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Genetic Diversity of the Balkan Endemics Moehringia jankae Griseb. ex Janka and Moehringia grisebachii Janka (Caryophyllaceae) from Bulgaria using ISSR markers

Mariya Zhelyazkova[1], Svetlana Georgieva[1], Neli Grozeva[2]*

1 - Trakia University, Faculty of Agriculture, Department of Genetics, Breeding and Reproduction, Students' campus, 6000 Stara Zagora, BULGARIA
2 - Trakia University, Faculty of Agriculture, Department of Biology and Aquaculture, Students' campus, 6000 Stara Zagora, BULGARIA
[*]Corresponding author: m.jelqzkova@uni-sz.bg

Abstract. Eleven populations of the endangered plant *Moehringia jankae* and twenty-eight of the rare plant *Moehringia grisebachii* were collected across its natural range from Bulgaria. Their genetic diversity was investigated through fifteen selected Inter Simple Sequence Repeats (ISSR) primers. The ISSR primers produced a total of 285 bands, of which 275 were polymorphic and 10 – monomorphic. Capability of the primers was assessed through the high mean values for the polymorphic information content (0.78), effective multiplex ratio (14.73), resolving power (27.90) and marker index (11.36). Based on the obtained mean values of the molecular data the species *M. grisebachii* (effective number of alleles = 1.39, Shannon's information index = 0.38, expected heterozygosity = 0.24, Nei's genetic diversity = 0.25, gene flow= 0.65) demonstrated higher genetic diversity than the species *M. jankae* (effective number of alleles = 1.28, Shannon's information index = 0.26, expected heterozygosity = 0.17, Nei's genetic diversity = 0.23, gene flow = 0.52). These results were supported by Analysis of molecular variance (AMOVA), showing higher variability within populations of *M. jankae* (90%) and *M. grisebachii* (62%), than among populations - 10% and 38%, respectively, and 25% among both species. Neighbor joining and principal coordinate analysis (PCoA) grouped the thirty-nine studied populations by species and region of spread. The data are applicable in conservation programs for protecting and keeping of both species. The registered genetic similarity between the populations of the two species (from Eastern Balkan Range) does not exclude the possibility of hybridization between their natural populations.

Key words: *Moehringia jankae*, *Moehringia grisebachii*, ISSR markers, endemic, genetic diversity.

Introduction

Recent studies of the Bulgarian flora are based, in part, on the problems of conservation of the natural gene fund of plants, the distribution and condition of the Bulgarian and Balkan endemic plant populations, along with the rare and endangered species, and measures for their protection. The Bulgarian flora includes 270 Balkan endemic species belonging to 116 genera and 35 families (Petrova & Vladimirov, 2010). In Bulgaria endemism reflects on the specific and genetic characteristics of the flora. The endemic species, most commonly, have a limited area of distribution, relatively small and

fragmented populations. Knowing their morphological characteristics, ecological requirements, history, and population structure, in order to protect and preserve them is directly related to knowing their genetic diversity.

According to Martini (1990) the majority of European endemics of genus *Moehringia* were steno endemics, widely distributed in the peripheral mountain ranges around the Mediterranean. *Moehringia jankae* is a protected species according to the Bulgarian legislation (Biological Diversity Act, 2002), it's part of the Red Data Book of Bulgaria (Stoeva, 2015) in the category endangered. The species is protected under the Bern convention (1979) and is included in European Red List of Vascular plants (Bilz et al., 2011) under category Data Deficient (DD). *Moehringia grisebachii* is part of the Red Data Book of Bulgaria in the category endangered (Stoyanov, 2015). Until now the Bulgarian populations of the two endemic species have been the subject of morphological and karyological studies (Zhelyazkova et al., 2019b, 2020a, b; Grozeva et al., 2020).

This study aims to determine the intrapopulation, interpopulation and between species genetic diversity of *Moehringia jankae* and *Moehringia grisebachii* in Bulgaria through ISSR markers.

Due to the higher annealing temperature and the longer sequence of the ISSR primers, they can provide reliable and reproducible bands from RAPD (Nagaoka & Ogihara, 1997; Wolfe et al., 1998; Goulão et al., 2001; Qian et al., 2001). Compared to other markers (RFLP, SSR и AFLP) the prime cost of the analysis is lower (Yang et al., 1996; Wang et al., 2008). ISSR is technically simpler than many other marker systems (Bornet & Branchard, 2001), because it does not require preliminary information about the genome sequence. The ISSR also have disadvantages, as dominant nature, requirement for the high quality of genomic DNA and sometimes have less specificity to the genome (Sarwat, 2012).

Many authors report that high levels of polymorphism, detected with the use of ISSR, confirm that these markers are highly informative for the investigation of genetic parameters of endemic species (Xie et al., 2005; Arzate-Fernández et al., 2005; Cao et al., 2006; Lu et al., 2006; Meloni et al., 2006; Zhang et al., 2006; Xia et al., 2007; Trindade et al., 2012; Zhelyazkova et al., 2019a).

Material and Methods

Plant material

The leaf samples of eleven populations of M*oehringia jankae* and twenty-eight of *Moehringia grisebachii* were included in this study. Plant materials were collected from different parts representing the spread of the species in Bulgaria (Table 1). The Plant samples were identified according to Flora of PR Bulgaria (Kuzmanov & Kožuharov, 1966), Conspectus of the Bulgarian vascular flora (Assyov & Petrova, 2012), Key to the Plants of Bulgaria (Delipavlov & Cheshmedzhiev, 2003) and then were placed in silica gel and stored at -18°C for subsequent DNA extraction.

ISSR assay

Total genomic DNA was extracted using the modified protocols of Plant DNA Preparation Kit (Jena Bioscience). In the study of the genetic diversity of *M. jankae* and *M. grisebachii* were tested 20 ISSR primers. For this study were chosen 15 of them. The selection of these primers was done mainly on the base of literature data from similar studies on the species *M. jankae*, as well as studies on the *Moehringia*, and the family Caryophyllaceae shown on Table 2.

DNA quality and yield have been established by Nano Vue Plus spectrophotometer and Agarose gel (1%) electrophoresis, visualized on Transilluminator (BioImaging System). DNA samples with purity from 1.6 – 1.9 (260/280 nm) were used for PCR amplification.

The PCR amplifications were performed in a total volume of 25 µl, containing 1 µl genomic DNA, 12.5 µl Red Taq DNA Polymerase 2×Master Mix, 1.5 µl Primer (Bioneer) and 10 µl nuclease free ddH2O (Sigma). Amplification was carried out following a protocol by Pourhosseini et al. (2018). PCR was optimized by modification of annealing temperature to specific annealing temperature (sTa°) until maximum results were reached with each separate primer. The recording of ISSR-PCR amplified products was performed through the horizontal electrophoresis, on 1.5% agarose gel with 1×TBE buffer for 50 min at 80 V/cm. Gels were comprised 7 µl of product mixed with 1.5 µl loading buffer and 100+ DNA-ladder (100 – 3000 bp) and then were stained with fluorescent nucleic acid dye GelRed® (Biotium, USA). The presence (1) and absence (0) of bands were recorded with the help of Electrophoresis Gel Imaging Analysis System (Bio-Imaging Systems, Israel).

Statistical assay

Capability of primers was determined by calculating the following parameters: polymorphic information content (PIC) (Botstein et al., 1980; Roldan-Ruiz et al., 2000), effective multiplex ratio (EMR) (Powell et al., 1996; Nagaraju et al., 2001), marker index (MI) (Varshney et al., 2007) and resolving power (RP) (Prevost & Wilkinson 1999).

GenAlEx (Peakall & Smouse, 2006) ver. 6.5 was used for the parameters: different number of alleles (Na), effective number of alleles (Ne), Shannon's Information Index (I), expected (He) and unbiased (uHe) expected heterozygosity, percentage of polymorphic bands (PPB). Principal coordinate analysis (PCoA) and Analysis of Molecular Variance (AMOVA) were constructed in this statistical package, too.

Mantel test was performed in GenAlEx 6.5 to examine the correlation between geographic (in kilometres) and genetic distance (pairwise GD).

Gene follow (Nm) and Nei's Genetic diversity (H) were calculating with software package PopGene ver. 1.32.

Neighbor joining analysis was conducted using MEGA version 4 (Tamura et al., 2007).

Results

ISSR primers

The fifteen ISSR primers used in this study produced total of 285 bands, of which 275 bands were polymorphic and 10 bands were monomorphic. Eight of all used primers were 100% polymorphic. Minimum (10) and maximum (25) number of bands were obtained with ISSR primers (AC)8G and (AG)8YC, respectively. PIC were in correlation with high and medium polymorphism with values from 0.64 for primer (AG)8YT to 0.90 for (CA)8G, and mean value 0.78. Lowest Rp was recorded for primer (GACA)4 - 15.60, and the highest with primer (AC)8T – 43.41, with mean value 27.9. The mean value of MI was 11.36, it was lowest (8.95) for primer (AC)8G and highest (17.54) for (AC)8T. According to the received results the most effective "marker-primer" system (EMR) was for primer (AC)8T with 20.32, mean value for all primers 14.73 (Table 3). ISSR polymorphism for both species found with the different primers is shown on Fig. 1.

Moehringia jankae

In 11 populations of *Moehringia jankae* the mean frequency of loci was 0.36, ranging between 120 – 2000 bp. Minimum effective number of alleles was 1.13 (ATC6) and maximum 1.46 (GACA4), with average 1.28. The lowest value for Shannon information index was 0.11 and highest 0.42 with primers ATC6 and AG8YC, respectively, and average value 0.26. The value for expected and unbiased expected heterozygosity varied from 0.07 – 0.08 (ATC6) to 0.28 - 0.29 (AG8YC), with average 0.17 - 0.18 (Table 4).

Specific ISSR bands were found in some populations, as follows: with primer ATC6 (150 bp) in population Mj7, with primer CA8RG (550 bp) in population Mj8, with primer ATG6 in populations Mj1 (280 bp) and Mj3 (320 bp), with primer AC8T in population Mj1 (550 bp) and population Mj8 (1400 bp).

Table 1. Location of studied populations of *Moehringia jankae* and *Moehringia grisebachii*.

***Moehringia jankae* (Mj), Eastern Balkan Range, Sinite Kamani Natural Park**	**Latitude/Longitude (Altitude, m)**
Mj1 Kaloyanovi kuli area	N 42° 42.755´ E 26° 23.015´(756)
Mj2 Haiduschka pateka area east of Karandila hotel	N 42° 42.704´ E 26° 22.261´(889)
Mj3 Micro dam area	N 42° 42.790´ E 26° 22.612´(972)
Mj4 350 m. south of hotel complex Karandila	N 42° 42.709´ E 26° 22.355´(933)
Mj5 450 m. southwest of hotel complex Karandila	N 42° 42.712´ E 26° 22.252´(908)
Mj6 The rocks south-east of Kamilata area	N 42° 42.593´ E 26° 22.196´(851)
Mj7 The rocks between Karandila and Kamilata area	N 42° 42.726´ E 26° 22.349´(952)
Mj8 The north of Kamilata area	N 42° 42.673´ E 26° 22.217´(866)
Mj9 The rock formation in Kamilata area	N 42° 42.603´ E 26° 22.180´(857)
Mj10 The east of Kamilata area	N 42° 42.647´ E 26° 22.198´(869)
Mj11 rock formations near Karandilska polyana	N 42° 42.873´ E 26° 22.452´(955)
***Moehringia grisebachii*, Eastern Balkan Range, Sinite Kamani Natural Park (MgSl)**	
MgSl1 The east of Haiduschka pateka	N 42° 42.785´ E 26°21.349´(921)
MgSl2 The south-east of Karandila hotel	N 42° 42.851´ E 26°22.447´(971)
MgSl3 Kaloyanovi kuli area	N 42° 42.833´ E 26°23.169´(685)
MgSl4 The west of Karandilska polyana	N 42° 42.818´ E 26°22.482´(965)
MgSl5 Gornaka area	N 42° 42.828´ E 26°23.735´(920)
MgSl6 Haiduschka polyana	N 42° 42.290´ E 26°21.655´(641)
MgSl7 The north of Micro dam area	N42° 42.815´ E 26°22.647´(951)
MgSl8 The east of Micro dam area	N 42° 42.818´ E 26°22.482´(975)
MgSl9 The south of Karandilska polyana	N 42° 42.828´ E 26°22.530´(956)
MgSl10 Kamilata area	N 42° 42.595´ E 26°22.181´(838)
MgSl11 Around hotel complex Karandila	N42° 42.871´ E 26°22.447´(938)
MgSl12 High East Rocks - Alpine climbing route	N42° 42.706´ E 26°22.349´(913)
MgSl13 Between Kamilata and hotel Karandila	N42° 43.082´ E 26°22.157´(909)
MgSl14 Bellow hotel complex Karandila	N42° 42.786´ E 26°22.360´(919)
***Moehringia grisebachii*, Sredna gora Mts, (MgR)**	
MgR1 Orlite Peak	N42° 28.783´ E 25°06.896´(773)
MgR2 On the path towards Bratan peak	N42° 28.708´ E 25°07.427´(741)
MgR3 Big Rock east of Kara Dere	N42° 29.037´ E 25°05.170´(813)
MgR4 The rock formation Pravite Kamani	N42° 28.935´ E 25°05.290´(738)
MgR5 The northwest of Pravite Kamani	N42°28.845´ E 25°05.206´(602)
MgR6 The north of Chepilskata Cheshma	N 42°29.067´ E 25°07.421´(845)
MgR7 The rocks between Orlite and Popova Turla	N42° 28.794´ E 25°06.975´(786)
MgR8 On the path towards Pravite Kamani	N42° 28.831´ E 25°05.204´(638)
MgR9 The west part of Orlite Peak	N42° 28.783´ E 25°06.896´(773)
MgR10 The west of rock formation Pravite Kamani	N42° 28.929´ E 25°05.271´(725)
MgR11 Little Rock east of Kara Dere	N42° 29.052´ E 25°05.186´(821)
MgP Usoykata area	N42° 29.489´ E 24°48.011´(378)
***Moehringia grisebachii*, North-Eastern Bulgaria (MgSh)**	
MgSh1 The Madara rider	N 43°16.631´ E 27°07.181´(293)
MgSh2 The fortress above village Madara	N 43°16.599´ E 27°07.214´(392)

Table 2. Literature sources and sequence for used ISSR markers in the family Caryophyllaceae.

Sequence	Literature sources
AGAGAGAGAGAGAGAGC	Korkmaz & Dogan (2015); Hilooğlu et al.(2016)
AGAGAGAGAGAGAGAGYC	Peng Fu et al.(2008); Kołodziej et al. (2018)
ATCATCATCATCATCATC	Muller et al. (2015); Kołodziej et al. (2018)
GACAGACAGACAGACA	Minuto et al. (2006); Holobiuc et al. (2018); Kołodziej et al. (2018)
CACACACACACACACARG	-
GAGAGAGAGAGAGAGAYG	Minuto et al. (2006); Holobiuc et al. (2018);
ACACACACACACACACG	Minuto et al. (2006); Korkmaz & Dogan (2015); Holobiuc et al. (2018); Kołodziej et al. (2018)
AGAGAGAGAGAGAGAGYT	Fu et al. (2008); Kołodziej et al. (2018)
ATGATGATGATGATGATG	Minuto et al. (2006); Holobiuc et al. (2018); Muller et al. (2015)
GAGAGAGAGAGAGAGAC	Kołodziej et al. (2018); Korkmaz & Dogan (2015)
GAGAGAGAGAGAGAGAT	Holobiuc et al. (2018) Kołodziej et al. (2018)
GTGTGT GTGTGT GTGTYC	Hilooğlu et al.(2016); Kołodziej et al. (2018)
ACACACACACACACACT	Korkmaz & Dogan (2015); Kołodziej et al. (2018);
AGAGAGAGAGAGAGAGG	Fu et al.(2008); Kołodziej et al. (2018)
CACACACACACACACAG	Korkmaz & Dogan (2015); Kołodziej et al. (2018)

Table 3. ISSR primers used for the assessment of the genetic diversity in 39 natural populations of *Moehringia jankae* and *Moehringia grisebachii* and their parameters: Specific annealing temperature (sTa°), Total bands (TB), Polymorphic bands (PB), Monomorphic bands (MB), Effective multiplex ratio (EMR), Polymorphic information content (PIC), Resolving power (Rp), Marker index (MI).

Primer	Sequence	sT_a°	TB	PB	MB	% PB	EMR	PIC	Rp	MI
(AG)8C	AGAGAGAGAGAGAGAGC	52.3	20	20	0	100	10.50	0.88	27.68	9.25
(AG)8YC	AGAGAGAGAGAGAGAGYC	55	25	25	0	100	12.84	0.84	34.72	10.83
(ATC)6	ATCATCATCATCATCATC	49	13	12	1	92.31	15.48	0.72	23.05	11.17
(GACA)4	GACAGACAGACAGACA	52	15	15	0	100	15.87	0.77	15.60	12.17
(CA)8RG	CACACACACACACACARG	56.8	22	22	0	100	14.14	0.82	34.68	11.56
(GA)8YG	GAGAGAGAGAGAGAGAYG	53	22	21	1	95.45	13.86	0.81	30.63	11.19
(AC)8G	ACACACACACACACACG	58	13	10	3	76.92	13.85	0.65	20.36	8.95
(AG)8YT	AGAGAGAGAGAGAGAGYT	55	15	14	1	93.33	18.36	0.64	18.37	11.70
(ATG)6	ATGATGATGATGATGATG	49.3	16	16	0	100	12.75	0.83	28.29	10.54
(GA)8C	GAGAGAGAGAGAGAGAC	50.4	20	19	1	95	14.25	0.76	27.07	10.83
(GA)8T	GAGAGAGAGAGAGAGAT	50	20	19	1	95	18.86	0.67	25.23	12.68
(GT)8YC	GTGTGTGTGTGTGTGTYC	58.3	17	17	0	100	16.94	0.72	26.04	12.20
(AC)8T	ACACACACACACACACT	56.5	24	24	0	100	20.32	0.86	43.41	17.54
(AG)8G	AGAGAGAGAGAGAGAGG	52.4	19	17	2	89.47	12.86	0.77	27.90	9.94
(CA)8G	CACACACACACACACAG	55.3	24	24	0	100	10.17	0.90	34.03	9.84
Total			285	275	10					
	Mean						14.73	0.78	27.9	11.36

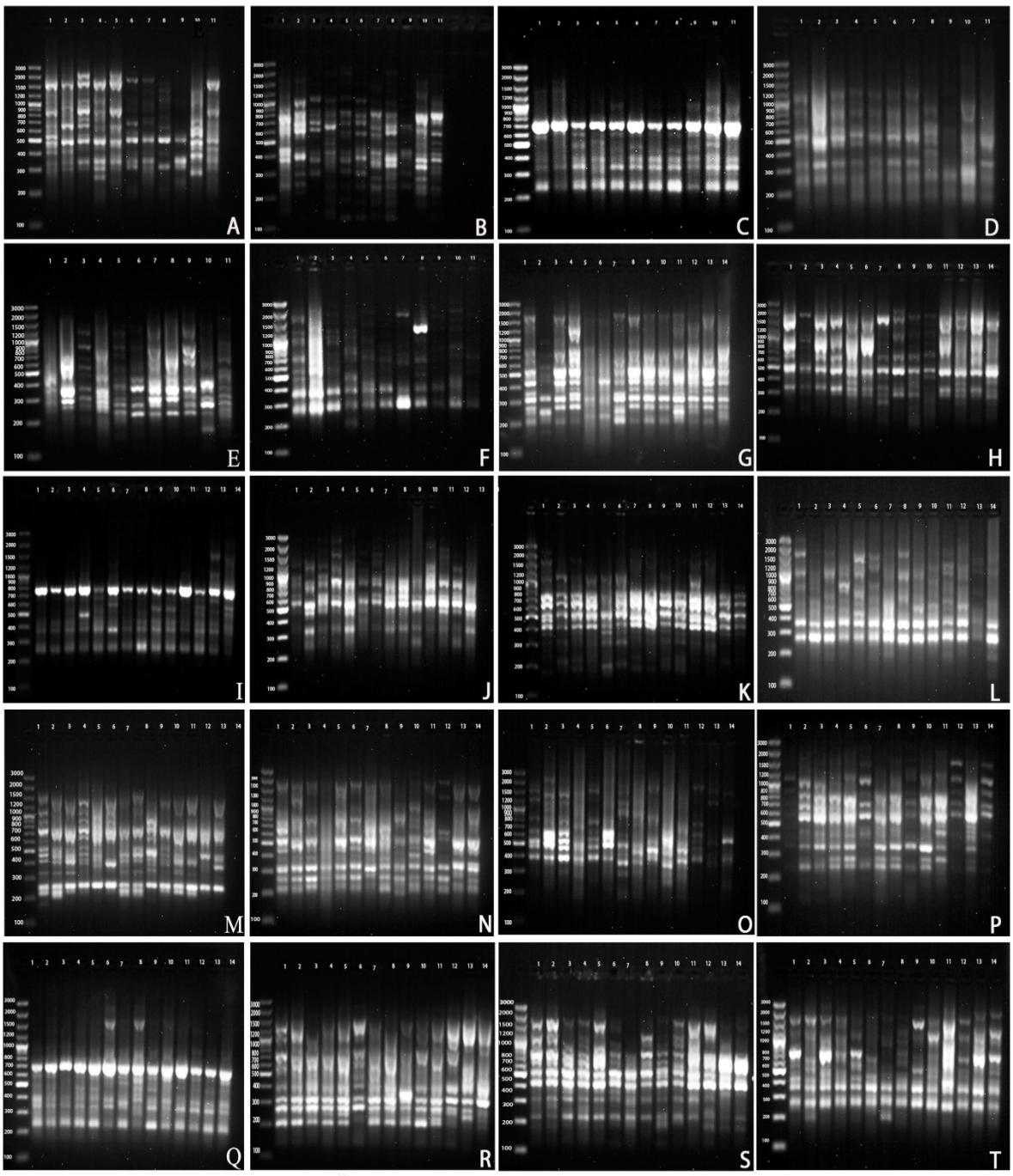

Fig. 1. ISSR genetic diversity in *M. jankae* (Mj1 - Mj11, A - F) and *M. grisebachii* (MgSl1 - MgSl14, G – L and MgR1 - MgR11, MgP - 12, MgSh 13–14, M - T), DNA Marker (Ladder 100 bp+); Primer (AC)8T - A, P; Primer (AG)8YC - B, J; Primer (AC)8G - C, K, Q; Primer (GT)8YC - D, R; Primer (GACA)4 - E; Primer (CA)8RG - F, N, T; Primer (AG)8C - G; Primer (GA)8C - H, O; Primer (CA)8G - I; Primer (GA)8T - L; Primer (AG)8YT - M, S.

Table 4. Genetic diversity in eleven populations of species *M. jankae* based on 15 ISSR markers. *Legend:* * p and q – Allele Frequency, Na – different and Ne – effective number alleles, I - Shannon's Information Index, He – expected and uHe - unbiased expected heterozygosity.

Primer	Size range,bp	Bands freq.	p	q	Na (mean)	Ne (mean)	I (mean)	He (mean)	uHe (mean)
*AG8C	200 - 1500	0.23	0.15	0.85	1.35	1.24	0.24	0.15	0.16
AG8YC	120 - 1000	0.41	0.27	0.73	1.64	1.47	0.42	0.28	0.29
ATC6	150 - 1200	0.32	0.28	0.72	0.69	1.13	0.11	0.07	0.08
GACA4	180 - 1200	0.47	0.34	0.66	1.60	1.46	0.39	0.26	0.27
CA8RG	200 - 2000	0.34	0.25	0.75	1.27	1.32	0.29	0.19	0.20
GA8YG	120 - 1200	0.32	0.22	0.78	1.38	1.37	0.33	0.22	0.23
AC8G	250 - 1000	0.42	0.38	0.62	0.77	1.14	0.12	0.08	0.08
AG8YT	180 - 700	0.44	0.35	0.65	1.13	1.29	0.25	0.17	0.18
ATG6	200 - 1000	0.26	0.15	0.85	1.50	1.35	0.33	0.21	0.22
GA8C	200 - 1200	0.37	0.31	0.69	1.00	1.21	0.19	0.13	0.13
GA8T	150 - 1300	0.38	0.31	0.69	1.10	1.25	0.22	0.15	0.15
GT8YC	180 - 1200	0.44	0.35	0.65	1.13	1.29	0.25	0.17	0.18
AC8T	250 - 1800	0.33	0.24	0.76	1.63	1.26	0.30	0.18	0.19
AG8G	150 - 1200	0.40	0.33	0.67	0.95	1.25	0.21	0.14	0.15
CA8G	180 - 2000	0.28	0.20	0.80	1.33	1.25	0.27	0.17	0.17
Grand mean		**0.36**	**0.27**	**0.73**	**1.23**	**1.28**	**0.26**	**0.17**	**0.18**
SD					0.05	0.02	0.02	0.01	0.01

For all studied populations of *M. jankae*, Nei's H index (1973) of genetic diversity on the base of 15 ISSR markers was 0.23 ± 0.18. The calculated flow of genes (Nm) between 11 populations of the species was 0.52.

The analysis of molecular variance (AMOVA) shows significant level of intrapopulation diversity in *M. jankae*, with p-value < 0.001, and value Fst = 0,099 (data not shown). The received results for за Ne, I, He and uHe, along with the value for Fst show low to medium level of genetic diversity in the studied populations of the species.

Moehringia grisebachii

In 28 populations of *M. grisebachii* the mean frequency of loci was 0.39, ranging between 120 – 3000 bp. Minimum effective number of alleles was 1.28 (AC8G) and maximum 1.60 (GA8T), with average 1.39. The lowest value for Shannon information index was 0.30 and highest 0.47 with primers AC8G and GA8T, respectively, and average value 0.38. The value for expected and unbiased expected heterozygosity varied from 0.19 (AC8G) to 0.33 (GA8T), with average 0.24 - 0.25 (Table 5).

Specific ISSR bands were found in some populations, as follow: Primer ATC6 has only ISSR band (1500 bp) in population MgR1; Primer CA8RG has only ISSR band (150 bp) in population MgSl14; Primer GA8YG has only ISSR band (450 bp) in population MgSl1; Primer AC8G has only ISSR band (300 bp) in population MgR8; Primer AG8YT has only ISSR band (450 bp) in population MgSl1; Primer GT8YC has only ISSR band (2000 bp) in population MgSl12; Primer AG8G has only ISSR band (900 bp) in population MgSl4.

For all studied populations of *M. grisebachii*, Nei's H index (1973) of genetic

diversity on the base of 15 ISSR markers was 0.25±0.17. The calculated flow of genes (Nm) between 28 populations of the species was 0.65.

The analysis of molecular variance (AMOVA) made on the base of 15 ISSR markers and the three main groups of the species MgSl, MgR, and MgSh showed a significant level of intrapopulation diversity in the studied populations of *M. grisebachii*, with p-value (< 0.001), and value Fst = 0,38 (data not shown). The Fst value shows a high level of genetic diversity beween the different groups of the populations of the species.

Between species genetic diversity

On the base of the molecular data generated from 15 ISSR markers in 39 populations, the calculated Nei D (0.115) and Nei I (0.892) for *M. jankae* and *M. grisebachii*, show that the two species are greatly similar to each other (Table 6). The genetic diversity according to the calculated parameters for Ne (1.293), I (0.270), He (0.176) and uHe (0.185), PPB (57.04) is lower for the species *M. jankae,* as is the number of bands (199).

The analysis of molecular variance (AMOVA) made on the base of 15 ISSR markers and a total of 39 populations of the species *M. jankae* and *M. grisebachii* showed significant level of within species diversity with p-value = 0.001, and value Fst = 0,25.

Specific ISSR loci monomorphic for the species *M. jankae* were registered with primers CA8RG (300bp), GA8T (1300 bp) and GT8YC (180 bp). These bands were missing in the species *M. grisebachii* and can be applied in further studies to differentiate between the two species. ISSR specific monomorphic band the species *M. grisebachii* is also registered with primer CA8RG (350 bp). In the populations of *M. grisebachii* in the different locations (MgSl, MgR and MgSh), are also seen ISSR specific band, for the separate groups but they are not shown in the genotype of all studied populations.

The specifics of the locations of the different populations is reflected in the PCoA analysis and the cluster analysis which differentiates the populations not only by species but by location as well, placing them in three separate clusters (Fig. 2 and 3).

The two analysis show that between populations of the two species *M. jankae* and *M. grisebachii* there is greater similarity that between the populations of *M. grisebachii* in the two main areas of the species – Eastern Balkan Range and Sredna gora mountain (Fig. 2, 3).

Mantel test analysis showed no correlation between geographic and genetic distance among *M. jankae* populations (r = 0.323, p = 0.1, Fig. 4a), whereas a significant correlation was found within *M. grisebachii* populations (r = 0.763, p = 0.01, Fig. 4b).

Table 5. Genetic diversity in twenty-eight natural population of species *M. grisebachii* based on 15 ISSR primers. *Legend:* * p and q - Allele Frequency, Na – different and Ne – effective number alleles, I - Shannon's Information Index, He – expected and uHe - unbiased expected heterozygosity.

Primer	Size range,bp	Bands freq.	p	q	Na (mean)	Ne (mean)	I (mean)	He (mean)	uHe (mean)
AG8C	180 - 1500	0.29	0.17	0.83	2.00	1.32	0.38	0.23	0.23
AG8YC	120 - 1500	0.30	0.18	0.82	1.84	1.39	0.39	0.25	0.25
ATC6	200 - 1500	0.47	0.35	0.65	1.77	1.39	0.38	0.24	0.25
GACA4	180 - 800	0.38	0.24	0.76	1.73	1.46	0.43	0.28	0.29
CA8RG	150 - 2800	0.37	0.24	0.76	1.68	1.44	0.39	0.26	0.26
GA8YG	120 - 2000	0.37	0.24	0.76	1.86	1.38	0.39	0.25	0.25
AC8G	250 - 1500	0.48	0.38	0.62	1.77	1.28	0.30	0.19	0.19
AG8YT	180 - 1500	0.53	0.40	0.60	1.87	1.43	0.41	0.27	0.27
ATG6	200 - 1500	0.35	0.23	0.77	1.63	1.41	0.38	0.25	0.25
GA8C	200 - 1500	0.39	0.26	0.74	1.85	1.44	0.40	0.26	0.27

GA8T	150 - 1500	0.56	0.40	0.60	1.80	1.60	0.47	0.33	0.33
GT8YC	250 - 3000	0.43	0.31	0.69	1.71	1.42	0.37	0.25	0.25
AC8T	200 - 2000	0.25	0.15	0.85	1.58	1.31	0.31	0.20	0.20
AG8G	200 - 1800	0.36	0.25	0.75	1.79	1.32	0.33	0.21	0.21
CA8G	180 - 2000	0.25	0.14	0.86	1.92	1.33	0.36	0.22	0.23
Grand mean		**0.39**	**0.26**	**0.74**	**1.79**	**1.39**	**0.38**	**0.24**	**0.25**

Table 6. Genetic diversity of *M. jankae* and *M. grisebachii* based on 15 ISSR primers. *Legend:* N – number of population, Na – different and Ne – effective number alleles, I - Shannon's Information Index, He – expected and uHe - unbiased expected heterozygosity, percentage of polymorphic bands (PPB), Nei D and Nei I – Genetic distance and identify, ()-SD.

Species	N	Na	Ne	I	He	uHe	PPB%	Bands	Nei D Mg	Nei I Mj
Mj	11	1.27 (0.05)	1.293 (0.021)	0.270 (0.016)	0.176 (0.011)	0.185 (0.012)	57.04	199	0.115	1.000
Mg	28	1.79 (0.03)	1.393 (0.019)	0.380 (0.014)	0.244 (0.010)	0.248 (0.010)	86.97	261	0.000	0.892

Table 7. Data of AMOVA analysis in studied species. *Legend:* *df – degree of freedom, SS – total sum of square, MS – midle square, Est. Var. – estimated variance.

Source of variation	df	SS	MS	Est. Var.	Variation%
Among the species	1	247.99	247.99	13.173	25
Within the species	37	1487.30	39.93	39.972	75
Total	38	1725.28		53.100	100

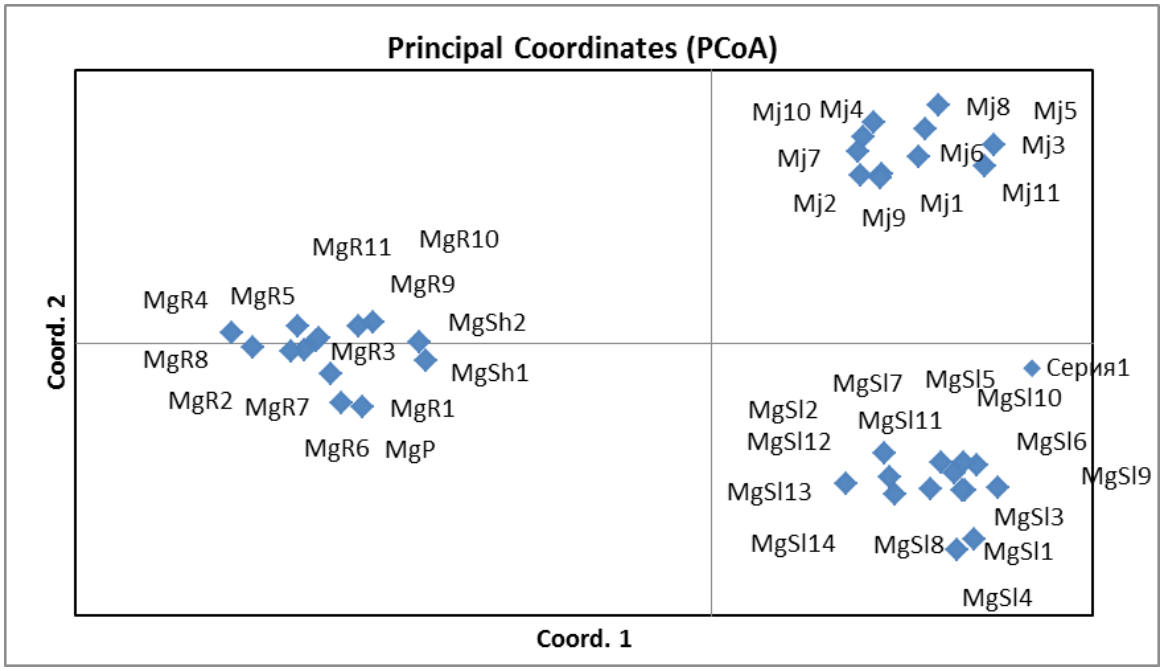

Fig. 2. Two-dimensional plot of PCoA of thirty-nine natural populations of *M. jankae* и *M. grisebachii* based on 15 ISSR primers

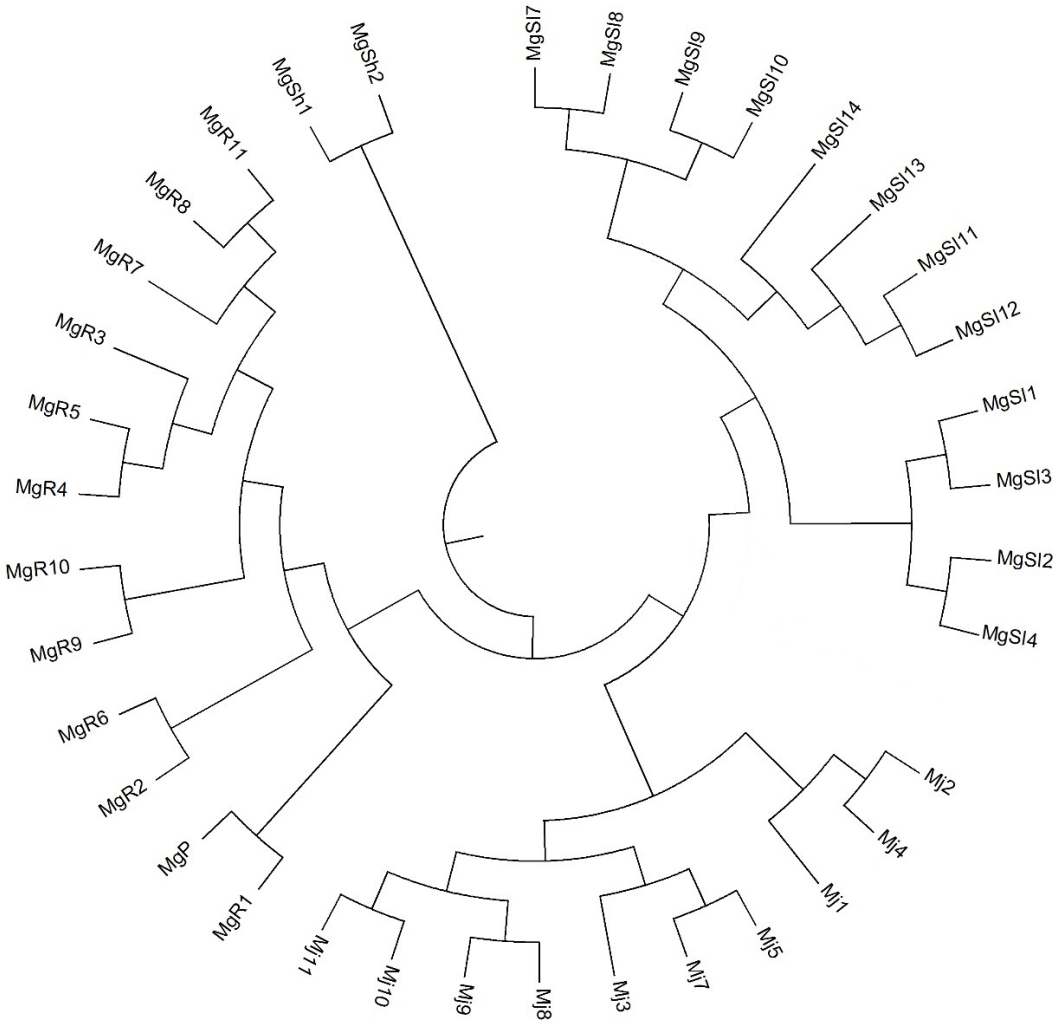

Fig. 3. Neighbor-joining based clustering of genetic diversity of *Moehringia jankae* Griseb. ex Janka and *Moehringia grisebachii* Janka using 15 ISSR primers.

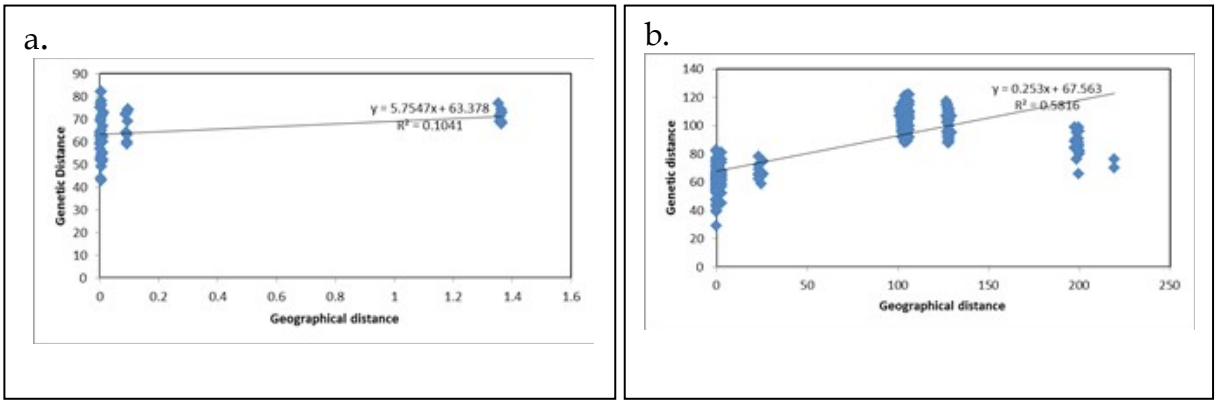

Fig. 4. Correlation between geographic distance (km) and genetic distance (pairwise GD) among 11 populations of *M. jankae* (a) and 28 populations of *M. grisebachii* (b).

Discussion

In this study the intrapopulation genetic diversity is leading in *M. jankae* (91%, Fst = 0.09) as well as in *M. grisebachii* (62%, F_{st} = 0.38). These results are similar to the ones received by Minuto et al. (2006) for *M. lebrunii* (G_{st} = 0.355) and *M. sedoides* (G_{st} = 0.255), whose genetic diversity was also studied with ISSR markers. The species *M. jankae*, similar to the data of Minuto et al. (2006) for the species *M. lebrunii*, has a small area of distribution and a significantly lowered number and size of the populations, which negatively affects the level of interpopulation diversity of the species. The level of genetic diversity inter and intra populations is directly related with the resistance of the species against long term biotic and abiotic changes in the environment (Soulé, 1980). In this study the species *M. grisebachii* shows higher level interpopulations diversity (F_{ST} = 0.38), which is partly due to the bigger area of distribution of the species and possibly better adaptivity. The higher values determined for effective number alleles, Shannon's Information Index, expected and unbiased expected heterozygosity, as well as percentage of polymorphic bands (PPB), in the species *M. grisebachii*, are possibly influenced by the larger number of studied populations (28), but in comparison between the two species are reliable, because there are no data for other distribution areas for the species *M. jankae*. In our previous research (Zhelyazkova et al., 2020a, b; Grozeva et al., 2020) for all studied populations of *M. jankae* and *M. grisebachii* was established diploid chromosome number 2*n*=24 and a karyotype of metacentric and submetacentric chromosomes, with metacentric ones being dominant. The karyological analysis of both species from Eastern Balkan Range doesn't make a definite differentiation between their populations.

The distribution in separate clusters of the total 39 populations according to location and species is seen in the PCoA analysis and the cluster analysis. This confirms the hypothesis that the specific conditions of the location influence the differences between populations, shown through specific loci, for each species as well as each region they inhabit.

Interesting here is that the species *M. jankae* shows more genetic similarity with the populations of *M. grisebachii* on the territory of Eastern Balkan Range, Sliven, than is seen between all populations of *M. grisebachii* in their two main areas of distribution – Sredna gora Mountain and Eastern Balkan Range. In a previous study was found similarity in the changeability of the karyotype of *M. jankae* and *M. grisebachii*, as well, and according to Zhelyazkova et al. (2020a) 5 different karyotypes are repeated in a total of 20 populations of the two species, distributed on the territory of Eastern Balkan Range. Our field researchers (Grozeva et al, 2016) showed that some of the populations of the two species grow close to each other. In subsequent unpublished studies, it was observed that in these populations of *M. grisebachii*, hairing of stems with multicellular non-branched straight hairs varies from weak to abundant hairing. *M. jankae* and *M. grisebachii* can be distinguished on the base of hairing of stem, leaves and flower petioles in *M. grisebachii*, because *M. jankae* is glabrous.

These results and the present results raise the question of a possible hybridization between natural populations of the two species distributed in joint territory. The genetic diversity between *M. jankae* and *M. grisebachii* was confirmed by the calculations Nei genetic distances (Nei D = 0.115) and Nei genetic identity (Nei I = 0.892). On the other side in our results is confirmed their identity as separate species and the level of similarity between then could be due to the level of diversification to the specific habitat. Most representatives from the genus *Moehringia* have specific requirements to the habitats (Fior & Karis, 2007), and this allows for the isolation of the separate populations (Akeroyd & Preston, 1981; Fior & Karis 2007;

Minuto et al., 2006; Lorite et al., 2018). Akeroyd & Preston (1981) reports that most of the colonies for *M. minutiflora* are topographically isolated. This is seen in *M. jankae* and *M. grisebachii* and confirmed by the low flow of genes between populations (Nm = 0.52 и 0.62). Akeroyd (1981) does not have proof for the reproductive level of isolation of the colonies but allows that small flowers and barely visible could lead to a high level of self-pollination. He also supposes that due to the morphology of the species there it is very possible that there is some level of cross pollination between plants from the same colony as they grow with the branches of an individual reaching to those of other individuals. In studies of the morphology of the species *M. grisebachii* Zhelyazkova et al. (2019b) reports for a higher intrapopulation genetic diversity. In a study of the morphology of *M. fontqueri*, *M. glochidisperma* and *M. intricata*, Lorite et al. (2018) reports that *M. glochidisperma* showed differences which according to the authors are contributed by the isolation of the species as endemic for North Morocco (Valdés et al., 2002). In the survival and distribution of species of the genus *Moehringia* which grow on rocks similar to *M. jankae* and *M grisebachii* is considered that the spread of the seeds through ant colonies breaks the isolation of the different populations and is more likely than the spread of flower pollen (Akeroyd & Preston, 1981; Casazza et al., 2008). When the geographical distance between populations increases, the genetic differentiation often increases as well. This is shown by the Mantel test in the present study with statistical significance (p=0.01) in *M. grisebachii*, and no statistical significance in *M. jankae* populations (p=0.1). In a number of studies (Li & Jin, 2008; Sheeja et al., 2009; Ng & Tan, 2015) Inter-simple sequence repeat (ISSR) are not only successfully applied for the study of the genetic structure and diversity in plant species but are a more effective marker than RAPD markers (Fernández et al., 2002; Behera et al., 2008). In our study the effectiveness of ISSR to find polymorphism in 11 populations of *M. jankae* reached 95.24% PB, and 100% PB in 28 populations of *M. grisebachii* and was proven with the recorded high values of the parameters characterising each primer.

Conclusions

The conducted study on the genetic diversity in *M. jankae* and *M. grisebachii* shows that in populations with similar conditions and geographical closeness the individuals have bigger similarity in the genotype. The established greater genetic similarity between the populations of *M. jankae* and *M. grisebachii* from the Eastern Balkan Range than between all studied populations of *M. grisebachii* from Sredna gora Mountain and Eastern Balkan Range does not exclude the possibility of possible hybridization between natural populations of the two species distributed in joint territory. The results confirm that the level of genetic diversity is directly related to the size and area of distribution of the species. The Balkan endemic *M. jankae*, distributed only in Eastern Balkan Range, shows lower level of genetic diversity. In order to protect and conserve this species is necessary the development of in situ and ex situ conservation programmes. Efforts should be aimed to support seed reproduction and increase the number of individuals in the population. The anthropogenic impact must be reduced by limiting access to their habitats.

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