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Zihao Wang

Ludong University

Hao Zhang

Ludong University

Boqiang Tong

Institute of Forestry

Biao Han

Institute of Forestry

Dan Liu

Institute of Forestry

Ping Zhang

Ludong University

Dechang Hu (✉ hudch78@163.com)

Ludong University

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The study on genetic diversity and genetic structure of *Juglans mandshurica* in Shandong Province based on EST-SSR

Zihao Wang¹, Hao Zhang¹, Boqiang Tong², Biao Han², Dan Liu², Ping Zhang¹, Dechang Hu^{1,*}

(1 College of life science, Ludong University, Shandong Yantai China, 264025; 2. Shandong Forest and Grass Germplasm Resources Center, Shandong Jinan China, 250014)

Abstract: *Juglans* plant with important ecological and economic value is endangered in China. And the basis for its protection is the genetic diversity assessment. In the present study, the genetic diversity and the genetic structure of 134 samples from 9 natural *J. mandshurica* populations with 2 related species were surveyed by EST-SSR markers. The results showed that (1) 77 alleles were amplified within 9 EST-SSR markers which had high polymorphism ($PIC = 0.5070$). Compared with other *Juglans* species in China, the genetic diversity of *J. mandshurica* population in Shandong areas was relatively low ($H_o=0.3333$, $I=0.7051$). (2) The AMOVA analysis showed that 89% variation occurred within the population of *J. mandshurica* in Shandong areas, and 11% occurred among the populations. The low genetic differentiation ($F_{ST}=0.103$) and the high gene flow ($N_m= 2.894$) among populations may be related to the long-distance transmission of pollen. The results of Mantel test ($r=-0.0870$, $p=0.4320$) showed that there was no significant correlation between geographic distance and genetic distance. (3) UPGMA cluster analysis divided 8 *J. mandshurica* populations in Shandong areas into two groups. The results of STRUCTURE analysis suggested interspecific introgression exists between *J. mandshurica* and common walnuts (*J. regia* L.). These genetic data would be great significance for biodiversity conservation for endangered *J. mandshurica*.

Keywords: *Juglans mandshurica*; EST-SSR; Genetic diversity; Genetic structure

Zihao Wang and Hao Zhang were contributed equally to this paper.

* Corresponding author. E-mail: hudch78@163.com.

1. Introduction

Juglans mandshurica Maxim, which belongs to the *Juglans* L. in the family Juglandaceae, is mainly distributed in North China, Northeast China, the Korean Peninsula and the Far East like Russia(Bai et al. 2010). The species of *J. mandshurica*, *J. cathayensis* Dode, *J. ailantifolia* Carr. and *J. hopeiensis* Hu all belong to the Sect. Cardiocaryonn (Hu et al. 2015). *J. mandshurica* is a multi-purpose species with high ecological and economic value. As an important timber tree, along with *Fraxinus mandshurica* Rupr and *Phellodendron amurense* Rupr, it is known as the three broad-sclerophyll species in Northeast China. And its leaves, roots and seeds had been applied in medical sciences such as the development of drugs for cancer treatment (Xu et al. 2010). Due to the destruction of habitats and over exploitation, the population of *J. mandshurica* has declined sharply. It had been listed as the protected wild plant species by Beijing, Jilin, Hebei and Shandong in the China Information System of Chinese Rare and Endangered Plants (ISCREP) (<http://www.iplant.cn/rep/>).

The research on genetic diversity and genetic structure is the basis for the conservation and utilization of *J. mandshurica*. And to formulate scientific conservation strategies, clarifying the causes of endangerment as well as estimating the ability of the species to face the environmental changes and the population evolution potential are essential. With the development of biological studies, some molecular markers such as inter simple sequence repeats (ISSR) and amplified fragment length polymorphism (AFLP) had become the widely adopted methods for plant genetic diversity and genetic structure research (Nasim et al. 2020; Wu et al. 2020). Microsatellite markers (SSRs) also proved to be an ideal molecular marker due to its high polymorphism, high reproducibility and interspecific codominance (Liu et al. 2019). Expressed sequence tag SSRs (EST-SSRs) is designed based on simple repetitive sequences in the transcription region. Compared with genomic SSR markers (gSSRs), it owns the advantages of lower cost and higher level of transferability between species (Xiang et al. 2018), which had been largely adopted in the study of genetic diversity of *J. mandshurica* and other *Juglans* plants. Hu et al. (2016) studied the diversity of 48 *J. mandshurica* individuals collected from Jilin, Hebei and Beijing based on 20 pairs of EST-SSR primers and high genetic diversity was exhibited. Torokeldiev et al.(2019) analyzed the genetic diversity of *J. regia* L. populations in the Kyrgyz Republic through SSR markers, which showed that the population differentiation was small. Dang et al. (2019) researched on the gene penetration of *Juglans* spp. with the application of EST-SSR

markers and the research showed that the gene penetration was common within the same group of the *Juglans* genus.

However, studies on the genetic diversity of *J. mandshurica* populations in Shandong areas are few due to the limitations of location and quantity of the sample. Field investigations found that some natural populations of *J. mandshurica* are preserved in areas like Mount Tai, Mount Culai, Mount Meng, Mount Lao and Mount Kunyu in Shandong area, showing the characteristics of fragmented distribution. Morphological discrepancies occurred in leaves and fruit shapes among individuals in different populations. Facing the man-made destruction and environment deterioration seriously, the species is endangered and resource protection is urgent. At present, the unclearness of the genetic diversity and the genetic structure of *J. mandshurica* populations in Shandong area has become a restraint for recognizing the population situation and causes of endangerment as well as formulating the protection strategies. In this study, we detected the genetic diversity on 122 *J. mandshurica* individuals in 8 natural populations in Shandong area and 1 in Jilin area, using 9 EST-SSR primers pairs. The species of *J. regia* and *J. cathayensis* var. *formosana* were taken as the out-group. The genetic relationships among the populations in Shandong areas were discussed to provide the theoretical basis for the protection and development of *J. mandshurica*.

2. Materials and Methods

2.1 Sample collection and DNA extraction

A total of 134 individuals were sampled, including 122 genotypes of *J. mandshurica*, 9 genotypes of *J. cathayensis* var. *formosana* (originated from Fujian province, preserved in National Walnut Germplasm Conservation Center, Shandong Tai'an) and 3 genotypes of *J. regia* (common walnut) from Shandong areas. The material information was showed in Table1. The samples of *J. mandshurica* were divided into 9 populations according to the distribution. Eight populations were in Shandong areas, named as TS, CL, MS, YT, WL, LS, KY and QS (including of 111 individuals) respectively, and one was in Jilin areas, named as JL (including of 11 individuals). The samples of collection sites Mt. Yangtian and Mt. Lu were treated as YT population, yet Mt.Daze and Mt. Lao as LS population because of closer distance.

Fresh leaves from each test samples were collected and preserved in liquid nitrogen. Genomic DNA

was extracted according to the CTAB method (Doyle and Doyle, 1990) and purity were detected by spectrophotometer and gel electrophoresis.

2.2 EST-SSR primer synthesis and PCR amplification

Based on the SSR primer sequence and amplification conditions of the genus *Juglans* by Dang et al.(2015), 9 EST-SSRs (sequences were listed in Table 2) were synthesized by Shanghai Biotech Biotechnology Co, Ltd. and applied to the research on the genetic diversity of *J. mandshurica* and its related species. The PCR amplification products were subjected to capillary electrophoresis on the 3730XL sequencer, and the original data was obtained with the evaluation of the fragment length by GeneMapper v3.7 software.

2.3 Genetic diversity analysis

According to the biological information obtained, relevant software was used to analyze the genetic diversity. The percentage of polymorphic loci (PPL), the number of observed alleles (N_a), number of effective alleles (N_e), observed heterozygosity (H_o), expected heterozygosity (H_e), Shannon information index (I), genetic similarity coefficient, genetic distance and inbreeding coefficient (F_{IS}) were calculated by POPGENE version 1.32. Power Marker version 3.25 software was used to calculate the polymorphic information content (PIC) and to draw the UPGMA phylogenetic tree between individuals. Used GenAlEx version 6.503 to calculate Gene flow (N_m) and genetic differentiation coefficient (F_{ST}) and to perform the analysis of molecular variation (AMOVA). The UPGMA phylogenetic tree was constructed by MEGA X (Sudhir et al. 2018) based on genetic distance between populations. IBD (Isolation by Distance) was applied to analyze the correlation between population genetic distance and geographic distance by Mantel test.

2.4 Population genetic structure analysis

STRUCTURE version 2.3.4 (Pritchard et al. 2000) was used to perform Bayesian cluster analysis on 11 populations. The K value was set from 2 to 10, and each K value setting was repeated 10 times. The length of burn-in period value and Markov chain Monte Carlo (MCMC) were both set to 100,000. We uploaded the STRUCTURE calculation results to Structure Harvester online (http://taylor0.biology.ucla.edu/struct_harvest/) (Earl et al. 2012) to determine the best K value of different populations. With the usage of CLUMMP (Jakobsson et al. 2007) for repeated sampling and analysis of Clusters, Graphpad Prism 8.0 was used to draw the STRUCTURE genetic structure

116 diagram.

117 **3. Results**

118 **3.1 EST-SSR analysis**

119 In the present study, 77 alleles were detected by the 9 EST-SSR loci (showed in Table 3), and
120 averages of 8.5556 alleles were observed at each locus. The polymorphic information content (PIC)
121 varied from 0.2621 (JC4535) to 0.7163 (JC8125), with an average of 0.5070. The results showed that 9
122 primers pairs had high polymorphisms. The observed heterozygosity (H_O) value ranged from 0.0970
123 (JC1151) to 0.7313 (JC2995), with an average of 0.3972. The expected heterozygosity (H_E) ranged
124 from 0.2861 (JC4535) to 0.7571 (JC8125), with an average of 0.5490. Most of the loci showed positive
125 F_{IS} value, while only JC2995 and JC3304 had negative values which were -0.3761 and -0.0744,
126 respectively. The locus gene flow ranged from 0.2317 (JC1151) to 1.4389 (JC2995) with an average of
127 0.6532.

128 **3.2 Population genetic diversity**

129 Based on the genetic information obtained by EST-SSRs, the genetic diversity analysis on the
130 population level were carried out (Table 4). The results indicated that the discrepancy existed in the
131 genetic variation among the populations of *J. mandshurica* in Shandong areas, and the overall
132 diversity level was low ($H_O=0.3633$, $I=0.7488$). The value of observed alleles (N_a), expected alleles
133 (N_e), expected heterozygosity (H_E), and the percentage of polymorphic loci (PPL) at the population
134 level of *J. mandshurica* in this area were 3.2917, 1.9989, 0.4502 and 86.11%, respectively. Among the
135 populations in Shandong areas, TS and CL had higher genetic diversity, while the genetic diversity of
136 WL and MS was lower. TS had the highest N_a (5.7778) and I (1.0207) value, and CL had the highest
137 H_O (0.5169) and H_E (0.5241). The PPL of both populations were 100%. WL had the lowest N_a (2.0000)
138 and I (0.4873) value and MS had the lowest H_O (0.2000) and H_E (0.3010) value. The PPL in these two
139 populations were 77.78% and 55.56%, respectively. The average private allele (N_P) value was 0.2779,
140 of which private alleles existed in populations KY, QS, LS, TS and WL.

141 **3.3 Cluster analysis**

142 Nei's genetic identity and genetic distance was adopted to evaluate the genetic relationships

among 11 genus *Juglans* populations (Table 5). Populations TS and CL exhibited the highest genetic identity (0.9574) and the shortest genetic distance (0.0435), while populations HT and HDY showed the lowest genetic identity (0.2606) and the longest genetic distance (1.3449). The UPGMA phylogenetic tree among populations was constructed based on genetic distance (Figure 2). The results showed that populations HT (*J. regia*) and HDY (*J. cathayensis* var. *formosana*) were clustered separately, 9 populations of *J. mandshurica* could be divided into two main clusters. Cluster I was composed of WL, YT, TS and CL, while Cluster II included JL, KY, QS, LS and MS.

The UPGMA phylogenetic tree of all sample individuals was constructed based on genetic distance (Figure 3). The results showed that the clustering relationships of 121 *J. mandshurica* samples were complicated. There was no obvious correlation between geographical distance and genetic distance. Mantel test analysis of geographic distance and genetic distance between populations were $r = -0.0870$ and $p = 0.4320$, which also verified that there was no significant correlation in between.

The average values of gene flow (N_m) and genetic differentiation index (F_{ST}) among the 8 *J. mandshurica* populations in Shandong areas were 2.894 and 0.103 respectively (Table 6). The TS and CL populations showed the largest gene flow (12.463) and the smallest genetic differentiation coefficient (0.020), while the WL and QS populations presented the smallest gene flow (1.032) and the largest genetic differentiation coefficient (0.195). The results of AMOVA (Table 7) showed that 89% of the total genetic population variation occurred within the population, while 11% of the variation happened between the populations, implying that the genetic variation of the *J. mandshurica* population mainly exists in the population.

3.4 Population genetic structure

The optimal K value was 4 determined by Structure Harvester, indicating that there should be 4 basic gene pools in the surveyed populations in the study. All accessions could be divided into 4 gene clusters. The STRUCTURE results (Figure 4) showed that the genetic structure of *J. mandshurica* was mainly composed of two gene pools (Yellow and Green), and the species of *J. cathayensis* var. *formosana* (Red) and *J. regia* (Blue) as out-group had significant difference compared with *J. mandshurica*. According to the percentage of yellow/green gene pools, nine of *J. mandshurica* populations were divided into two groups. One group consisted of TS, CL, YT and WL (Yellow) and the other consisted of KY, QS, MS, LS and JL (Green). The STRUCTURE grouping result was

consistent with the UPGMA clustering result. It should be noted that parts of individuals belongs to *J. mandshurica* had the same genetic composition as that of *J. regia* (Blue gene pool). Among that, the proportion of the blue gene pool had exceeded 60% of its own genetic structure, such as genotypes TS08 and TS26 in the TS population, and CL03 and CL14 individuals in the CL population. The STRUCTURE results indicated that there had interspecific hybridization between *J. mandshurica* and *J. regia*.

4. Discussion

Genetic diversity is the guarantee of long-term survival of species (Lu et al. 2020). The fact shows that the more abundant the genetic diversity is, the stronger the adaptability of species to the environment will be, while the lower the genetic diversity will lead to the decline of adaptability and greater extinction risk. In this study, 9 EST-SSRs were used to study the genetic diversity of 8 *J. mandshurica* populations in Shandong, and the observed heterozygosity (H_o) and expected heterozygosity (H_e) were 0.3633 and 0.4502 respectively. The heterozygosity index could effectively assess the genetic diversity of species (Montgomery and Nicholas 1989). Dang et al. (2015) surveyed the genetic diversity of 15 *J. cathayensis* populations in China based on EST-SSRs, and found that the average observed heterozygosity was 0.475. Hu et al. (2016) detected the average observed heterozygosity of *J. mandshurica* populations in Hebei and Jilin was 0.627 used by SSR markers. The genetic diversity of *J. mandshurica* population of Shandong is higher than that of JL population of Jilin in this study ($H_o=0.3333$, $H_e=0.4367$), but is lower than other *Juglans* species in the previously reported. The influencing factors of genetic diversity include reproductive system, gene mutation, natural selection, population isolation and habitat fragmentation caused by environmental change and human disturbance (Wen et al. 2010). Human activities should reduce the effective population size and the effective recombination rate of alleles, so the genetic diversity of *J. mandshurica* in Shandong Province was relatively low (Li et al. 2011). In addition, the characteristics of wind-pollinated and self-compatibility can also increase the inbreeding frequency and decrease heterozygosity of the alleles, thus resulting in a lack of sufficient heterozygosity to maintain high genetic diversity. There is obvious inbreeding in *J. mandshurica* populations in Shandong (F_{IS} value is 0.2).

The genetic variation had mainly occurred within the population of cross-pollinated species (Zhou et

al. 2020). In this study, the results of AMOVA analysis showed that 89% of the total variations of *J. mandshurica* occur within the population. The F_{ST} of the 8 populations in this area is 0.103, which was greater than the genetic analysis of *J. mandshurica* populations in Northeast China and the Korean Peninsula ($F_{ST}=0.054$) by nSSR markers (Wang et al. 2016). When $F_{ST} > 0.250$, it was considered that there was a large genetic differentiation between populations (Wright et al. 1978). So, we infer that genetic differentiation was slight to some extent among *J. mandshurica* in Shandong area. Previous studies have shown that many factors may affect the genetic differentiation between species, such as gene flow, reproduction mode, and natural environment (Manners et al. 2013). When the gene flow is greater than 1.000, it could effectively eliminate the influence of genetic drift (Wright et al. 1990). The average N_m of Shandong' populations is 2.894, indicating that there was gene exchange among populations. The seeds of *Juglans* plants have relatively large quality, which means they cannot be spread over long distances by wind, birds, etc., but their pollen can transmit through long distances to achieve gene exchange between populations in different locations (Victory et al. 2006). Therefore, *J. mandshurica* in Shandong areas may eliminate the geographical isolation through long-distance pollen transmission, so as to reduce the genetic differentiation among populations.

Phylogenetic relationships play an essential role in the study of species evolution and become an important part of evolutionary biology. In the UPGMA clustering results of this study, *J. regia* (HT) was clustered separately, which can be clearly distinguished from *J. mandshurica* and *J. cathayensis* var. *formosana* (HDY), demonstrating that the latter two were relatively close, but far from the former. This conclusion was consistent with the traditional classification of morphology. From the results of Bayesian STRUCTURE analysis, some *J. mandshurica* individuals showed heterozygous genetic information of *J. mandshurica* and *J. regia*, which demonstrates that there was gene introgression between them. Because of the sympatric distribution of the two species, natural hybridization to a certain extent affected the overall gene composition of *J. mandshurica*. Considering the homogeneity of the ecological environment, the differences in phenotypic traits such as leaves and fruit types among *J. mandshurica* individuals in Shandong areas may be related to this.

Genetic diversity provides the material basis for species adaptation and evolution and is related to the formulation of endangered species protection and rehabilitation strategies (Elam 1993). According to this study, a protection strategy should be adopted combining in-situ and ex-situ conservation of natural *J. mandshurica* populations in Shandong area. In the aspect of in-situ protection, it is strictly

forbidden to cut down them and destroy the ecological environment. Ecological protection and restoration work should be carried out in areas with small number of individuals and serious ecological damage. Populations with high genetic diversity and large number of private alleles, such as KY, QS, CI and TS, can provide priority protection. If ex-situ conservation is to be performed, resources with relatively genetic relationship should be selected based on the genetic distance of populations obtained by SSR markers. And the seeds should be collected to establish a germplasm bank or for provenance test. The genetic data of *J. mandshurica* in Shandong areas obtained by this study can provide a theoretical basis for the biodiversity conservation of this species.

5. Conclusions

In the present study, the genetic diversity and genetic structure were surveyed by EST-SSR markers for the basis of biodiversity conservation for endangered *J. mandshurica*. The results showed that EST-SSR markers had high polymorphism among 134 samples from 9 natural *J. mandshurica* populations with 2 related species. The genetic diversity of *J. mandshurica* population in Shandong areas was relatively low. Total 89% variation occurred within the population, and 11% among the populations of *J. mandshurica* by AMOVA analysis. The low genetic differentiation and the high gene flow among populations may be related to the long-distance transmission of pollen. There was no significant correlation between geographic distance and genetic distance based on the results of Mantel test. The results of STRUCTURE analysis suggested interspecific introgression exists between *J. mandshurica* and common walnuts (*J. regia* L.).

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Conflict of Interest

The authors declare that they have no conflict of interest.

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Figures

Figure 1

Geographic locations of 8 *J. mandshurica* of Shandong Prov.in this study

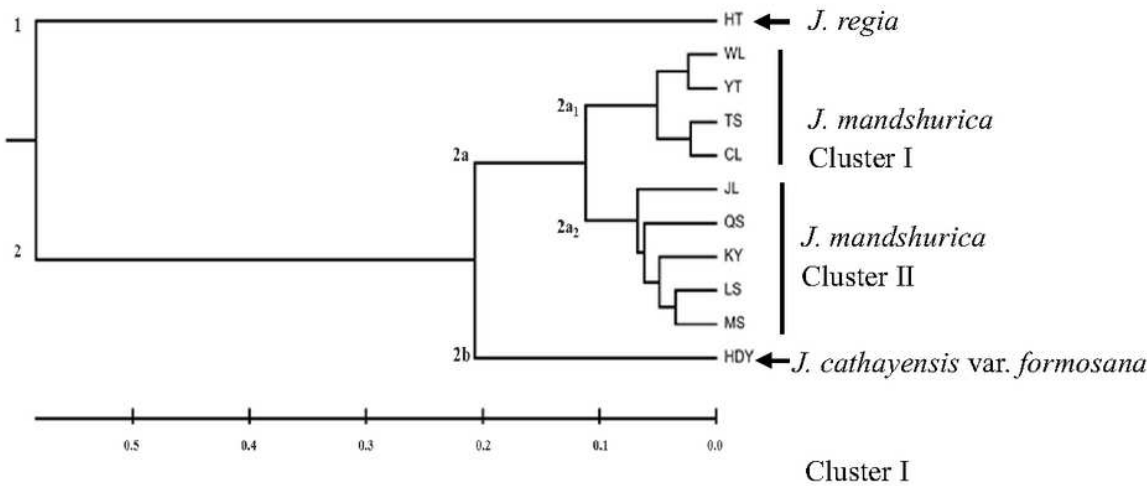


Figure 2

UPGMA-based cluster analysis of the 11 populations of *Juglans* L.

Figure 3

UPGMA-based cluster analysis of the 134 individuals from 11 populations of *Juglans* L.

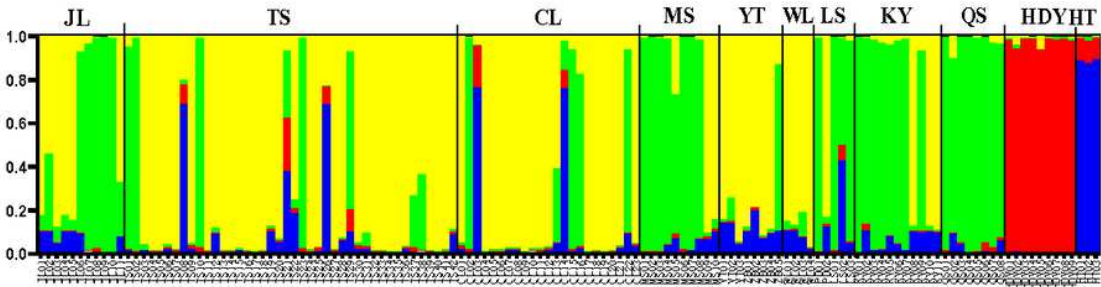


Figure 4

Estimated genetic structure of the 11 populations of *Juglans* L. based on STRUCTURE analysis (K = 4)

Supplementary Files

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