



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

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INTRODUCTION

The Bulgarian flora includes 270 endemic Balkan species belonging to 116 genera and 35 families. 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. *Moehringia jankae* is a protected species according to the Bulgarian legislation, it's part of the Red Data Book of Bulgaria in the category endangered. *Moehringia grisebachii* is part of the Red Data Book of Bulgaria in the category endangered. Until now the Bulgarian populations of the two endemic species have been the subject of morphological and karyological studies .

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

MATERIALS AND METHODS

The leaf samples of eleven populations of *M. jankae* and twenty-eight of *M. grisebachii* were included in this study. Plant materials were collected from different parts representing the spread of the species in Bulgaria (Figure 1).

Total genomic DNA was extracted using the modified protocols of Plant DNA Preparation Kit (Jena Bioscience). 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). 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).



Fig.1. Location of *M. jankae* (Mj) and *M. grisebachii* (Mg), as follows: Mj and MgSl – Eastern Balkan Range, MgR – Sredna gora Mts, MgSh – Northeastern Bulgaria.

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 1). ISSR polymorphism for both species found with the different primers is shown on Figure 2.

Table 1. ISSR primers used for the assessment of the genetic diversity in 39 natural populations of *M. jankae* and *M. 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
(AC)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	CACACACACACACARG	56.8	22	22	0	100	14.14	0.82	34.68	11.56
(CA)8YG	GAGAGAGAGAGAGAYG	53	22	21	1	95.45	13.86	0.81	30.63	11.19
(AC)8C	ACACACACACACACCG	58	13	10	3	76.92	13.85	0.65	20.36	8.95
(AG)8YT	AGAGAGAGAGAGAGAYT	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	GTGTGTGTGTGTGTGYC	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										
	Mean		285	275	10		14.73	0.78	27.9	11.36

Moehringia jankae

In 11 populations of *M. jankae* the mean frequency of loci was 0.36, ranging between 120 – 2000 bp. Minimum effective number (Ne) of alleles was 1.13 (ATC6) and maximum 1.46 (GACA4), with average 1.28. The lowest value for Shannon information index (I) was 0.11 and highest 0.42 with primers ATC6 and AG8YC, respectively, and average value 0.26. The value for expected (He) and unbiased expected heterozygosity (uHe) varied from 0.07 – 0.08 (ATC6) to 0.28 - 0.29 (AG8YC), with average 0.17 - 0.18 (Table 2).

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 3).

RESULTS AND DISCUSSION

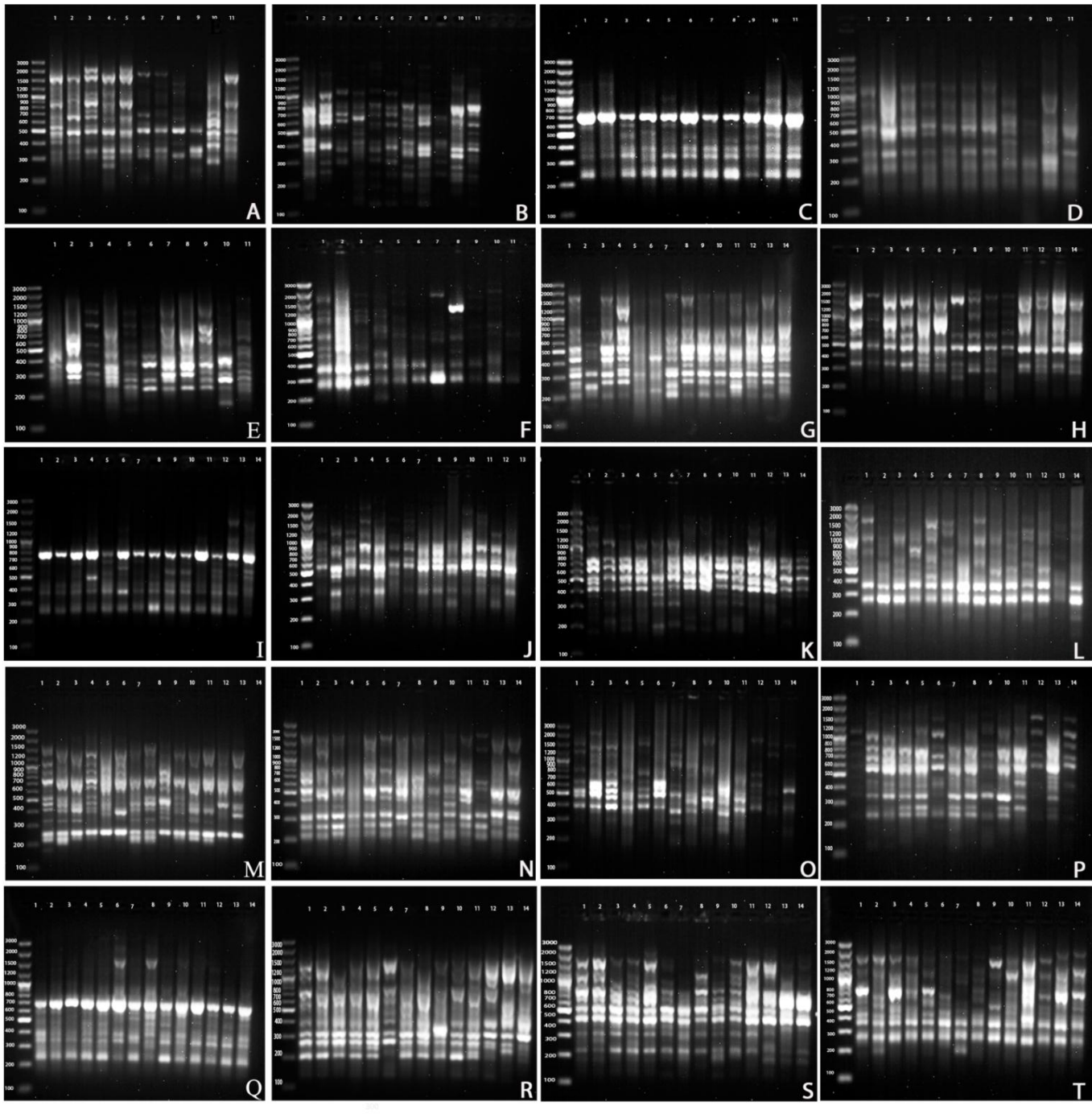


Fig. 2. ISSR genetic diversity in *M. jankae* (Mj1 - Mj11, A - F) and *M. grisebachii* (MgSl1 - MgSl14, G - L and MgR1 - MgR11, MgP - 12, MgSh 13-14, M - T), DNA Marker (Ladder 100 bp+); Primer (AC)8T - A, P; Primer (AG)8YC - B, J; Primer (AC)8G - C, K, Q; Primer (GT)8YC - D, R; Primer (GACA)4 - E; Primer (CA)8RG - F, N, T; Primer (AC)8C - G; Primer (GA)8C - H, I; Primer (CA)8G - J; Primer (GA)8T - L; Primer (AG)8YT - M, S.

Table 2. Genetic diversity in eleven populations of *M. jankae* based on 15 ISSR primers

Primer	Size range, bp	Bands freq.	p	q	Na (mean)	Ne (mean)	I (mean)	He (mean)	uHe (mean)
*AC8C	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	0.37	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
SD					0.05	0.02	0.02	0.01	0.01

Table 3. Genetic diversity in twenty-eight populations of *M. grisebachii* based on 15 ISSR primers

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
GA8YC	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

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 4). 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) showed significant level of within species diversity with p-value = 0.001, and value Fst = 0.25 (Figure 3).

Table 4. Genetic diversity of <i>M. jankae</i> and <i>M. grisebachii</i> based on 15 ISSR primers										
Species	N	Na	Ne	I	He	uHe	PPB %	Bands	Nei D	Nei I
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

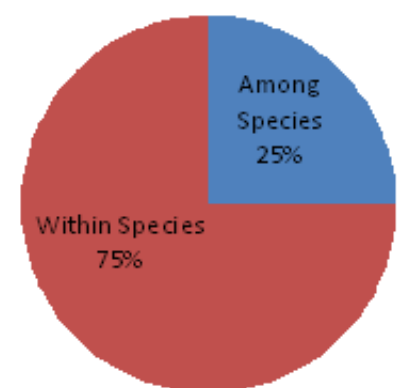


Fig. 3. Percentages of Molecular Variance

The conducted study on the genetic diversity in *M. jankae* and *M. grisebachii* shows that similar conditions are reflected with a bigger similarity in the genotype of populations from close species (Figure 4).

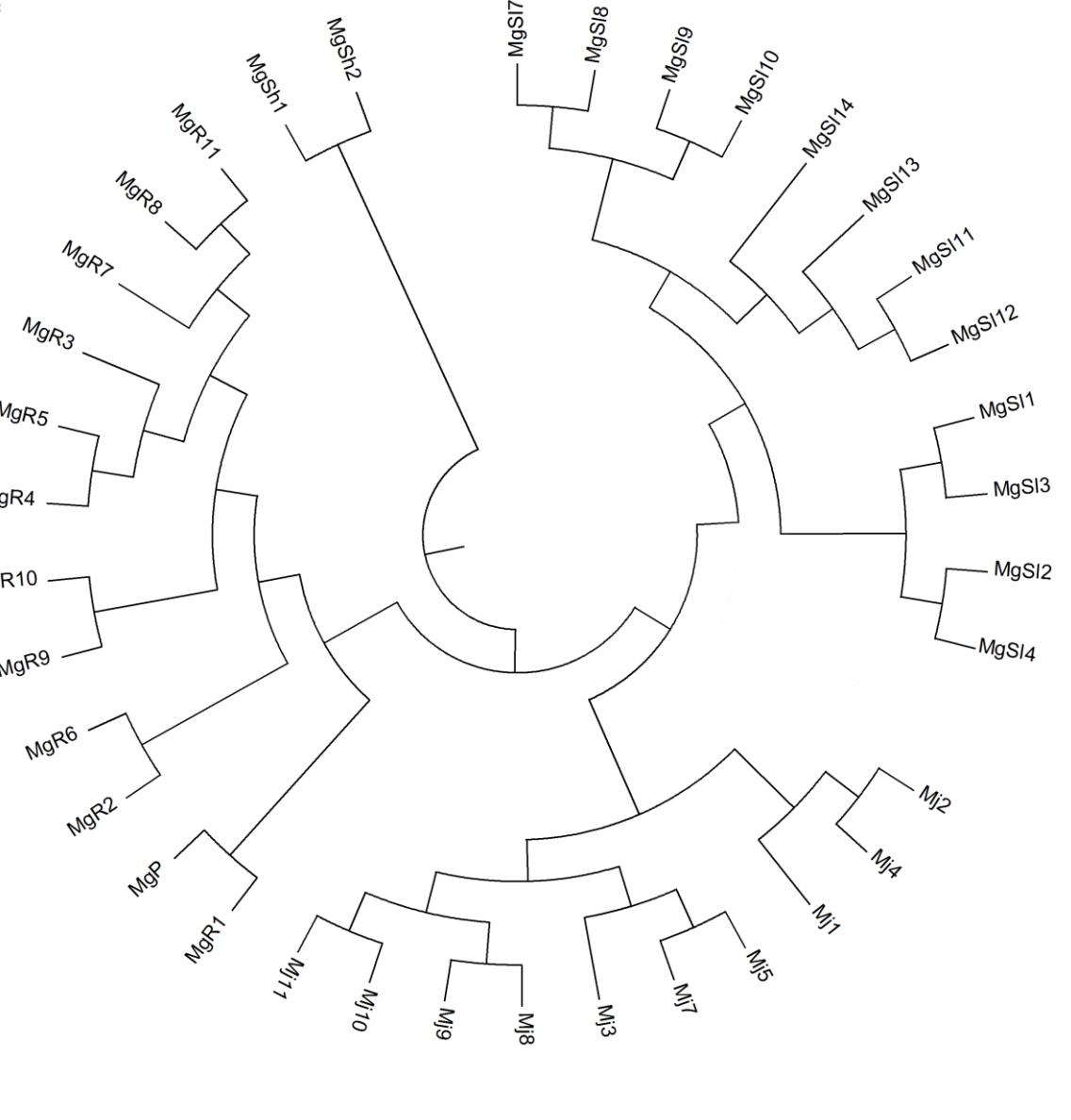


Fig. 4. Neighbor-joining based clustering of genetic diversity of *M. jankae* and *M. grisebachii* using 15 ISSR primers.

Conclusions

The results received by us confirm that the level of genetic diversity is directly related to the size and area of distribution of the species, like 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 to be developed conservation programmes.