International Journal of Modern Agriculture, Volume 10, No.1, 2021
ISSN: 2305-7246

GENETIC DIVERSITY OF BUXUS HYRCANA (POJARK) POPULATIONS USING RAPD MOLECULAR MARKERS

Mana Hosseini, Reza Azizinejad and Vahid Zarrinia

[1] M.A. Student of Agricultural Biotechnology, Department of Biotechnology and Breeding, Faculty of Agricultural Sciences and Food Industry, Science and Research Branch, Islamic Azad University, Tehran, Iran.
[2] Assistant Professor, Department of Biotechnology and Breeding, Faculty of Agricultural Sciences and Food Industry, Science and Research Branch, Islamic Azad University, Tehran, Iran.
[3] Assistant Professor, Department of Plant Pathology, Faculty of Agricultural Sciences and Food Industry, Science and Research Branch, Islamic Azad University, Tehran, Iran.

Abstract

Categorized in the *Buxaceae* family and known as a broad-leaved and evergreen, *Buxus hyrcana Pojark* is one of the most valuable native shrubs in the Hyrcanian forests in northern Iran. However, fire blight disease which is a dangerous disease, threatens the biological survival of this native plant, exposing it to the perils of extinction. In this study, the genetic diversity of 72 plant species collected from 4 regions of Neka, Varki, Gileh and Rostamkola was examined respectively based on their RAPD molecular markers. As the results from molecular studies and software analysis suggested, a great deal of genetic affinity was noted between populations 1 and 4 (Neka and Rostamkola) and populations 2 and 3 (Varki and Gileh). Also, the variance analysis showed that 4% diversity was seen in the population species while 96% of this diversity was seen in the population species. In fact, genetic diversity in the populations was very low and the highest diversity in the species was noted in each population, which, considering the intrinsic or extrinsic resistance in some *Buxus hyrcana Pojark* species against the fire blight disease, contribute significantly to identifying resistant species of this plant against this disease.

Keywords; Boxwood blight, Genetic diversity, *Buxus hyrcana Pojark*, Intrinsic resistance, RAPD.

Introduction

The hyrcanian region is a green strip of long-lived forests, located on the northern Mt. Alborz ranges, along southern boundaries of the Caspian Sea spanning the northern provinces of Iran such as Gilan, Mazandaran and Golestan. From biodiversity and vegetation point of view, the Hyrcanian region is very valuable and is exclusively home to well-known native species such as *Buxus hyrcana Pojark*. The Hyrcanian forest, nationally important, also faces many perils threatening the vegetation and ultimately biodiversity of this region (Tohidifar, et al, 2016). For example, the pathogen fungus Bocxwood blight in *Buxus hyrcana Pojark*, scientifically known as *Cylindrocladium buxicola* (Henricot, B. and Culham, A., 2002), causes serious damages to this plant in the Hyrcanian forests, endangering its genetic diversity (Esmaeilnezhad, et al, 2020). Boxwood blight kills young plants and weakens old plants (Shishkoff, et al. 2015). The disease usually appears as dark spots on *Buxus* leaves and stems, causing the leaves to deform and the plant to die, so identifying *Buxus* species resistant to fire blight is necessary to protect the plant's survival (Kramer, et al.) al, 2020). Also, susceptibility diversity between and within different *Buxus* species is one of the biggest challenges when identifying sources of resistance to this disease (LeBlanc, et al, 2018).

One of the appropriate ways to study the genetic diversity of *Buxus hyrcana (Pojark)* populations is via molecular markers. These markers help us better understand the distribution and extent of genetic variation within and between species and serve as valuable tools for assessing plant genetic resources (Porth, I. and El-Kassaby, YA, 2014). Most molecular studies examine genetic relationships, the extent of genetic diversity and differentiation in *Buxus hyrcana Pojark* species (Esmaeilnezhad, et al, 2020).

Since boxwood blight is a dangerous disease and threatens the biological survival and longevity of this native shrub, this study which concerned the genetic diversity of *Buxus hyrcana Pojark*, aimed to assess the relationship between phenotypic traits of resistance to this disease with a resulting pattern of genotypic diversity in the *Buxus hyrcana Pojark* populations using RAPD markers.

Materials and Procedure:
To conduct this research, 4 regions of Neka, Varki, Gileh and Rostamkola were selected. Leaves of young tree trimmings with healthy appearance and identical crown height from different areas under study were taken as samples and rootstocks were randomly selected in each area. The species were completely healthy free of any diseases showing no signs of disease for the past six months. Although the distance between the trees did not follow a specific pattern, the average distance was about 20 meters. In order to extract DNA and determine genetic diversity using RAPD molecular markers, the leaves collected from each rootstock were placed in aluminum foils inside a liquid nitrogen tank and transferred to a laboratory and kept at -80°C until the extraction stage. Then, in the laboratory, as many as 18 species from each region and a total of 72 plant species from these four regions were used for this research.

DNA Extraction:
Transformed C-TAB method was used for DNA extraction. In this way, the dry material and then the solution were poured into a pre-autoclaved glass and shaken until completely dissolved. Then it was placed inside a laboratory water bath and after that, the samples were taken out of the -80°C freezer (in the shortest possible time) and 700 ml of extraction buffer was added to each sample. The tubes were capped and shaken inversely to get a green mass remain inside. The tubes were then placed in a rack and placed in the water bath of 65°C for one hour. In this part, it was better for the water bath to be equipped with a stirrer, otherwise, once every 15 minutes for an hour, the rack could be removed from the water bath with the tubes shaken inversely by hand. At the water bath timing ended, the samples were placed in powdered ice for 5 minutes. A chloroform/isoamyl 24:1 solution (24 chloroforms and 1 isoamyl) was already prepared in advance (Isoamyl was used to prevent chloroform foaming). After 5 minutes, as the samples were in ice, 700 ml (blue tip) of chloroform/isoamyl solution was added to the tubes. The tubes were put inside the rack and placed on a stirrer at 182 to 150 rpm for 30 minutes. The tubes were then centrifuged at 12,000 rpm for 20 minutes. As the previous stage ended, the samples were observed in two phases; the inferior phase turning dark green and the supernatant phase oily and pale. Then, with great care and without shaking the tubes, the supernatant phase was carefully separated using a blue sampler tip and placed tip inside a new tube of the same name. Then 650 µl of 100% Isopropanol was added to each tube. (100% Isopropanol causes DNA deposition). The tubes were capped and shaken inversely 10 times. The tubes were re-centrifuged at 12,000 rpm for 30 minutes. After the centrifuging stage, the supernatant phase of the tubes was slowly discarded (Pellets should not be discarded in here). Then 650 µl of 70% alcohol was added to each tube and the samples were centrifuged at 12,000 rpm for 12 minutes. This was repeated, then the supernatant alcohol phase was separated and the tube was exposed to air for 30 minutes to 1 hour where it couldn't be subjected to traffic in order that the pellets would dry.
In the last step, 200 µl of TE buffer was added to each tube, and at the end, the samples were refrigerated at 4°C for one night, and transferred to a -20°C freezer the next day. However, for quality assessment, the extracted DNAs were taken on 0.8% agarose gel and the quality of the DNA was ensured.

Polymerase Chain Reaction (PCR):
AMPLIQON company kit (PCR master mix (2X) RED kit) was used to prepare the PCR reaction mixture. Materials used in a PCR reaction (7.5 µl of PCR master mix, 1.5 µl of primer, 2 µl of DNA, 4 µl of H2O) made the mixture. PCR was performed using the BIO RAD T100 thermal cycler. After the PCR step, the 1.5% gel was prepared for electrophoresis, then 3-5 µl of each sample was stained with 2 µl of loading buffer (under buffer conditions (TBE 1X) and the product was electrophoresed on 0.1% agarose gel with a voltage of 114 V. After the electrophoresis, the gels were photographed with a UV trans-illuminator.
The steps and conditions of polymerase chain reaction are listed in Tables (1 to 3):

Table 1 - Materials and concentrations required for PCR

Material	Base concentration	Amount for each 15 µl reaction
Master Mix	2X	7.5 µl
Primer	3 µmol	1.5 µl
DNA	4 nanograms	2 µl
ddH2O		4 µl
Total		15 µl

Table 2 - Steps, heat and time required in PCR

Stage conducted	Heating	Timing
Denaturation	94	3mins
Denaturation	94	30 sec.
Anling temperature	32	1 min
Propagation temperature	72	2 mins
Back to stage 2 (39 times)		
Final expansion	72	5 mins
Storage	20	Indefinite

Table 3 - Name and sequence of primers used

No.	Sequence (3.....5)	Primers	Connection temperature
1	CATCCCCCTG	OPB03	36
2	TGCGCCCTTC	OPB05	36
3	GGTGACGCAG	OPB07	36
4	TGGGGGACTC	OPB09	36
5	GGACCCAACC	OPD02	36
6	TGAGCGGACA	OPD05	36
7	TTGGCACGGG	OPD07	36

Since the data were qualitative, to score the data from the electrophoresis, the bands were scored in form of 0 (no band) and 1 (presence of band). The number and percentage of polymorphic markers, polymorphism information, Nei's genetic diversity (h), Shannon's index (I), Nei's genetic distance (D), total genetic diversity (H_t), diversity between populations (G_{st}), and diversity within populations (H_{st}) were calculated using PopGene Version 1.32 software.

Ntsys Version PC-2.02e software was used to analyze the clusters of samples within the studied habitats. Also, Gen Alex software and Mantel test were performed to confirm the reliability of the dendrogram shape from Ntsys.

Results:
Of the 7 RAPD markers used, all 7 primers were able to generate a scoring banding pattern. An example of an electrophoretic pattern of one of these primers is shown in Figure (1).

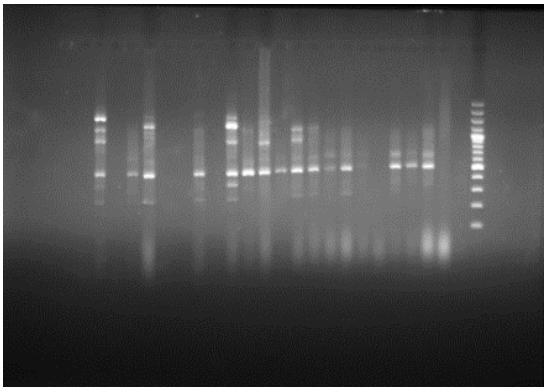

Figure 1 - Electrophoretic pattern of primer OPB03 in samples of Buxus population in Neka

In the first part, the four regions studied, i.e.: Neka, Varki, Gileh and Rostamkola were examined in terms of polymorphic position and the following results were obtained in terms of polymorphic samples of each region. (Table 4)

Table 4 - Number and percentage of polymorphic loci

Name of the region	Number of polymorphic loci	Percentage of polymorphic loci
1- Neka	90	91.98
2- Varki	85	86.73
3- Gileh	86	87.76
4- Rostamkola	92	93.88
5- Total	98	100

The mean Nei's genetic index was 28.30, and the mean Shannon's diversity index was 43.73. The highest Shannon and Nei's genetic index pertained to the Neka population (Table 5)

Table -5: Minimum, maximum and mean Nei 's genetic index and Shannon's diversity index

	Shannon's index (I)%			Nei's generic diversity (h) (%)		
Population	Min.	Max.	Mean	Min.	Max.	Mean
Neka	5.48	49.85	29.07	12.83	69.17	44.14
Varki	5.19	49.97	28.28	12.30	68.98	42.87
Gileh	5.48	49.85	24.65	12.83	69.17	38.36
Rostamkola	5.48	49.85	26.25	12.83	69.17	41.00
Total		28.30			43.73	

Based on the Nei's genetic affinity coefficient, the four populations under study had a great deal of genetic affinity, among which two populations of 1 and 4 (Neka and Rostamkola) and two populations of 2 and 3 (Varki and Gileh) were mostly affinitive to each other (Table 6).

Table 6 - The extent of genetic distance and affinity between populations using the Nei's genetic index (1978)

POP	Neka	Varki	Gileh	Rostamkola
Neka	***	0.966	0.970	0.985
Varki	0.033	***	0.987	0.974
Gileh	0.0297	0.0131	***	0.982
Rostamkola	0.0143	0.0259	0.0172	***

Based on cluster analysis by UPGMA method, two populations of 1 and 4 (Neka and Rostamkola) were placed in a group together, indicating their greater genetic affinity, and two populations of 2 and 3 (Verki and Gileh) were placed in a separate group.

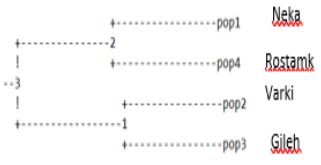

Figure 2 - Grouping of four populations studied by UPGMA method based on the Nei's genetic distance

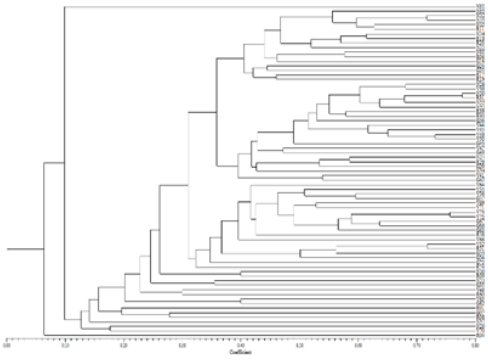

Figure 3 - Grouping of samples of four populations of Neka (V), Varki (V), Gileh (G) and Rostamkola (R) based on Euclidean Affinity coefficient

Discussion:

Because of the importance and national status of Buxus hyrcana Pojark as a native plant and the need to preserve it biologically, the genetic diversity of *Buxus hyrcana* populations collected from four regions of Neka, Varki, Gileh and Rostamkola in the northern forests of Iran was examined using RAPD molecular markers. In general, according to the results from laboratory studies, a great genetic affinity was observed between two populations of 1 and 4 (Neka and Rostamkola) and populations of 2 and 3 (Verki and Gileh), and also according to software analysis, 4% of the diversity between the populations and 96% of this diversity were observed among the species.

According to the studies conducted in this field (Kandahari et al., 2013), an examination of genetic diversity of *Buxus hyrcana* populations in forest areas of the northern region using RAPD molecular markers, indicated greater intra-population genetic diversity in total genetic diversity (90% to 10%) than inter-population genetic diversity; this is while studies confirmed the use of RAPD markers to assess the genetic population of *Buxus hyrcana* species in the northern forests of the country to be an effective tool. Also, in another study by Shanjani, et al. (2018), the examination of genetic differentiation between healthy species and those affected by boxwood blight suggested various amounts of genetic changes in the tested Buxus genome sequence, targeted and used by RAPD and ISSR markers. They also stated that genetic diversity was relatively greater with the greatest diversity

occurring within the population. Significant intra-population diversity and greater genetic diversity in healthy trees compared to affected ones suggested that efforts should focus on conserving the remaining trees in each population. Moreover, the examination of the effect of blight disease on the physiological and genetic traits of *Buxus* susceptibility can lead to the identification of new mechanisms that may be useful for creating resistance against living stresses.
Also, the variance analysis showed that 4% diversity was seen in the population species while 96% of this diversity was seen in the population species. In fact, genetic diversity in the populations was very low and the highest diversity in the species was noted in each population, which, considering the intrinsic or extrinsic resistance in some *Buxus hyrcana Pojark* species against the boxwood blight disease, contribute significantly to identifying resistant species of this plant against this disease.

Acknowledgments
This article is the result of a Master's thesis. Therefore, the authors express their gratefulness to the staff at the Zakaria Razi Laboratory Complex, especially the Biotechnology Laboratory at the Islamic Azad University, Science and Research Branch of Tehran.

References

1. Tohidifar, M., Moser, M., Zehzad, B. and Ghadirian, T., 2016. BIODIVERSITY OF THE HYRCANIAN FORESTS.
2. Henricot, B. and Culham, A., 2002. Cylindrocladium buxicola, a new species affecting Buxus spp., and its phylogenetic tatus. Mycologia, 94(6), pp.980-997.
3. Esmaeilnezhad, K., Mehrjerdi, M.Z. and Neghab, M.G., 2020. Genetic diversity and structure of Buxus hyrcana (Pojark) populations in the Caspian forests of northern Iran revealed by ISSR markers. Biologia, pp.1-10.
4. Shishkoff, N., Daughtrey, M., Aker, S. and Olsen, R.T., 2015. Evaluating boxwood susceptibility to Calonectria pseudonaviculata using cuttings from the National Boxwood Collection. Plant Health Progress, 16(1), pp.11-15.
5. Kramer, M., Guo, Y. and Pooler, M., 2020. Ranking Resistance of Buxus Cultivars to Boxwood Blight–an Integrated Analysis. Journal of Environmental Horticulture, 38(2), pp.50-55.
6. LeBlanc, N., Salgado-Salazar, C. and Crouch, J.A., 2018. Boxwood blight: an ongoing threat to ornamental and native boxwood. Applied microbiology and biotechnology, 102(10), pp.4371-4380.
7. Porth, I. and El-Kassaby, Y.A., 2014. Assessment of the genetic diversity in forest tree populations using molecular markers. Diversity, 6(2), pp.283-295.
8. Kandahari, V., Payam Noor, V., Ahmadikhah, A. and Pahlavani, M., (2013). Investigating genetic diversity between and within Buxus populations in the northern Iranian forests using RAPD molecular markers. 5(2), pp. 207-218
9. Shanjani, P.S., Javadi, H., Rasoulzadeh, L. and Amirkhani, M., 2018. Evaluation of genetic differentiation among healthy and infected Buxus hyrcana with boxwood blight using RAPD and ISSR markers. New Zealand Journal of Forestry Science, 48(1), p.15.