to analyze the correlation between geographical distance and genetic distance for the different populations.

RESULTS

Polymorphisms and genetic diversity in Job's tear populations

A total of 100 widespread ISSR primers were tested prior to the genetic diversity analysis. Ten ISSR primers that gave rise to clear, identifiable and polymorphic bands were selected for further analyses. In total, 116 polymorphic loci were generated from the 10 primers. The 10 primers result in the generation of 116 fragments, of which 98 were polymorphic (84.48%); the percentage range for the polymorphic bands generated from each ISSR primer ranged from 60% to 100%. In each ISSR reaction, the total number of amplified fragments ranged from 7 (UBC-844) to 14 (UBC-815, UBC-825, UBC-840 and UBC-857), with a mean number of 11.6 loci per primer (Table 2, Figure 1, Figure 2, Figure 3, and Figure 4).

Popgene 1.32 software was used to analyze the genetic diversity index for each population of Job's tears. The main genetic parameters are reported in Table 3. Following ISSR analysis, the NPB was 98, the PPB was 84.48%, N_a was 1.8448, the N_e was 1.5277, the h was 0.3049 and the I was 0.4525 at the species level.

The values for each index at the population level were as follows: NPB varied from 54 for the Hubei population to 95 for the Guizhou population; the mean value was 76.13. The PPB varied from 46.55% for the Hubei population to 81.90% for the Guizhou population; the mean value was 65.63%. The N_a varied from 1.4655 for the Hubei population to 1.8190 for the Guizhou population; the mean value was 1.6559. The N_e varied from 1.3242 for the Hubei population to 1.5412 for the Guizhou population; the mean value was 1.4453. The h value varied from 0.1842 for the Hubei population to 0.3113 for the Guizhou population; the mean value was 0.2524. The I varied from 0.2701 for the Hubei population to 0.4589 for the Guizhou population; the mean value was 0.3706. The highest genetic diversity was observed in the Guizhou population (PPB = 81.90%, h = 0.3113, I = 0.4589), whereas the lowest genetic diversity was found in the Hebei population (PPB = 46.55%, h = 0.1842, I = 0.2701).

The eight populations were sequenced according to the detected polymorphic loci, the results for the populations were as follows: Guizhou (NPB = 95, PPB = 81.90%) > Yunnan (NPB = 86, PPB = 74.14%) > Chongqing (NPB = 80, PPB = 68.97%) > Hunan (NPB = 79, PPB = 68.10%) > Qianxinan Institute (NPB = 76, PPB = 65.52%) > Sichuan (NPB = 70, PPB = 60.34%) > Guangxi (NPB = 69, PPB = 59.48%) > Hubei (NPB = 54, PPB = 46.55%).

Genetic differentiation among Job's tears populations

Genetic differentiation analysis of 8 Job's tears populations revealed that the total gene diversity (H_t) was 0.2950, the gene diversity with provenance (H_s) was 0.2524, the coefficient of gene differentiation (G_{ST}) was 0.1443 and the estimate of gene flow from G_{ST} was 2.9654 (Table 4). The results revealed that 14.43% of the total genetic variation occurred among populations, while 85.57% of the total genetic variation occurred within populations. These results reveal that genetic variation within populations accounts for the largest proportion of genetic variation. The results also reveal that the estimate of gene flow from G_{ST} (N_m) was greater than one ($2.9654 > 1$); This indicates that the genetic differentiation among populations of Job's tears is not obvious.

To further test the difference between different Job's tear populations we utilized the molecular variance (AMOVA) method. The results revealed that 1.77% of the total variation occurred among the populations, 98.23% of the total variation occurred within the populations, and the variation among the populations and within the populations was significant ($P < 0.05$) (Table 5).

The results were similar using both the AMOVA and POPGENE methods. Both methods revealed that there was limited genetic differentiation among the Job's tears populations and the genetic variation predominantly existed within the populations of Job's tears.

Nei's unbiased measure of genetic distance and genetic identity

Nei's unbiased measure of genetic distance and genetic identity was utilized to further elucidate the genetic relationship among the Job's tears populations (Table 6). The results revealed that the genetic distance ranged from 0.0095 to 0.0948, while the genetic identity ranged from 0.9095 to 0.9905. The Guizhou and Chongqing populations were the least genetically different (0.0095) with a genetic identity value of 0.9905. Conversely, the largest genetic distance (0.0948) occurred between Hubei and Hunan populations with a genetic

identity value of 0.9095. These results indicated that the closest genetic relationship occurred between the Guizhou and Chongqing populations while the most distant genetic relationship occurred between the Hubei and Hunan populations. The results also showed that the closest genetic relationship occurred between the Sichuan and Guangxi populations with a genetic distance value of 0.0168 and a genetic identity value of 0.9833. Conversely, the most distant genetic relationship occurred between the Sichuan and Hubei populations with a genetic distance value of 0.0909 and a genetic identity value of 0.9131. The genetic distance between Yunnan and Qianxinan Institute was 0.0173; this indicates that a closer genetic relationship existed between Yunnan and Qianxinan Institute.

Cluster analysis among populations of Job's tears

Relationships between populations were further illustrated by a dendrogram using UPGMA algorithm based on Nei's genetic distance (Figure 5). The illustrated dendrogram grouped the 8 populations into 2 main clusters. Cluster I comprised 5 populations that were further delineated into two sub-clusters. Within cluster I, Guangxi and Sichuan populations constituted one sub-group. Chongqing, Guizhou and Hunan populations clustered into the other sub-group, with Chongqing and Guizhou appearing to be closer to each other than the other populations. The other 3 populations grouped together in cluster II and were further grouped into 2 sub-clusters. Within cluster II, Yunnan and Qianxinan Institute populations constituted one sub-group, while the Hubei population formed a separate sub-group.

Bayesian cluster analysis

STRUCTURE computations do not require prior knowledge of the genetic background of the population; therefore, a STRUCTURE computation analysis was considered an ideal tool for population genetic structure analysis^[22]. The logarithmic rate of change of ΔK can more accurately reflect the true value of K , and the genetic structure of 94 Job's tears individuals were analyzed using the following formula:

$$\Delta K = m(|L(K + 1) - 2L(K) + L(K - 1)|)/s[L(K)]$$

In this formula, $L(K)$ or $\ln P(D)$ represents the “ K ” Corresponding logarithm, “ s ” is the standard deviation, and “ m ” is the average. This formula revealed the most suitable number of subgroups to be 2 (Figure 6). In total, the 94 accessions of Job's tears can be grouped into 2 subpopulations, G_1 (red) and G_2 (green) (Figure 7). Following analysis of membership

fractions, the accessions with a probability of $> 70\%$ were assigned to corresponding subgroups, while other accessions were categorized as mixed subpopulations (Figure 7). Twenty-two accessions (23.40%) were assigned to subpopulation G_1 from Chongqing (3), Guizhou (11), Hunan (2), Guangxi (1), Qianxinan Institute (1), Sichuan (1) and Yunnan (3). Subpopulation G_2 consisted of 61 accessions (64.89%) which were collected from Chongqing (4), Guizhou (20), Hunan (2), Hubei (2), Guangxi (4), Qianxinan Institute (8), Sichuan (6) and Yunnan (15). The mixed subpopulation contained 11 (11.7%) accessions from Guizhou (5), Hunan (3), Hubei (2) and Yunnan (1). From figure 7, we observed that some accessions including Y49, Y23, Y50, Y9, Y28, Y18, Y86, Y54, Y29, Y47 and Y53 shared identity with both the first group (red) and the second group (green).

Cluster analysis in individuals of Job's tears

A dendrogram based on the genetic distance matrix of the SRAP data was generated using the UPGMA algorithm (Figure 8). In this dendrogram, the 94 accessions of Job's tears were clustered into 2 clusters (Cluster I and Cluster II). The clustering results that were generated according to genetic distance were generally consistent with the results from the STRUCTURE analysis. Cluster I contained 24 accessions of Job's tears: 3 from Chongqing, 12 from Guizhou, 3 from Hunan, one from Guangxi, one from Qianxinan Institute, one from Sichuan and 3 from Yunnan. This group consisted of all of the accessions from group G_1 (Y1, Y2, Y7, Y8, Y12, Y13, Y14, Y19, Y21, Y24, Y27, Y31, Y36, Y38, Y46, Y48, Y58, Y61, Y75, Y84, Y90 and Y93) and 2 accessions from the mixed subpopulation (Y49 and Y23). Cluster II contained 70 accessions and was further divided into 3 subgroups (IIa, IIb and IIc). Subgroup IIa which consisted of IIa₁ and IIa₂ included 24 accessions: 2 from Chongqing, 9 from Guizhou, 2 from Hunan, 2 from Hubei, one from Guangxi, 2 from Qianxinan Institute and 6 from Yunnan. IIa₂ subgroup consisted of 9 accessions from mixed subpopulation (Y18, Y47, Y9, Y28, Y50, Y29, Y86, Y54 and Y53). Subgroup IIb comprised 22 accessions: one from Chongqing, 4 from Guizhou, 2 from Hubei, 2 from Guangxi, 5 from Qianxinan Institute, 2 from Sichuan and 8 from Yunnan.

Correlation analysis of genetic distance and geographical distance

Based on the latitude and longitude of each location, the geographical distance between the populations was measured by GPS, and the genetic distance was calculated using

POPGENE3.2 software. Results of the Mantel test revealed no significant correlation between genetic distance and geographical distance ($r = 0.055$; $P > 0.05$) (Figure 9)

DISCUSSION

The genetic diversity of different organisms is predominantly affected by biological characteristics including life-style, breeding methods, eco-environmental factors, related human activities and the evolutionary history of the species. Evolution of a species determines how well a given organism will survive over time. However, the diversity of a population occurs following adaptation to the changing surroundings during the process of evolution; the diversity level can reflect the evolutionary potential of the species^[23]. In this study, 10 pairs of ISSR primers were selected to analyze the genetic diversity of 8 populations of Job's tears (94 individuals in total). The results showed that 116 loci were amplified using the 10 primer pairs. Of these loci, 98 resulted in polymorphic bands and the polymorphism rate was 84.48%; on average 9.8 polymorphic loci were amplified per primer pair. The ISSR marker is highly polymorphic in Job's tears suggesting that it is more suitable for analyzing the genetic diversity of this plant.

The genetic diversity data of 8 populations in Chongqing, Guizhou, Hunan, Hubei, Guangxi, Qianxinan Institute, Sichuan and Yunnan were analyzed. The results revealed that the percentage of polymorphic loci of the 8 populations varied from 46.55% to 81.90%. The highest percentage of polymorphic loci was observed for the Guizhou population (81.90%), the lowest percentage was observed for the Hubei population (46.55%), and the average percentage of polymorphic loci for the 8 populations was 65.63%. This indicates that the level of genetic variation in Job's tears varies according to geographical location. At the same time, the results also implied that there is reasonably high genetic diversity in Job's tears in the Guizhou population. The latter population also exhibited relatively high levels of adaptability. These observations suggest that the climate in Guizhou is more suitable than other populations in this study for the growth of Job's tears^[24].

At the species level, the N_a was 1.8448, the N_e was 1.5277, h was 0.3049 and I was 0.4525; this reveals that the 8 populations of Job's tears exhibit rich genetic diversity. The pattern of genetic diversity in the 8 populations was as follows: Guizhou > Yunnan > Chongqing > Hunan > Qianxinan Institute > Sichuan > Guangxi > Hubei. Thus, the greatest

degree of genetic diversity was observed in the Guizhou population, while the Hubei population had the lowest genetic diversity. There are two possible reasons for this result. First, Southwest China is the origin and main producing area in relation to Job's tears, and associated germplasm resources are richer than those observed in other regions^[25]. Secondly, the largest number of samples was collected in Guizhou, while the lowest number was collected in Hubei.

Gene differentiation and gene flow indices are important when evaluating the genetic structure of a species. In the current study, following analysis of the ISSR marker, we observed that the GST value in Job's tears was 0.1443. According to genetic differentiation data, 14.43% of the total genetic variation occurred among the populations and 85.57% of the genetic variation occurred within the populations. This indicates that variation is more prevalent within populations while there are only minor variations among populations. Gene flow, which depicts the gene movement within and between populations, is negatively correlated with gene differentiation^[26] and is extremely important for population transfer and plant evolution^[27]. According to the value of Nm, Govindaraju^[28] divided gene flow into 3 levels: high level ($Nm > 1$), medium level ($0.25 < Nm < 0.99$), and low level ($Nm < 0.25$). Following analysis of the ISSR marker, we observed that gene flow (Nm) was 2.9654 (>1). This indicates that there is no obvious genetic differentiation among populations of Job's tears. This is likely because the spread of Job's tears pollen relies on the wind and the geographic distance between populations (minimum is 49.0 km) is generally significant. These factors hinder genetic exchange between populations, thereby causing inter-population genetic differentiation to be greater than intra-population genetic differentiation.

According to the results of AMOVA, we observed 98.23% genetic variation within the Job's tears populations, while there was only 1.77% genetic variation among populations. These results are consistent with the genetic differentiation results where intra-population genetic variation was greater than inter-population genetic variation. In fact, this phenomenon was also observed for other species, with Allnutt et al.^[29] using RAPD markers to study diversity in *Pilgerodendron uviferum*; the results showed that 81.4% of the genetic variation occurred within the analyzed populations. Wang et al.^[30] detected 10 populations of *Taihangia rupestris* and observed that 73.51% of the variation existed within the analyzed populations.

The results showed that the genetic distance among the eight populations of Job's tears ranged from 0.0095 to 0.0948, while the genetic identity ranged from 0.9095 to 0.9905. The genetic distance between the Guizhou and Chongqing populations was 0.0095 and the genetic identity was 0.9905, whereas the genetic distance between the Hubei and Hunan populations was 0.0948 and the genetic identity was 0.9095. This indicates that the closest genetic relationship occurred between the Guizhou and Chongqing populations. Conversely, the most distant genetic relationship occurred between the Hubei and Hunan populations. Further, this also shows that there are significant differences in relation to the genetic distance and genetic identity among the 8 populations. The results of an UPGMA cluster analysis showed that the Guizhou and Chongqing populations, the Guangxi and Sichuan populations, and the Yunnan and Qianxinan Institute populations grouped together, respectively. This suggests that there is a close genetic relationship between populations that group together (Figure 5). This is consistent with the genetic distance results.

The Bayesian cluster and UPGMA cluster analyses suggested the existence of two clusters or subpopulations. Despite minor differences, the results were largely consistent. Bayesian cluster analysis can be used to infer genetic structure; this strategy is also used to analyze which populations might be present and to estimate the ancestry of the sampled individuals^[31]. In this study, Bayesian cluster analysis grouped the 94 accessions of Job's tears into 2 groups: G_1 and G_2 (Figure 7). The dendrogram constructed using the UPGMA clustering algorithm grouped the 94 accessions of Job's tears into 2 clusters; this was in accordance with the results of the Bayesian cluster analysis. All accessions from the G_1 and G_2 subgroups were present in cluster I and II, respectively. Apart from Y23 and Y49, the accessions in cluster I were the same as those in group G_1 (Y1, Y2, Y7, Y8, Y12, Y13, Y14, Y19, Y21, Y24, Y27, Y31, Y36, Y38, Y46, Y48, Y58, Y61, Y75, Y84, Y90 and Y93). The accessions of the mixed subgroup were clustered into 2 clusters (cluster I and cluster II_{a2}) following UPGMA. Two accessions (Y23 and Y49) from the mixed subgroup were clustered into cluster I and 9 accessions (Y18, Y47, Y9, Y28, Y50, Y29, Y86, Y54 and Y53) were clustered into cluster II_{a2} (Figure 8). Following comparison with the results generated by the Bayesian cluster analysis, the accessions in the mixed group were not identified in the UPGMA tree. Therefore, Bayesian cluster analysis can not only assign each individual to a

hypothetical ancestral cluster(s) without any prior information, but it also reveals admixtures that are not obvious using distance-based clustering methods^[22].

In general, clustering is carried out according to the location associated with sample collection. However, in this study some samples from the same population clustered in different groups while samples from different populations clustered in the same group. For example, in the Chongqing population, Y1, Y2, Y7 clustered in the first group (red), while Y3, Y4, Y5, Y6 clustered in the second group (green). Y51, Y52, Y57, and Y57 from the Guangxi population and Y62, Y63, Y64, Y65, Y66, Y67, and Y68 from the Qianxinan Institute population clustered in the same group (green).

According to the theory of distance isolation, if there is a balance between gene flow and genetic drift, the genetic distance is positively correlated with the geographical distance^[32]. When the gene flow rate is very small, genetic drift causes genetic variation^[33-34]. In the present study, we did not observe a correlation between genetic distance and geographical distance ($r = 0.055$, $P = 0.782$). This suggests that gene flow and genetic drift among populations were not balanced, and that genetic drift plays a greater role in population structure than gene flow.

CONCLUSIONS

1. The 10 pairs of ISSR primers that were used in this screening study resulted in the amplification of 98 polymorphic DNA fragments (84.48%) with an average of 9.8 polymorphic bands per primer. This indicates that the selected primers can be used to assess polymorphism levels and are suitable for genetic diversity analysis for Job's tears.

2. Analysis of genetic diversity and genetic structure of 8 populations of Job's tears. The results showed that there is a high level of genetic diversity in the Job's tears populations that were analyzed in this study. The Guizhou population exhibited the highest genetic diversity, while the Hubei population had the lowest genetic diversity.

3. Both the genetic distance and UPGMA cluster analyses for the 8 Job's tears populations revealed that there was a close genetic relationship between the Guizhou and Chongqing, the Guangxi and Sichuan, and the Yunnan and Qianxinan Institute populations, respectively.

4. In the current study, we observed following ISSR marker analysis that the GST value

in Job's tears was 0.1443; this indicated that 14.43% of the total genetic variation occurred among the populations, while 85.57% of the genetic variation occurred within the populations. The results of an AMOVA showed that 98.23% of the genetic variation occurred within the populations, while the genetic variation among populations was only 1.77%. Both GST and AMOVA results revealed that total variation was most prevalent within Job's tears populations while only minor variations existed among populations.

5. Bayesian cluster and UPGMA cluster analyses produced similar results in relation to the Job's populations. Ninety-four Job's tears accessions were grouped into 2 clusters. Cluster I contained 24 Job's tears accessions: 3 from Chongqing, 12 from Guizhou, 3 from Hunan, one from Guangxi, one from Qianxinan Institute, one from Sichuan and 3 from Yunnan. Cluster II contained 70 accessions: 4 from Chongqing, 24 from Guizhou, 4 from Hunan, 4 from Guangxi, 4 from Guangxi, 8 from Qianxinan Institute, 6 from Sichuan and 16 from Yunnan. At the same time, the results show that accessions predominantly clustered in accordance with the collection areas analyzed.

6. The results of the Mantel test showed that there was no correlation between geographic distance and genetic distance among the populations of Job's tears ($r = 0.055$, $p = 0.782$). We also observed that genetic drift plays a greater role in population structure than gene flow.

DECLARATIONS

(1) Ethics approval and consent to participate

Not applicable.

(2) Consent for publication

Not applicable.

(3) Experimental research and field studies on plants

Experimental research and field studies on plants (either cultivated or wild) complied with relevant institutional, national, and international guidelines and legislation. And the permissions or licenses to collect plants described in a manuscript were obtained. All wild plants described in a manuscript are deposited in Institute of Subtropical Crops Research, Guizhou Academy of Agricultural Sciences.

(4) Availability of data and materials

The datasets used and/or analysed during the current study are available from Lingling Yang.

(5) Competing interests

The authors declare that they have no competing interests.

(6) Funding

This work was sponsored by Guizhou talent base construction [No. QRLF (2016) 22] and Guizhou Key Technologies R&D Program Qiankehezhicheng[2016]2569.

(7) Authors' contributions

Gang Shen finished the experiment. Lingling Yang completed data analysis and wrote the main manuscript text. Shen and Yang are the authors contributed equally to the work. Teerayoot Girdthai is corresponding author. Fanzhi Liu provides project funds. Yuhua Fu assisted in data analysis and provides project funds. Qingyang Meng assisted in the experiment.

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(8) Acknowledgements

The authors would also like to thank Suranaree University of Technology and Guizhou Academy of Agricultural Sciences for partly supported and the technical assistance.

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Figure Legends

Figure 1 Inter simple sequence repeat (ISSR) profiles for 24 Job's tears genotypes following amplification with UBC857 primers and agarose gel electrophoresis.

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Figure 9 MANTEL test plots of the genetic distance and geographical distance. The vertical axis represents the genetic distance, and the abscissa axis represents the geographical distance.

Table 1 The codes and collection place of Job's tears accessions used in this study.

Code	Collection place	type	Code	Collection place	type
Y ₁	Qianjiang Chongqing	Wild	Y ₄₈	Cili Hunan	Wild
Y ₂	Qianjiang Chongqing	Wild	Y ₄₉	Cili Hunan	Wild
Y ₃	Qianjiang Chongqing	Wild	Y ₅₀	Cili Hunan	Wild
Y ₄	Banan Chongqing	Wild	Y ₅₁	Enshi Hubei	Wild
Y ₅	Nanchuan Chongqing	Wild	Y ₅₂	Shizong Hubei	Cultivated
Y ₆	Nanchuan Chongqing	Cultivated	Y ₅₃	Xianfeng Hubei	Wild
Y ₇	Wansheng Chongqing	Wild	Y ₅₄	Xianfeng Hubei	Wild
Y ₈	Changshun Guizhou	Wild	Y ₅₅	Longling Guangxi	Cultivated
Y ₉	Changshun Guizhou	Wild	Y ₅₆	Longling Guangxi	Wild
Y ₁₀	Changshun Guizhou	Wild	Y ₅₇	Xilin Guangxi	Cultivated
Y ₁₁	Zunyi Guizhou	Wild	Y ₅₈	Baise Guangxi	Cultivated
Y ₁₂	Huaxi Guizhou	Wild	Y ₅₉	Tianlin Guangxi	Cultivated
Y ₁₃	Huaxi Guizhou	Wild	Y ₆₀	Qianxinan Institute1	Cultivated
Y ₁₄	Huaxi Guizhou	Wild	Y ₆₁	Qianxinan Institute2	Cultivated
Y ₁₅	Huaxi Guizhou	Wild	Y ₆₂	Qianxinan Institute3	Cultivated
Y ₁₆	Suiyang Guizhou	Wild	Y ₆₃	Qianxinan Institute4	Cultivated
Y ₁₇	Ziyun Guizhou	Cultivated	Y ₆₄	Qianxinan Institute5	Cultivated
Y ₁₈	Yuping Guizhou	Wild	Y ₆₅	Qianxinan Institute6	Cultivated
Y ₁₉	Meitan Guizhou	Wild	Y ₆₆	Qianxinan Institute7	Cultivated
Y ₂₀	Wangmo Guizhou	Wild	Y ₆₇	Qianxinan Institute8	Cultivated
Y ₂₁	Wangmo Guizhou	Wild	Y ₆₈	Qianxinan Institute9	Cultivated
Y ₂₂	Wangmo Guizhou	Wild	Y ₆₉	Zizhong Sichuan	Wild
Y ₂₃	Wangmo Guizhou	Wild	Y ₇₀	Zizhong Sichuan	Wild
Y ₂₄	Wangmo Guizhou	Wild	Y ₇₁	Jianyang Sichuan	Wild
Y ₂₅	Qianlong Guizhou	Cultivated	Y ₇₂	Jianyang Sichuan	Wild
Y ₂₆	Puding Guizhou	Cultivated	Y ₇₃	Jianyang Sichuan	Wild
Y ₂₇	Puding Guizhou	Wild	Y ₇₄	Chengdu Sichuan	Wild

Y ₂₈	Puding Guizhou	Wild	Y ₇₅	Tianjin Sichuan	Wild
Y ₂₉	Sinan Guizhou	Cultivated	Y ₇₆	Maguan Yunnan	Cultivated
Y ₃₀	Yinjiang Guizhou	Cultivated	Y ₇₇	Maguan Yunnan	Cultivated
Y ₃₁	Yinjiang Guizhou	Wild	Y ₇₈	Qiubei Yunnan	Cultivated
Y ₃₂	Wuchuan Guizhou	Wild	Y ₇₉	Xichou Yunnan	Wild
Y ₃₃	Wuchuan Guizhou	Wild	Y ₈₀	Luoping Yunnan	Cultivated
Y ₃₄	Wuchuan Guizhou	Wild	Y ₈₁	Luoping Yunnan	Cultivated
Y ₃₅	Ceheng Guizhou	Wild	Y ₈₂	Luoping Yunnan	Cultivated
Y ₃₆	Ceheng Guizhou	Cultivated	Y ₈₃	Luoping Yunnan	Cultivated
Y ₃₇	Ceheng Guizhou	Cultivated	Y ₈₄	Quejing Yunnan	Wild
Y ₃₈	Xingren Guizhou	Cultivated	Y ₈₅	Wenshan Yunnan	Wild
Y ₃₉	Xingren Guizhou	Cultivated	Y ₈₆	Wenshan Yunnan	Cultivated
Y ₄₀	Xingrenxiashan Guizhou	Cultivated	Y ₈₇	Shizong Yunnan	Wild
Y ₄₁	Xingrenxiashan Guizhou	Cultivated	Y ₈₈	Shizong Yunnan	Cultivated
Y ₄₂	Xingyi Guizhou	Cultivated	Y ₈₉	Fuyuan Yunnan	Cultivated
Y ₄₃	Guanling Guizhou	Cultivated	Y ₉₀	Fuyuang Yunnan	Cultivated
Y ₄₄	Yongshun Hunan	Wild	Y ₉₁	Fuyuan Yunnan	Cultivated
Y ₄₅	Sanzhi Hunan	Wild	Y ₉₂	Kunming Yunnan	Wild
Y ₄₆	Sanzhi Hunan	Wild	Y ₉₃	Dali Yunnan	Wild
Y ₄₇	Sanzhi Hunan	Wild	Y ₉₄	Lijiang Yunnan	Wild

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578 **Table 2** Inter simple sequence repeat (ISSR) primers and amplification results

Code of the Primer	Sequence (3'–5')	Optimal annealing temperature	Total number of amplified bands	Number of polymorphic bands (NPB)	Percentage of polymorphic loci (PPB)
815	CTC TCT CTC TCT CTC TG	46.9	14	14	100%
818	CAC ACA CAC ACA CAC AG	56.0	13	13	100%
825	ACA CAC ACA CAC ACA CT	52.2	14	12	85.7%
836	AGA GAG AGA GAG AGA GYA	53.9	11	7	63.6%
840	GAG AGA GAG AGA GAG AYT	48.1	14	14	100%
841	GAG AGA GAG AGA GAG AYC	54.7	8	5	62.5%
844	CTC TCT CTC TCT CTC TRC	49.8	7	7	100%
856	ACA CAC ACA CAC ACA CYA	54.7	10	6	60%
857	ACA CAC ACA CAC ACA CYG	56.2	14	11	78.6%
859	TGT GTG TGT GTG TGT GRC	55.2	11	9	81.8%
Total			116	98	84.48%
Average		53.0	11.6	9.8	

579 Y = (C, T), R = (A, G)

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581 **Table 3** Genetic diversity index for 8 populations of Job's tears

Populations	Sample Size	NPB	PPB	Na	Ne	h	I
Chongqing	7	80	68.97%	1.6897	1.4847	0.2727	0.3984
Guizhou	36	95	81.90%	1.8190	1.5412	0.3113	0.4589
Hunan	7	79	68.10%	1.6810	1.4729	0.2663	0.3898
Hubei	4	54	46.55%	1.4655	1.3242	0.1842	0.2701
Guangxi	5	69	59.48%	1.5948	1.4175	0.2342	0.3428
Qianxinan Institute	9	76	65.52%	1.6552	1.4299	0.2434	0.3586
Sichuan	7	70	60.34%	1.6034	1.3977	0.2264	0.3342
Yunnan	19	86	74.14%	1.7414	1.4944	0.2807	0.4130
Average	11.75	76.1 3	65.63%	1.6559	1.4453	0.2524	0.3706
Species level	94	98	84.48%	1.8448	1.5277	0.3049	0.4525

582 NPB = Number of polymorphic bands, PPB = Percentage of polymorphic bands, Na =
583 Observed number of alleles, Ne = Effective number of alleles [Kimura and Crow (1964)], h =
584 Nei's (1973) gene diversity, I = Shannon's Information index [Lewontin (1972)]

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587 **Table 4** Genetic differentiation among eight populations of Job's tears

All population	Size	Ht	Hs	Gst	Nm*
Mean	94	0.2950	0.2524	0.1443	2.9654
St. Dev		0.0329	0.0250		

588 Ht = total gene diversity, Hs = gene diversity with provenance, Gst = coefficient of
589 gene differentiation, *Nm = estimate of gene flow from Gst.

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Table 5 Results of analysis of molecular variance of ISSR data of Job's tears

Source of variation	d.f.	Sum of squares	Variance components	Percentage of variation	<i>P</i>
Among populations	7	138.738	0.29966	1.77	<0.05
Within populations	86	1433.868	16.67288	98.23	<0.05
Total	93	1572.606	16.97254		

595 **Table 6** Nei's unbiased Measures of genetic distance (below diagonal) and genetic identity
596 (above diagonal)

Population	Chongqing	Guizhou	Hunan	Hubei	Guangxi	Qianxinan Institute	Sichuan	Yunnan
Chongqing	****	0.9905	0.9681	0.9365	0.9812	0.9612	0.9487	0.9774
Guizhou	0.0095	****	0.9747	0.9447	0.9757	0.9631	0.9687	0.9804
Hunan	0.0324	0.0256	****	0.9095	0.9317	0.9224	0.9227	0.9478
Hubei	0.0656	0.0569	0.0948	****	0.9417	0.9743	0.9131	0.9644
Guangxi	0.0190	0.0246	0.0708	0.0601	****	0.9780	0.9833	0.9742
Qianxinan Institute	0.0395	0.0376	0.0808	0.0261	0.0223	****	0.9454	0.9828
Sichuan	0.0527	0.0318	0.0805	0.0909	0.0168	0.0562	****	0.9545
Yunnan	0.0228	0.0198	0.0536	0.0363	0.0261	0.0173	0.0465	****

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Figure 8

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Figure 9

MANTEL test plots of the genetic distance and geographical distance. The vertical axis represents the genetic distance, and the abscissa axis represents the geographical distance.