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BIOLOGICAL PROPERTIES OF WALNUT GENOTYPES IN KAZAKHSTAN

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Abstract
Walnut, which is known for its polymorphism, has long been growing in the Sairam-Ugam region. This is a biological characteristics study of walnut genotypes cultivated in the Almaty and Turkestan regions. In 2018, out of 137 seedlings of local walnut, 19 genotypes had good winter hardiness. After pruning of damaged branches, genotypes X1 / 4, X5 / 5, X15 / 5 and X3 / 2 showed a high shoot-forming ability. The height of 3-year-old trees was within the range of 2.10-3.50 m (2.83 m in average, p = 0.05) and the crown width was 1.30-3.36 m (2.33 m in average, p = 0.05). In 4 summer trees, the height was 2.50-4.50 m (3.53 m in average, p = 0.05), and the crown width was from 1.8 to 5.4 m (3.66 m in average, p = 0.05). In 2019, 200 Turkish walnut genotypes were found to have I degree of winter hardiness. Among them, 24 genotypes began to bear fruit, but the presence of diseases was found in 6 trees. In microsatellite analysis, depending on the primer pairs used and the walnut genotype, the sizes of the allelic loci WGA001, WGA005, WGA009, WGA069, and WGA202 varied within 178-192 bp, 240-252 bp, 237-252 bp, 160-184 bp, and 259 to 295 bp, respectively. An expanded study of other SSR loci in local Kazakh genotypes will provide a complete picture of the genetic diversity of the J. regia nut.

Keywords: Juglans spp., walnut, genotypes, SSR-markers, frost-hardiness, microsatellites, height of tree

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INTRODUCTION

The most common introduced species from the Juglandaceae family A. Rich. ex Kunth refers to the walnut [1 Kairova M., 2019]. The value of nut species is determined by the use of their phytomass in medicine, fruits rich in lipids in the food industry, the formation of valuable wood, as well as phytomeliorative use [2 Pomogaybin A.V., 2016]. It has been established that, in comparison with hazelnuts, it is the walnut that has the most pronounced antioxidant properties [3 Dmitrieva AN, 2015]. In addition, species of deceptive walnut Juglans fallax Dode and walnut J. regia L. were used as dye plants [4 Korolyuk E.A. 2003].

Researchers previously isolated from 7 to 9 species of nuts with 4 centers of origin: Central Asian (Tajikistan, Kyrgyzstan, Uzbekistan, Afghanistan), Near East (Greece, Turkey, Iran, Caucasus), Sino-Japanese and North American [5 Cherepanov S.K., 1995]. French scientist L.A. Dode divided the genus Juglans into 44 species, which are distributed in 4 sections [6 Dode L.A., 1906]. Due to the strong variability of the morphological and biological characteristics of the walnut, an unsuccessful attempt was made to divide it into two separate species J. fallax Dode and J. regia L. [7 Bikirov Sh.B., 2013]. Two species J. sieboldiana Maxim (Japanese or Siebold walnut) and J. cordiformis Maxim were distinguished separately, but at the moment they are referred to the same species of ailantholus nut J. ailanthifolia Carr. [8 Gorokhova S., 2009]. In general, the taxonomy of plants in the Juglandaceae family is not fully

established.

Many researchers agree that it was from the Central Asian region that the process of spreading the walnut to eastern and central Europe, as well as to the eastern part of India and China, began [9 Breton C., 2004]. In Central Asia, there are 3 centers of walnut distribution. The main large and continuous walnut massif in Central Asia is located in the Western Tien Shan [10 Murodov NS, 2011]. In Soviet times, scientists considered natural forests found on the territory of Kazakhstan, where walnuts act as a forest-forming species [11 Strela TE, 1990]. However, the study of pollen and charcoal in the bottom sediments of lakes and peat bogs adjacent to the walnut forests of Kyrgyzstan showed that the age of J. regia trees is no more than 2000 years [12 Beer R., 2008].

In walnut forests, walnuts are characterized by a high degree of polymorphism [13 Zapryagaeva VI, 1964]. Of interest to breeders are the natural forms of the nut growing in the Western Tien Shan, in the mountains of the Syrdarya Karatau, in the most original region of Central Asia and Kazakhstan [14 Kamelin R.V., 1990]. Moreover, the Kazakh-Ugam area is called the most northern zone of natural habitat of walnut J. regia L., where cold-resistant plants grow [15 Dzhangaliev AD, 2008].

Various methods are used to assess the genetic diversity and relatedness of varieties and populations of Persian walnut, including isozyme analysis, restriction fragment length polymorphism (or RFLP), RAPD, and ISSR markers [16 Dangl G.S., 2005]. Based on microsatellite markers, a structural analysis of the French collection of nut germplasm was carried out, including 253 samples of world origin [17 Bernard A., 2018]. Genome sequencing of the Chandler walnut cultivar led to the discovery of the second homologue of the JrPPO2 polyphenol oxidase gene [18 Martınez-Garcıa P.J., 2016]. The recently developed SLAF-seq method makes it possible to obtain a large number of markers for the detection of nut resistance genes to the serious disease of anthracnose [19 Zhu Y., 2015]. In general, the study of the diversity of walnut genotypes makes it possible to assess their potential for breeding [20 Martínez M.L., 2010]. The purpose of these studies was to study the biological characteristics and genotyping of J. regia L. walnut plants growing in the southern regions of Kazakhstan.

MATERIALS AND METHODS

Plant materials

Walnut plantations were located in the Almaty and Turkestan regions of Kazakhstan. Phenological observations and biometrics of walnut tree plantations were carried out from 2018 to 2019 according to the generally accepted methodology [21 Program, 1999]. The study of the biological characteristics of plants was carried out on selected 46 different local genotypes and Turkish walnut cultivars cultivated in private companies of Khizhkov (Enbekshikazakh disctrict, Almaty region) and Sary-agash Zher Syiy (Saryagash district in Turkistan region, Turkestan region).

DNA isolation

The collection of samples of young leaves of walnuts was carried out on the territory of walnut orchards in the spring and summer. Isolation of genomic DNA from leaf samples was performed in 3-5 replicates using the commercial DNeasy Plant Mini Kit (Qiagen, cat # 69104). DNA concentration was determined spectrophotometrically on a Nanodrop 1000 instrument (ThermoScientific, USA). Visual assessment of the quality of DNA samples was carried out using the electrophoresis in 0.9% of agarose method (Amresco RA, cas # 9012-36-6).

PCR procedure

DNA loci were amplified by PCR in ThermoCycler Eppendorf (Germany). Six SSR-primers WGA001, WGA009, WGA069, WGA202, WGA005 and WGA027, developed for locus amplification of J. nigra, and then used for varieties of J. regia, were assayed in different walnut genotypes growing on plantation of south and southeastern Kazakhstan [16 Dangl G.S., 2005, 22 Foroni I., 2006]. PCR reactions were carried out in 25 µl volume, consisting of 1x PCR buffer (20 mM Tris- Cl pH 8.4, 50 mM KCl, ThermoScientific), 2.0 mM MgCl2, 0.2 mM dNTPs (Ref# R0241, ThermoScientific), 0.2 µM of each primer (ordered from Sigma-Aldrich, Germany), 0.25 units of Taq-DNA recombinant polymerase (Ref# EP0402, ThermoScientific) and 10-25 ng of genomic target DNA.

A touch down PCR was used according to the protocol previously described [23 Ruiz-Garcia L., 2011]. PCR amplification consist of 5 minute for initial denaturation at 940C, 35 cycles of 45 sec at 940C, 45 sec at annealing temperature specific to each pair primer, and 45 сек at 720C, a final step including 10 minute at 720C. Fractionation of PCR products was done by gel-electrophoresis in 3.0% MetaPhor agarose (Lonza, cat# 50181) in 0.5x TBE buffer (Applichem, USA) at 120V in during 60 minutes. The GeneRuler 100 bp (Ref# SM0241, ThermoScientific) and 50 bp ladder (Invitrogen, Lithuania, Ref# 10416014) were used as the DNA length reference. PCR amplicons were compared to the DNA ladder by gel scanner (GelDoc, BioRad).

RESULTS

In 2014, the cultivation and sale of walnuts were mainly carried out by subsidiary farms of the population of the Turkestan region [24 Grigoruk V.V., 2016]. According to the Agency on Statistics of the Republic of Kazakhstan, the area of walnut crops in 2017 amounted to 1415.1 hectares [25 Kairova G.N., 2018].

The southern regions of Kazakhstan have agricultural landscapes most suitable for growing nuts. Thanks to the state program ‘Zhangak 2050’ in the territory of Almaty and Turkestan regions, in 2017 private agricultural enterprises are expanding walnut plantations. Thus, more than 95% of the country's walnuts are grown in the Almaty region of the South-Eastern Kazakhstan and Turkestan regions of the South Kazakhstan, as well as a small part in personal household plots of Zhambyl and Kyzylorda regions (figure 1).

Almaty region is located in the extreme south-east of the Republic of Kazakhstan and borders on China and Kyrgyzstan. Along of 17 districts of Almaty region, common known industrial plantations of walnut are locating in Enbekshikazakh and Zhambyl districts (stat.gov.kz).

In the Enbekshikazakh region, 53 km from the city of Almaty, on a mountain plain, there is a peasant farm called ‘Khizhkov’. This territory is characterized by a continental climate, low air humidity, abundant sunshine, short but rather cold winters. The first sowing of J. regia nuts, collected near the city of Kaskelen in Almaty region, was carried out in 2015 on an area of 12 hectares (reported by agronomist Maratzhan Kasymov from Khizhkov company). In 2019, the collection of walnuts in the Khizhkov farm was replenished with varieties from Kyrgyzstan and local genotypes from the Turkestan region. At the moment here in the nursery J. regia trees are from 2 to 5 years old.

Determination of the general degree of freezing of trees is necessary to assess the winter hardiness of walnut seedlings grown in the conditions of South and South-East Kazakhstan [26 Samigullina NS. 2006]. Table 1 presents data from 2018 to 2019, on the study of the biological characteristics of 3-4-year-old walnut trees in the Khizhkov farm.

Figure 1. Map showing south regions of Kazakhstan where is planting a walnut

Table 1. Biological characteristics of walnut's genotypes on plantation of Khizhkov company in 2018 - 2019

	Characteristics, in 2018					Characteristics, in 2019				
Genotype	Age, year	Height, m *	Crown width, m *	Infection	General degree of freezing	Height, m *	Crown width, m *	Infection	General degree of freezing	Fruiting degree
X 1/1	3	3,00	3,00	-	III	3,15	3,23	-	0	1
X 1/2	3	3,00	3,36	-	III	3,26	3,00	-	0	1
X 1/4	3	2,10	2,03	-	III	3,00	3,30	-	I	-
X 1/5	3	2,50	2,09	-	III	3,60	3,30	-	I	-
X 1/7	3	2,50	1,60	-	III	3,16	2,20	-	I	-
X 1/13	3	2,50	1,80	-	III	2,60	1,98	-	I	1
X 1/21	3	2,50	1,30	-	III	2,70	2,90	-	II	-
X 2/1	3	3,50	2,10	-	III	3,80	2,70	-	0	-
X 2/2	3	3,20	1,80	-	III	3,95	4,00	-	0	-
X 2/4	3	N/A	N/A	N/A	N/A	4,10	4,50	-	I	-
X 2/26	3	N/A	N/A	N/A	N/A	3,85	4,20	-	II	-
X 3/2	3	3,50	2,10	-	III	4,15	5,00	-	I	-
X 3/3	3	3,20	1,80	-	III	3,90	4,50	-	II	-
X 3/4	3	N/A	N/A	N/A	N/A	4,10	5,00	-	I	-
X 4/2	3	3,50	3,30	-	III	4,50	5,40	-	0	1
X 4/7	3	N/A	N/A	N/A	N/A	2,90	3,70	-	I	1
X 5/3	3	3,00	3,00	-	III	N/A	N/A	N/A	N/A	N/A
X 5/4	3	3,00	3,36	-	III	3,00	3,50	-	0	-
X 5/5	3	2,10	2,03	-	III	3,90	4,10	-	0	-
X 5/15	3	2,10	2,03	-	III	N/A	N/A	N/A	N/A	N/A
X5/16	3	2,50	1,30	-	III	N/A	N/A	N/A	N/A	N/A
X5/19	3	3,00	3,00	-	III	N/A	N/A	N/A	N/A	N/A
X5/20	3	3,00	3,36	-	III	N/A	N/A	N/A	N/A	N/A
Average	-	2.83±0.110	2.33±0.170			3.53±0.138	3.70±0.235			
SD	-	0.468	0.723			0.567	0.969			
N/A - data not available; - not damaged or no fruits; * - p=0.05; ** - p=0.001										

In total, after the winter period, in 2018 out of 137 seedlings, 118 walnut genotypes had severe and very severe damage, which corresponds to the 4 and 5 degrees of freezing according to the standard scale [21 Program, 1999]. The remaining 19

seedlings had good frost-hardiness, in which there was no freezing of the bark on the trunk and wood, but damaged tree branches were revealed. These 19 studied genotypes had III degree of freezing, in which the damaged branches turn brown and need pruning (figure 2 A, B).

Figure 2. Walnut's local genotypes on plantation of Khizhkov company in 2018

When studying the index of freezing of the nut, it was found that the genotypes X1 / 4, X5 / 5 and X15 / 5, after cutting off the branches, had 9 restored shoots, and the nut X3 / 2 had only 6 additional new shoots. According to the method of assessing the initial material, these local genotypes of J. regia have a high (> 6 points) shoot-forming ability [27, 28 Zhernovoy AS, 1966. Nguyen Thi Tuk, 2015]. All other genotypes of 3-year-old trees had an average shoot-forming ability, having restored 3-5 branches each.

In 2018, in the nursery, the height of 3-year-old trees was within 2.10-3.50 m (2.83 ± 0.110 m in average, SD = 0.468, p = 0.05) and crown width 1.30-3.36 m (2.33 ± 0.170 m in average, SD = 0.723, p = 0.05). In addition, depending on the genotype of the nut, the annual growth of tree branches was 112-213 cm (153.05 ± 9.001 cm in average, SD = 38.187, p = 0.001), while the trunk diameter of the trees was 7-16 cm (9.32 ± 0.527 cm in average, SD = 2.237, p = 0.05). In 2019, the trees under study had a height of 2.60-4.50 m (3.53 ± 0.138 m in average, SD = 0.567, p = 0.05) and a crown width from 1.98 to 5.40 m (3.70 ± 0.235 m in average, SD = 0.969, p = 0.05) (table 1).

The annual growth of tree branches in 2019 was 70-315 cm (190.44 ± 15.635 cm in average, SD = 64.466, p = 0.001).

Compared to 2018, the next year the overall degree of freezing of branches was lower (I-II degree), while 7 nut genotypes X1 / 1, X1 / 2, X2 / 1, X2 / 2, X4 / 2, X5 / 4 and X5 / 5 were completely free of damage, which is associated with the warmer winter of 2019. So, according to the data of the meteorological station (WH-1081PC Wireless Weather Station, China), located near the Khizhkov farm, in January and February 2018 the average monthly temperature was -9.40C and -2.50C, while in 2019 the temperature was -4, 50C and -6.20C, respectively. In Kazakhstan, the winter of 2018 compared to 2019 was very cold, so even in the southeastern part of the country (in the Almaty region), the daily air temperature dropped to -320C. As a result, in 2018, all Chandler walnut seedlings (private communication with agronomist of the company) died on the plantations of the Limited Liability Partnership 'Baimene' located on the outskirts of the city of Almaty (former state farm Alatau).

As you know, according to the time when nut varieties enter fruiting, they are divided into early-fruiting (entering the fruiting phase before 6 years of age), with an average fruiting period (at the age of 6-8 years) and late entering fruiting (more than 8 years) [21 Program, 1999]. Phenological observations in 2019 made it possible to classify local genotypes X1 / 1, X1 / 2, X1 / 13, X4 / 2 and X4 / 7 as early-growing trees. At the age of 4 years, these nut genotypes entered the fruiting phase and gave the first harvest in the range of 0.09-0.18 kg of nuts per tree.

In Kazakhstan, in terms of walnut cultivation area, the second place is occupied by the Turkestan region (formerly South Kazakhstan region). It is believed that the foothills of the Tyulkubas and Tolebi regions are favorable for walnuts, since here in winter the temperature is higher than the critical level and low wind activity [25 Kairova G.N., 2018]. In addition to walnuts, pistachios, almonds, hazelnuts and pecans are widely grown on an area of about 400 hectares in the Turkestan region in private subsidiary farms [www agroinfo.kz].

Limited Liability Partnership 'Saryagash Zher Syiy' (Saryagash Zher Syiy) has plantations of walnuts and other fruit crops, which are located 106 km from the city of Shymkent, in the Saryagash district, Turkestan region (figure 3).

Figure 3. Walnut's forms on plantation of Saryagash Zher Syiy in 2018 (T. Yegizbayeva on the photo)

Local varieties and genotypes of walnuts grow here, as well as young seedlings brought from Iran, China, Kyrgyzstan, Moldova, Russia and Turkey. The age of walnut trees cultivated in Limited Liability Partnership 'Saryagash Zher Syy' is 2-17 years. In 2015, 200 walnut seedlings brought

from Turkey were planted on a pilot site. In the spring-summer period of 2019, a study of the biometric indicators of these Turkish varieties of walnut was carried out (table 2).

Table 2. Biological characteristics of walnut's genotypes on plantation of Sary-agash Zher Syiy company in 2019

Genotype	Age, year	Characteristics			Infection	Fruiting degree	General degree of freezing
		Height, m	Barrel diameter, m	Crown diameter, cm			
FT1/2	5	3,30	7	1,50	-	2	I
FT1/13	5	2,10	8	1,10	-	3	I
FT2/12	5	2,20	5	1,00	-	3	I
FT2/14	5	2,10	5	1,10	-	2	I
FT3/2	5	3,00	7	2,10	-	2	I
FT4/10	5	2,20	5	0,80	-	3	I
FT4/11	5	2,60	9	1,70	-	3	I
FT4/12	5	2,70	7	1,80	-	3	I
FT5/3	5	3,60	11	2,60	-	2	I
FT5/6	5	3,70	7	1,90	-	2	I
FT5/8	5	2,80	6	1,50	-	3	I
FT5/10	5	3,30	10	2,10	-	3	I
FT5/15	5	2,20	6	1,10	+	3	I
FT6/3	5	2,80	9	1,70	-	2	I
FT6/12	5	2,50	7	2,80	-	2	I
FT6/15	5	2,00	5	0,90	-	2	I
FT7/2	5	3,30	5	0,40	-	2	I
FT7/16	5	2,80	4	0,50	-	3	I
FT7/20	5	3,00	12	2,40	-	2	I
FT8/8	5	2,50	10	1,80	+	2	I
FT8/9	5	1,90	7	1,90	+	3	I
FT8/12	5	2,60	9	1,60	+	3	I
FT8/15	5	2,70	6	1,80	+	2	I
FT8/18	5	3,20	8	2,20	+	2	I
Average		2.71±0.107	7.29±0.441	1.60±0.132			
SD		0.513	2.116	0.631			
+ damaged; - not damaged or no fruits							

In 2019, almost all Turkish cultivars were found to have I degree of winter hardiness, that is, very weak freezing at which apical buds and male catkins are damaged in a small part (about 10%) of fruit shoots [21 Program, 1999]. Of these 200 walnut trees, only 24 genotypes were selected for study, which at the age of 5 began to bear fruit (early-fruiting walnut). As shown in table 2, these early fruiting forms had low to medium fruiting (grades 2 and 3, respectively). However, diseases have been found in some Turkish walnut genotypes (see Figure 4). Visual assessment (information from an expert in plant protection) of trees FT5 / 15, FT8 / 8, FT8 / 9, FT8 / 12, FT8 / 15, FT8 / 18 showed the presence of diseases marssoniosis and bacteriosis [29 Khokhryakov MK, 2003].

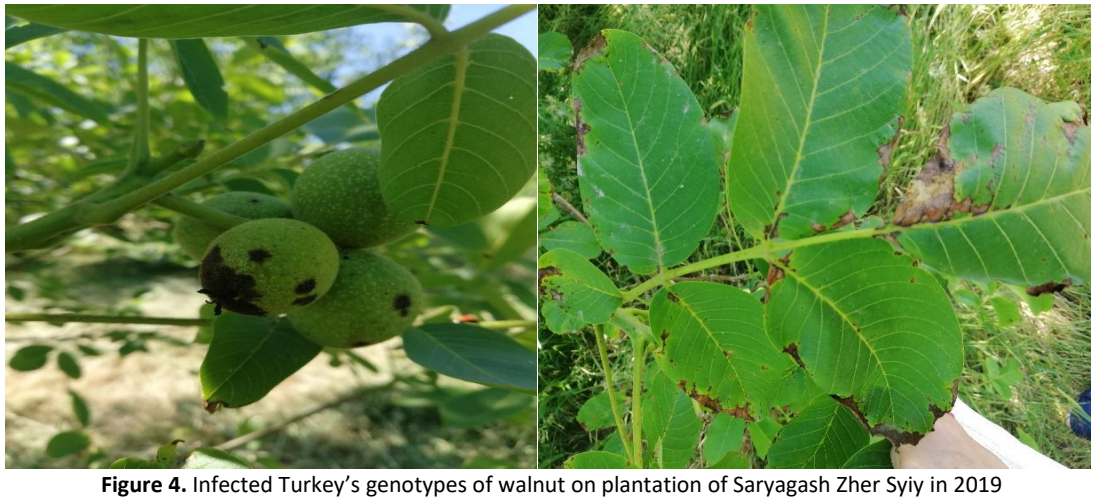


Figure 4. Infected Turkey's genotypes of walnut on plantation of Saryagash Zher Syiy in 2019

From table 2 it can be seen that in this nursery the height of 5-year-old J.regia trees was within 1.90-3.70 m (2.71 ± 0.107 m in average, SD = 0.513), and the crown width was 0.40-2, 80 m (1.60 ± 0.132 m in average, SD = 0.631). At the same time, the diameter of the trunk (stem) in Turkish genotypes was 4-12 cm (7.29 ± 0.441 cm in average, SD = 2.116).

SSR microsatellite markers are widely used to identify walnut plants [16 Dangl G.S., 2005, 22 Foroni I., 2006, 23 Ruiz-Garcia L., 2011]. Wegrzyn J.L. and his colleagues found that Juglans regia contains a large number of microsatellite

loci in comparison with other plant species [30 Wegrzyn, J.L., 2014]. Therefore, for microsatellite analysis of local walnuts, plant material of various genotypes cultivated in the companies Khizhkov and LLP Saryagash Zher Syyy was collected.

To optimize the reaction (Polymerase chain reaction, PCR diagnostics) and amplification of the WGA001, WGA005, WGA09, WGA027, WGA069 and WGA202 loci, a simple method (Polymerase chain reaction, PCR diagnostics) was used with a gradient of primer annealing temperatures (primer annealing) within 49- 630C (data not shown). The gel electrophoretogram (figure 5) shows that (Polymerase chain reaction, PCR diagnostics) amplification of the WGA202 locus proceeds well at the entire temperature range of 58-630C.

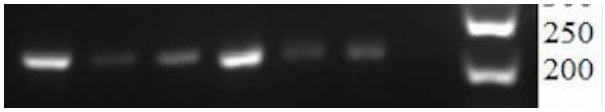


Figure 5. Gel lectrophoresis of gradient-PCR amplified loci WGA202 in walnut T7-10 genotype: 1-10 tracks – at t = 58; 58,6; 58,9; 59,7; 60,3; 60,7; 61,4; 62,1; 62,6 and 630C, relatively, K – negative control, M - GeneRuler 100 bp

Figure 6 shows the result of a simple (Polymerase chain reaction, PCR diagnostics) with amplification of the WGA202 locus at a primer annealing temperature of 580C (primer annealing temperature). Specific and unique alleles of loci WGA220 were observed in these twelve and others walnut genotypes growing on plantations of Khizhkov and Saryagash Zher Syiy companies. However, we can see that the fractionation of SSR-PCR products in 2% standard agarose gels was not very well because it is impossible to distinguish DNA fragments with very close sizes.

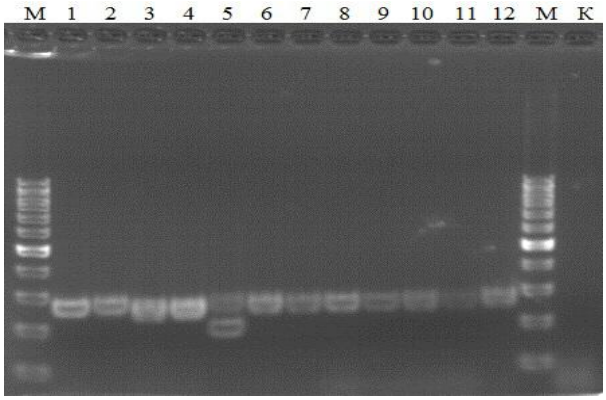


Figure 6. Gel electrophoresis of PCR amplified loci WGA202 in walnut genotypes: 1 - x8/3, 2 - x6/2, 3 - x2/2, 4 - x5/3, 5 - x7/6, 6 - x4/2, 7 - x1/1, 8 - x1/2, 9 - x2/1, 10 - x3/1, 11 - x3/2, 12 - T2-10, respectively. K – negative control, M - GeneRuler 100 bp (ThermoScientific)

Using a simple method (Polymerase chain reaction, PCR diagnostics) of the WGA005 locus and annealing temperature of 580C (primer annealing temperature), we obtained DNA fragments for six Kazakh walnut genotypes x8 / 3, x6 / 2, x2 / 2, x5 / 3, x7 / 6 and x4 / 2 (figure 7). Further separation (Polymerase chain reaction, PCR diagnostics) of - products was performed in 3% gel of MetaPhore agarose. Generally, loci WGA005 of 20 local Kazakh and foreign walnut genotypes were amplified with sizes of PCR products ranging from 240 to 252 bp as mentioned by Foroni I. [22 Foroni I., 2006].

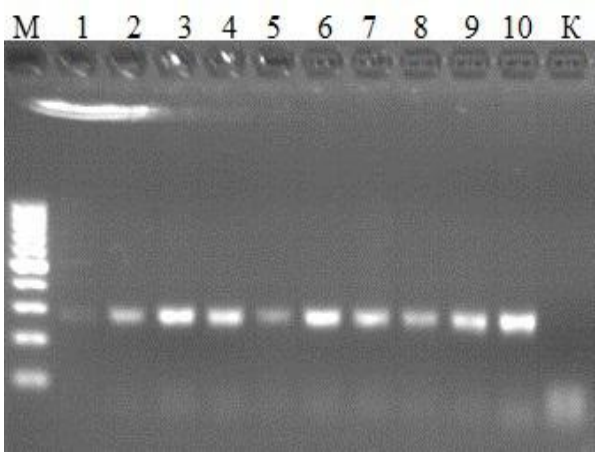
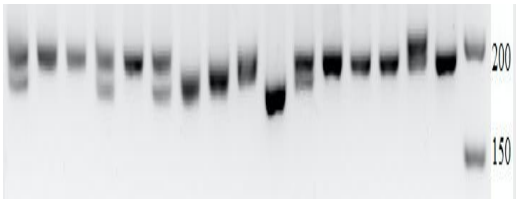


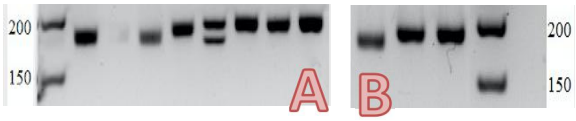
Figure 7. Fractionation of WGA005 PCR products in 3% of MetaPhore agarose. Plant samples: 1 - x8/3, 2 - x6/2, 3 - x2/2, 4 - x5/3, 5 - x7/6, 6 - x7/6, 7 - x4/2, M - 50 bp DNA ladder (Invitrogen) (from left to right)

In next series of our experiments, 35 cycles of the touch down PCR consisted of annealing temperature 580C at 45 sec for 1st cycle with reducing by 0.20C on following 2-15 cycles, and continuing by 550C at 45 sec for next 20 cycles, were carried out to anneal the primer pairs specific to loci WGA001, WGA009 and WGA069 [23 Ruiz-Garcia L., 2011]. The fractionation of obtained PCR products was done by using 3% Metaphore agarose gel. As shown in figures 8, 9A and 9B, the PCR protocol mentioned by Ruiz-Garcia L., allowed to amplify loci WGA001 and give specific amplicons for the each studied walnut genotype [23 Ruiz-Garcia L., 2011]. In this case however, additional DNA products with sizes about 250 bp and 400 bp for loci WGA001 and WGA009, respectively were observed but that are not clear visible (data not shown). The studied 27 of local Kazakh and foreign Turkish and Chinese walnut genotypes had seven alleles of loci WGA001 ranging in 178-192 bp (see fig. 8, 9A



and 9B).

Figure 8. Fractionation of WGA001 PCR products in 3% of MetaPhore agarose. Plant samples: 1 - x1/1, 2 – x1/2, 3 – x2/1, 4 – x3/1, 5 – x3/2, 6 – T2-10, 7 – T7-10, 8 – x6/2, 9 – x2/2, 10 – x5/3, 11 – x7/6, 12 – x4/2, 13 - Ch01, 14 - Ch02, 15 - Ch03, 16 – Ch04, M - 50 bp DNA ladder (Invitrogen)



(from left to right)

Figure 9. Fractionation of WGA001 PCR products in 3% of MetaPhore agarose. 9A. Plant samples: 1 - FT8/9, 2 - FT7/2, 3 - FT7/16, 4 - FT5/10, 5 - FT4/11, 6 – FT1/13, 7 – Kz11, 8 – FT12, M - 50 bp DNA ladder (Invitrogen). 9B. Plant samples: 1 -Kz19, 2 -Kz20, 3 – Kz21, K – negative control, M - 50 bp DNA ladder (Invitrogen) (from left to right)

It is seen in 10A, 10B and 11 figures, that the same PCR protocol allowed to amplify loci WGA009 in 25 walnut

genotypes. WGA009 DNA amplification of local x1/1 and x1/2 genotypes was repeated twice with the same results (fig. 10A and 11) whereas the second repeat of PCR reaction gave an amplification of Turkish FT7/16 genotype and only DNA of FT7/2 genotype had no been amplified (fig. 10B and 11). In totally, the four different alleles 237-252 bp in length were found for these local and foreign genotypes.

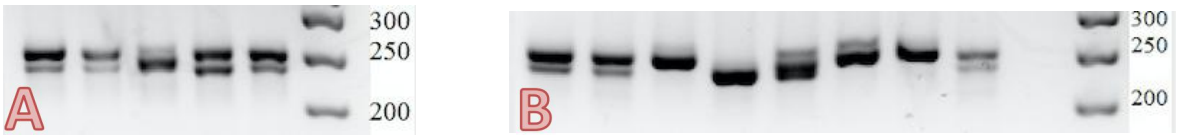
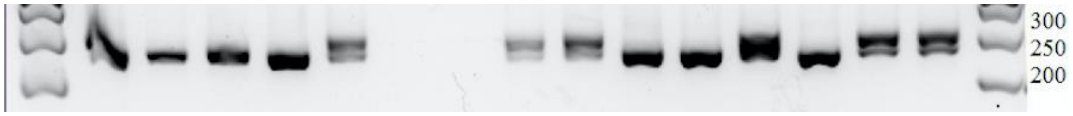
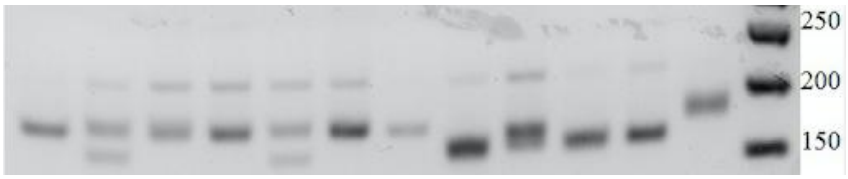


Figure 10. Fractionation of WGA009 PCR products in 3% of MetaPhore. 10A. Plant samples: 1 - x1/1, 2 – x1/2, 3 – x2/1, 4 – x3/1, 5 – x3/2, M - 50 bp DNA ladder (Invitrogen). 10B. Plant samples: 1 – T2-10, 2 – T7-10, 3 – x6/2, 4 – x2/2, 5 – x5/3, 6 – x7/6, 7 – x4/2, 8 – FT7/16, K - negative control, M - 50 bp DNA ladder (Invitrogen)

Figure 11. Fractionation of WGA009 PCR products in 3% of MetaPhore agarose. Plant samples: 1 - Ch01, 2 -Ch02, 3 - Ch03, 4 - Ch04,



5 - FT8/9, 6 - FT7/2, 7 - FT7/16, 8- FT5/10, 9 - FT4/11, 10 – FT1/13, 11 – Kz11, 12 – FT12, 13 – Kz22, 14 - x1/1, 15 – x1/2, M - 50 bp



DNA ladder (Invitrogen)

In touch down PCR for 26 walnut genotypes there was identified 5 alleles of loci WGA069 as shown in figures 12 and 13. The size of WGA069 alleles was in range 160-184 bp. Under using this PCR protocol, nonspecific DNA fragment about 220 bp in length was observed for loci WGA069. It is suggested that PCR protocol for loci WGA069 should be more properly optimized.

Figure 12. Fractionation of WGA069 PCR products in 3% of MetaPhore agarose. Plant samples: 1 - x1/1, 2 – x1/2, 3 – x2/1, 4 – x3/1, 5 – x3/2, 6 – T2-10, 7 – T7-10, 8 – x6/2, 9 – x2/2, 10 – x5/3, 11 – x7/6, 12 – x4/2, M - 50 bp DNA ladder (Invitrogen)

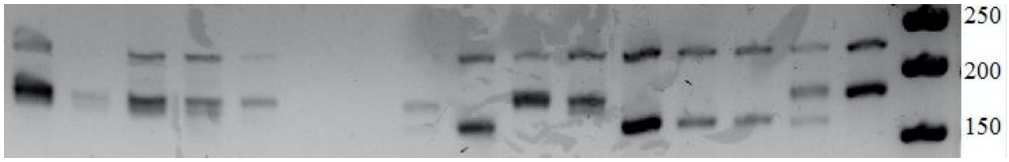


Figure 13. Fractionation of WGA069 PCR products in 3% of MetaPhore agarose. Plant samples: 1 - Ch01, 2 -Ch02, 3 - Ch03, 4 - Ch04, 5 - FT8/9, 6 - FT7/2, 7 - FT7/16, 8- FT5/10, 9 - FT4/11, 10 – FT1/13, 11 – Kz11, 12 – FT12, 13 - Kz19, 14 - Kz20, 15 - Kz21, 16 - Kz22, M - 50 bp DNA ladder (Invitrogen)

CONCLUSIONS AND DISCUSSIONS

Among the nut-bearing plants, the Juglans regia walnut is the most common crop that has long been growing in the southern regions of Kazakhstan [1 Kairova M., 2019]. Despite the contradictory data on the naturalness of walnut populations in Kazakhstan, scientists believe that polymorphic plants of the Sairam-Ugam area, located in the Turkestan region, are of great importance for selection and genetic research [15 Dzhangaliev AD, 2008]. Thanks to the state programs for the development of nut and berry industries 'Zhаңғақ-2050' and 'Zhidek-2050' in the territory of Almaty and Turkestan regions, private agricultural enterprises have created J. regia walnut plantations. In 2018-2019. a study of the biological characteristics of the growth and development of varieties and genotypes of walnut cultivated in two farms Khizhkov and Saryagash Zher Syiy was carried out.

In 2018, on the Khizhkov farm, out of 137 seedlings of local walnuts, 19 genotypes had good frost-hardiness with III degree of freezing of the branches. After pruning of damaged branches, walnut genotypes X1 / 4, X5 / 5, X15 / 5 and X3 / 2 showed a high shoot-forming ability. In the nursery, the height of 3-year-old trees was within 2.10-3.50 m (2.83 ± 0.110 m in average, SD = 0.468, p = 0.05) and the crown width was 1.30-3.36 m (2.33 ± 0.170 m in average, SD = 0.723, p = 0.05). In the next year, the studied genotypes were characterized by the I-II degree of freezing of branches, while 7 local genotypes X1 / 1, X1 / 2, X2 / 1, X2 / 2, X4 / 2, X5 / 4 and X5 / 5 completely there was no damage. This is possibly due to the warmer winter of 2019, so according to the Weather Station, the average monthly temperature in January 2018 was -9.40C, while in 2019 this figure was two

times lower (-4.50C). In addition, three of these 7 local genotypes X1 / 1, X1 / 2, X4 / 2, as well as X1 / 13 and X4 / 7 are attributed to early-fruiting nuts. In 2019, the trees under study had a height of 2.60-4.50 m (3.53 ± 0.138 m in average, SD = 0.567, p = 0.05) and a crown width from 1.98 to 5.40 m (3.70 ± 0.235 m in average, SD = 0.969, p = 0.05).

In 2015, 200 annual seedlings of Turkish varieties J. regia were planted on the experimental plot in Sary-agash Zher Syiy. In 2019, all 200 Turkish walnut genotypes had I degree of winter hardiness, with very little freezing of fruit shoots. Among Turkish accessions, only 24 genotypes at the age of 5 began to bear fruit and had 2 and 3 degrees of fruiting. However, visual inspection showed the presence of marssoniosis and bacteriosis in six Turkish accessions FT5 / 15, FT8 / 8, FT8 / 9, FT8 / 12, FT8 / 15 and FT8 / 18 [29 Khokhryakov MK, 2003]. In this nursery, the height of 5-year-old J. regia trees was within 1.90-3.70 m (2.71 ± 0.107 m in average, SD = 0.513), and the crown width was 0.40-2.80 m (1.60 ± 0.132 m in average, SD = 0.631).

Based on the study of biometric indicators of genotypes cultivated in both private plantations, it can be seen that walnut trees in Khizkov farm in height, crown width and trunk diameter significantly exceed Turkish genotypes from Limited Liability Partnership Saryagash Zher Syiy. This is despite the fact that in 2019 the studied Kazakh nut seedlings were 4 years old, and Turkish varieties were 5 years old. These differences, of course, can be associated with the fact that Kazakh walnut genotypes were obtained by planting the original seed material, which is more adapted to local soil and climatic conditions, while Turkish varieties were brought and planted as 1-year-old seedlings. In general, the researchers showed that the J. regia species have a very high winter hardiness in comparison with other types of nuts, and for the selection of promising forms for winter hardiness and other characteristics, it is necessary to study their natural populations [28 Nguyen Thi Tuk, 2015].

Microsatellite markers were used to study the genetic diversity of the walnut growing in the southern regions of Kazakhstan [22 Foroni I., 2006]. In microsatellite analysis of J. regia plants, a different methodological approach is used, which depends on the equipment of laboratories with modern equipment and reagents used [16 Dangl G.S., 2005, 17 Bernard A., 2018]. In our studies, we used two methods - simple PCR and touch down PCR.

A gradient PCR reaction was carried out to select the optimal annealing temperature for primers specific to 5 microsatellite loci WGA001, WGA005, WGA009, WGA069, and WGA202. In simple PCR technique, 3 alleles of loci WGA005 were amplified in 20 local Kazakh and foreign walnut genotypes with sizes of PCR products ranging from 240 to 252 bp [22 Foroni I., 2006, 31 Ahmed N., 2012]. Previously described the touch down PCR was used to amplify loci WGA001, WGA009 and WGA069 in different local Kazakh and foreign walnut genotypes [23 Ruiz-Garcia L., 2011]. The studied 27 of local Kazakh and foreign Turkish and Chinese walnut genotypes had seven alleles of loci WGA001 ranging in 178-192 bp. The same PCR protocol allowed to amplify loci WGA009 and the four different alleles 237-252 bp in length were found in the 25 local and foreign genotypes. In touch down PCR for 26 walnut genotypes there was identified 5 alleles of loci WGA069 with size in range 160-184 bp. The five alleles of loci WGA202 were found in 29 local Kazakh and foreign walnut genotypes with sizes of PCR products ranging from 259 to 295 bp [22 Foroni I., 2006]. However, all studied walnut accessions had clear unspecific DNA-amplification in the touch down PCR for loci WGA069. It is suggested to optimize an annealing temperature of primer pair specific to loci WGA069.

Thus, depending on the primer pairs used and the walnut genotype, the sizes of the allelic loci WGA001, WGA005, WGA009, WGA069, and WGA202 varied within 178-192 bp, 240-252 bp, 237-252 bp, 160-184 bp and 259 to 295 bp, respectively. An expanded study of other SSR loci in local Kazakh genotypes will provide a complete picture of the genetic diversity of the J. regia nut, which has long been growing along with Petunnikov's almonds, relict and Red Book pistachio Pistacia vera L. on the territory of Sairam-Ugam State National Natural Park, as well as in mountain forests of the Syrdarya Karatau in the Western Tien Shan [32 Chekalin S.V., 2011]. In addition, the study of the biological characteristics of natural populations of J. regia walnut is of relevance in the selection of winter-hardy genotypes and the successful conduct of selection tests.

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CONFLICT OF INTEREST

Authors declared not conflict of interests.

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