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COMPARISON OF DIFFERENT TYPES OF MOLECULAR MARKERS USED IN GENETIC DIVERSITY STUDIES OF BROOMRAPE FROM SERBIA

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Abstract

The purpose of this study was to investigate the utility of the different types of molecular markers in assessing the genetic diversity of seven Serbian broomrape (Orobanche cumana Wallr.) populations, such as ISSR and SSR. The discriminatory potential of SSR markers was on average lower (Rp=2.46) than for ISSR (Rp=13.85), indicating a large interpopulation genetic variability. According to the indices of genetic diversity, a higher intrapopulation molecular variability in the case of SSR markers (PIC=0.58; H=0.63) compared to ISSR (PIC=0.33) was revealed. AMOVA analysis also showed a high genetic diversity within populations with SSR markers (within pops 53% and among pops 47%) and the most genetic diversity among populations with ISSR (34% and 66%, respectively). These results showed a high degree of genetic variations among and within broomrape genotypes from Serbia which favor the evolution of more virulent physiological races of O. cumana. This study can serve as scientific support for future researches in monitoring and developing strategies to improve sunflower crops resistant to this pathogen.

Key words*: microsatellite markers, intra- and interpopulation variability, broomrape, populations.*

INTRODUCTION

Broomrape (*Orobanche cumana* Wallr.) began to affect sunflower fields from Serbia since 1950's, when sunflower cultivation area was suddenly expanded with more productive sunflower foreign varieties (Bošković, 1962). Two dominant races of broomrape have been identified in Serbia, namely race B and E at the moment (Mihaljčević, 1996; Miladinović et al., 2014). Thus, the study of the current situation of *O. cumana* infestation of sunflower fields from Serbia has shown a stability and slower evolution of aggressiveness of this parasite.

In order to the development of effective sunflower breeding programs with broomrape resistance, pathogen control durable strategies, the modern methods of study quite effective as molecular markers are increasingly used (Calderón-González, 2021; Cvejić et al., 2020; Duca & Martea, 2020a; Sukhareva & Kuluev, 2018). At the moment there is a rich variety of molecular markers (RFLP, RAPD, SSR, AFLP, SCAR, ISSR etc.). Each of their have its own advantages and disadvantages, so the usefulness and applicability of a single marker system are not universal. The most appropriate genetic marker depends on the aim pursued, and also the presence of sufficient technical facilities, marker desirable properties (highly polymorphic, reproducible, abundance and evenly distributed throughout the genome etc.), and financial limitations.

In recent years, *Inter-Simple Sequence Repeat* (ISSR) technique based on the polymorphism of the nucleotide sequences in microsatellite regions distributed throughout the genome and with high reproducibility (92-95%) was developed. The ISSR sequences belong to a class of semi-arbitrary and multilocus markers (detect polymorphism in different genes or chromosomes) that are inherited dominantly according to Mendelian laws (Idrees & Irshad, 2014; Tsumura et al., 1996).

Simple sequence repeats (SSRs) represent the specific DNA sequences consisting of short (1-9 bp), tandemly repeated di-, tri-, tetra- or pentanucleotide motifs. SSR markers are multiallelic and codominant, so they can distinguish heterozygotes from homozygotes genotypes. These markers belong to the highly polymorphic due to the fact that the repeats are

from a few to more than ten alleles in each locus (monolocus) and widely dispersed across the genome (Sukhareva & Kuluev, 2018; Vieira et al., 2016).

Both molecular marker systems possess a high level of polymorphism therefore they are widespread applied in developing of genetic linkage map, estimating of gene flow, studying of genetic diversity, analysis of phylogenetic relationships and genetic structure of *O. cumana* populations (Benharrat et al., 2002; Calderón-González et al., 2019; Duca et al., 2019; Duca et al., 2017; Guchetl et al., 2014; Pineda-Martos et al., 2014a; Pineda-Martos et al., 2013).

The superiority of the ISSR and SSR methods over other molecular techniques in determining genetic diversity of the different species (rice, cotton, broomrape, nut, pine, narcissus etc.) in a great number of published works is reflected (Abbasi et al., 2017; Noormohammadi et al., 2013; Duca et al., 2019; Jia et al., 2011; Wu et al., 2004; Zangeneh & Salehi, 2019).

The purpose of this study was to determine and compare the effectiveness of two microsatellite marker systems, which are SSR and ISSR for estimating genetic variability of seven Serbian broomrape populations.

MATERIALS AND METHODS

Plant material. Seven populations consisting from 49 genotypes of *O. cumana* were used as experimental material. Broomrape populations were collected from infected sunflower (*Helianthus annuus* L.) fields in Serbia and kindly provided by Dr. Dragana Miladinović (Institute of Field and Vegetable Crops, Novi Sad, Serbia).

Seeds of *O. cumana* were germinated on sunflower roots in the greenhouse of the laboratory. Fresh tissue samples from each population were collected and they were stored at -80°C until DNA extraction.

DNA extraction. Total DNA was extracted using GeneJET Plant Genomic DNA Purification Mini Kit #K0791 according to the manufacturer's protocol (*Thermo Fisher Scientific*, USA). Quantity and quality of isolated DNA were determined by spectrophotometry (T60 UV-VIS) and, also checked by 1% agarose gel electrophoresis in 1xTAE buffer (Sambrook & Russell, 2001).

ISSR and SSR amplification. Fourteen ISSR primers listed in Table 1 were used. The amplifications were performed with a thermocycler Genset 9700 (*Applied Biosystems*) according to the program: 95°C - 5 min; 35 cycles: 95°C - 30 sec, 45°C - 45 sec, 72°C - 2 min; 72°C - 5 min.

The SSR amplifications were conducted using 15 specific markers (Table 1). The PCR program was: 95°C - 3 min; 35 cycles: 95°C - 30 sec, 57°C - 45 sec, 72°C - 1 min; 72°C - 5 min (Veriti-96 Well Thermal Cycler, *Applied Biosystems*).

The amplification products were separated by 2% agarose (ISSR) and 8% polyacrylamide (SSR) gel electrophoresis. The gels were visualized on transilluminator under UV radiation after staining with ethidium bromide. GeneRuler Express DNA Ladder, ready-to-use SM1553 (ISSR) and GeneRuler Low Range DNA Ladder SM1191 (SSR) (*Thermo Fisher Scientific*, US) were used to estimate the molecular weight of amplified products. The molecular analysis results were documented using gel documentation system Doc-Print VX2 (SXT-F20.M, France).

Data analysis. The DNA amplified fragment analysis and determination of allele sizes were performed with the Photo-Capt V. 15.02 and POPGENE V.1.32 software, and also Microsoft Excel Office 2010. *Analysis of molecular variance* (AMOVA) was carried out using GenAlEx 6.501 and Mantel test by means of XLSTAT version 2014.5.03. The *Polymorphic Information Content* (PIC) of each dominant ISSR marker was calculated according to Roldán-Ruiz I. (Roldán-Ruiz et al., 2000) as:

$$PIC_i = 2f_i(1 - f_i),$$

where f_i is the frequency of the amplified allele (band present), and $(1 - f_i)$ is the frequency of the null allele.

The PIC index for SSR markers was computed according to Botstein D. (Botstein et al., 1980):

$$PIC = 1 - \sum_{i=1}^{n} P_i^2 - \sum_{i=1}^{n-1} \sum_{j=i+1}^{n} 2P_i^2 P_j^2,$$

where *Pi* and *Pj* is the frequency of the amplified alleles (*i, j*) and *n* is the number of detected amplicons (https://gene-calc.pl/pic).

Resolving power (Rp) was calculated according to Prevost A. (Prevost & Wilkinson, 1999):

$Rp = \sum Ibi$, where *Ibi* describes the relative amplicon informativeness *i* and it is calculated as: $Ibi = 1 - \left[2 \times \left|0.5 - pi\right|\right]$, *pi* is the proportion of individuals with identified amplicon *i*.

Table 1. Nucleotide sequences of ISSR and SSR markers used in this study

No.	*Inter-Simple Sequence Repeat* (ISSR)			*Simple Sequence Repeats* (SSR)		
	Name	Sequence (5′→3′)	NB	Name	Sequence F 5′→3′ /R 5′→3′	NB
1.	BC807	agagagagagagagagt	17	Ocum-052	catgtctaagcttttggctcg/ caagacttggaacaagcaaatc	21/22
2.	BC810	gagagagagagagagat	17	Ocum-059	tcttgatttgtatatgtctgatgcaat/ atgctacaatagaaatacacaacgaac	27/27
3.	BC835	agagagagagagagagyc	18	Ocum-070	aagctgtaaacaatgcctgaa/ cctcctccagtaccactaggc	21/21
4.	BC841	gagagagagagagagayc	18	Ocum-074	cctaaaattgaaaccttaaggaaa/ actttccgtgagacggagtc	24/20
5.	BC857	acacacacacacacacyg	18	Ocum-075	tgtggatagagtataagctaccagttc/ ttcccgtagcttggagaatg	27/20
6.	$(CAA)_5$	caacaacaacaacaa	15	Ocum-081	ttacaaggtgaaaccaccca/ cagctactgtccgcaagaaa	20/20
7.	$(GACA)_4$	gacagacagacagaca	16	Ocum-087	ttctcgacagctttgggtaaa/ atgccaacttcgagtgatcc	21/20
8.	$(GATA)_4$	gatagatagatagata	16	Ocum-108	tcgttaataagtggttcacgaaaa/ tgactaaaaataaaatgtacgggtg	24/25
9.	$(CA)_6R^2G$	cacacacacacarg	14	Ocum-122	ggaatacatcattaaagtagttgtccg/ gaaggagtcattaaactccgtga	27/23
10.	$(CTC)_4R^2C$	ctcctcctcctcrc	14	Ocum-141	cagcaactgtttcttccatagag/ tccaagaagaggaaaagaagtga	23/23
11.	$(CAG)_5$	cagcagcagcagcag	15	Ocum-160	tgagggtttgtaaagtgggc/ cgtaccttatccctccgtca	20/20
12.	$(CT)_8TC$	ctctctctctctctctc	18	Ocum-174	caaccaacaaacaagtagtgacg/ tcttgcggcaaaaccatt	23/18
13.	$(CA)_6AC$	cacacacacacaac	14	Ocum-196	gtatgtgcgcccgtcttg/ ggggatgactgtgtttcgat	18/20
14.	$(AG)_8Y^3A$	agagagagagagagagya	18	Ocum-197	agagacggcatcatcaatca/ gtgatcgtgcaggcaccta	20/19
15.				Ocum-206	ccgattgctgtttatgttgtatt/ tgtaggagatgcccagttca	23/20

[1]NB = number of nitrogenous bases; [2]R= (A, G); [3]Y= (C, T).

RESULTS AND DISCUSSIONS

Investigation of the genetic diversity among seven *O. cumana* populations based on microsatellite markers has allowed for revealing both the significant differences and similarity of genotypes depending on the analysed population or type of primer.

ISSR genotyping. Fourteen ISSR primers generated a total of 195 amplicons, and each primer had 2-22 amplified fragments with an average of 13.93 bands/primer (Table 2).

The highest number of amplicons was produced by $(AG)_8YA$ (22), followed by BC835 (21), BC810 and BC807 (20), $(CAG)_5$ and $(CTC)_4RC$ (19), and the least by $(GATA)_4$ (2). The size of the amplified fragments ranged from 360 to 5667 bp. Percentage of polymorphism varied from 62.50% $(CA)_6AC$ to 100% (BC835,BC841, $(CAG)_5$, $(CT)_8TC$, $(CTC)_4RC$, $(GATA)_4$) with an average of 90.82% (Table 2).

ISSR markers utility was evaluated by calculating the *Polymorphic Information Content* (PIC) and *Resolving power* (Rp). The PIC values for all ISSR primers ranged from 0.19 for $(CA)_6AC$ to 0.40 for $(AG)_8YA$ (mean value 0.33), and have been within acceptable limits for dominant markers (up to 0.50). *Resolving power* (Rp), which indicates the discriminatory ability of the primer to distinguish the genotypes or individuals, was estimated for each marker. The highest Rp value of 23.71 was observed for primer $(AG)_8YA$ and the lowest of 0.78 at $(GATA)_4$ with an average Rp of 13.85.

According to the same ISSR markers, the similar results of PIC values (mean value 0.33) were identified in *O. cumana* populations from China

and Turkey. Another index like Rp was lower both in Turkish (12.42) and Chinese (7.24) broomrape populations (Duca & Bivol, 2021a; Duca et al., 2021b).

Thus, based on the ISSR markers assessment on their informativeness and efficiency by means of the statistical indices (number of total fragments from 9 to 22, percentage of polymorphism ≥92.86%, PIC ≥0.30, and Rp ≥10.49) for the Serbian broomrape populations the following primers were selected: (AG)8YA, (CTC)4RC, BC807, (CAA)5, BC841, (CAG)5, (CT)8TC, BC857, BC835 (Table 2).

Table 2. Characteristics of ISSR marker system used for evaluating genetic diversity of *O. cumana* populations

Marker	Fragment sizes (*bp*[1])	Total number of fragments (N)	Percentage of polymorphism, %	Polymorphic Information Content (PIC)	Resolving power (Rp)
$(AG)_8YA$	467-5000	22	95.45	0.40	23.71
BC807	585-3000	20	95.00	0.32	21.55
BC810	461-5500	20	80.00	0.23	19.76
BC835	480-5125	21	100.00	0.38	17.76
BC841	360-5385	13	100.00	0.37	14.41
BC857	385-2233	15	93.33	0.30	14.29
$(CA)_6AC$	798-2951	8	62.50	0.19	6.04
$(CA)_6RG$	443-1313	7	85.71	0.28	4.86
$(CAA)_5$	631-3183	14	92.86	0.33	16.08
$(CAG)_5$	623-5267	19	100.00	0.38	18.50
$(CT)_8TC$	897-5000	9	100.00	0.38	10.49
$(CTC)_4RC$	470-5667	19	100.00	0.36	19.22
$(GACA)_4$	1117-4326	6	66.67	0.23	6.41
$(GATA)_4$	1059-2376	2	100.00	0.27	0.78
Total	360-5667	195	-	-	-
Mean(±SD[2])	-	13.93	90.82	0.33(±0.01)	13.85(±1.88)

[1]bp = base pairs; [2]SD = Standard Deviation.

These primers $(CTC)_4RC$, $(CAG)_5$, $(AG)_8YA$, BC807, BC841, and $(CT)_8TC$ have found equally efficient to identify of genetic diversity also in the case with Turkish populations (N: 15-28 amplicons, percentage of polymorphism ≥88,89%, PIC ≥0.36, Rp ≥11.37) (Duca & Bivol, 2021a). But for the Chinese broomrape populations were chosen the lowest number of informative markers: BC841, BC857, $(CAA)_5$, $(CAG)_5$, $(AG)_8YA$ (N: 10-14 amplicons, percentage of polymorphism ≥60%, PIC ≥0.35, Rp ≥7.20) (Duca et al., 2021b).

SSR genotyping. Analysis of the genotyping results of 49 *O. cumana* accessions from Serbia has determined that from all 15 SSR microsatellite markers used in this study, the only Ocum-122 is a monomorphic in contrast to other 14 polymorphic markers. Ocum-122 generated a monoallelic profile for all studied genotypes, represented by one type of allele - 244 bp (Table 3). A total of 68 alleles using all studied microsatellite markers were detected. The number of alleles produced of each marker ranged from 1 (Ocum-122) to 11 (Ocum-197) alleles with a mean of 4.53. The average effective number of alleles per marker was 3.53, ranging from 1.00 at Ocum-122 to 5.81 at Ocum-059.

The genetic polymorphism and discriminatory power for SSR markers were determined using the same indices: PIC, Rp and also *Nei's genetic diversity* – H (relevant index for codominant markers). The calculated minimum values of PIC and H for each marker constituted of 0.08 and 0.08 respectively for Ocum-160 but maximum of 0.81 and 0.83 respectively for Ocum-059 with average values of 0.58 (PIC) and 0.63 (H) (Table 3). According to Botstein D. et al. (1980), the majority of the molecular markers used in the genotyping of Serbian broomrape populations proved to be the most informative (PIC >0.5), with the exception of the moderately informative Ocum-074 (PIC =0.41) and the least informative Ocum-160 (PIC =0.08). The similar PIC values for these markers were reported also by other authors (Duca & Martea, 2020a; Duca et al., 2021b; Pineda-Martos et al., 2014b; Ziadi et al., 2018).

The highest Rp values for a single marker were obtained by Ocum-197, with a value of 8.49, followed by Ocum-059 with a value of 5.67. In the case of this index, unlike the results obtained

Table 3. Characteristics of SSR marker system used for evaluating genetic diversity of *O. cumana* populations

Marker	Allele size range (bp^2)	Total number of alleles/ marker (N)	Effective number of alleles (Ne)	Polymorphic Information Content (PIC)[1]	Nei's genetic diversity (H)	Resolving Power (Rp)
Ocum-052	130-178	5	5.00	0.77	0.80	0.00
Ocum-059	85-136	8	5.81	0.81	0.83	5.67
Ocum-070	100-126	4	2.90	0.59	0.66	2.98
Ocum-074	101-114	3	2.08	0.41	0.52	1.96
Ocum-075	98-135	5	5.00	0.77	0.80	0.00
Ocum-081	90-110	3	3.00	0.59	0.67	0.00
Ocum-087	109-151	6	5.35	0.79	0.81	4.00
Ocum-108	143-196	5	3.82	0.70	0.74	2.00
Ocum-122	244	1	1.00	0.00	0.00	0.00
Ocum-141	192-226	4	3.66	0.68	0.73	2.29
Ocum-160	134-145	3	1.09	0.08	0.08	2.04
Ocum-174	190-211	3	2.99	0.59	0.67	3.43
Ocum-196	196-343	4	4.00	0.70	0.75	0.00
Ocum-197	96-173	11	4.67	0.75	0.79	8.49
Ocum-206	118-131	3	2.55	0.53	0.61	4.00
Total	85-343	68	-	-	-	-
Mean(±SD[3])	-	4.53(±2.42)	3.53(±1.49)	0.58(±0.25)	0.63(±0.26)	2.46(±2.44)

[1]Interpretation PIC: high informative level PIC > 0.5; moderate informative 0.25< PIC <0.5; slightly informative PIC <0.25 (Botstein et al., 1980).
[2]bp = base pairs; [3]SD = Standard Deviation.

at ISSR, five markers (Ocum-052, Ocum-075, Ocum-081, Ocum-122, Ocum-196) with a zero value of Rp were highlighted (Table 3). Thus, these markers did not contribute to the differentiation of Serbian broomrape populations. The discriminatory potential of SSR markers was on average lower (Rp =2.46) compared to that identified for ISSR markers (Rp =13.85).

Thus, based on the obtained results of the statistical parameters, the most efficient SSR markers that can be used in the genetic characterization of *O. cumana* populations were highlighted.

The highest values for all analyzed indices and the best potential in differentiation of the investigated broomrape genotypes were determined by the Ocum-059, Ocum-087 and Ocum-197 markers. At the same time, highly informative loci are also Ocum-052, Ocum-075, Ocum-108, Ocum-196, which characterized a wide genetic diversity (values PIC and H ≥0.7) within populations. It is important to note that Ocum-206 also showed a relevant discrimination capacity (Rp =4.00) for the studied genotypes.

The high level of polymorphism determined by SSR markers within populations and genotypes is due to the codominant and multiallelic nature of these microsatellite sequences. In other words, SSR observed polymorphism are the result of the polymorphism of tandemly repeated DNA sequences, identifying a larger number of alleles at a locus (Mason, 2015; Vieira et al., 2016). Also, SSR sequences detect homo- and heterozygous genotypes within population.

The analysis of molecular variance (AMOVA) based on ISSR data, confirmed that the highest percentage of variation of 66% was found among populations, and the lowest of 34% within Serbian populations with a significant genetic difference (P <0.001) (Figure 1).

AMOVA test, based on SSR data set, revealed that 47% of genetic variability was due to the differences among populations and 53% was due to the differences within populations (Figure 1). In four broomrape populations from Republic of Moldova about a high genetic diversity within populations (69%) and a low genetic diversity among populations (31%) were reported by Duca M. and colab. (Duca et al., 2020b). Similar results for the populations of *O. cumana* from Turkey were described, which demonstrated that the differences within populations (66%) were greater than the differences between populations (34%) (Bilgen et al., 2019). Guchetl S. et al. (2014) also

reported that a high proportion of the genetic diversity of 78% was within populations and the 22% was due to variation between populations (Guchetl et al., 2014).

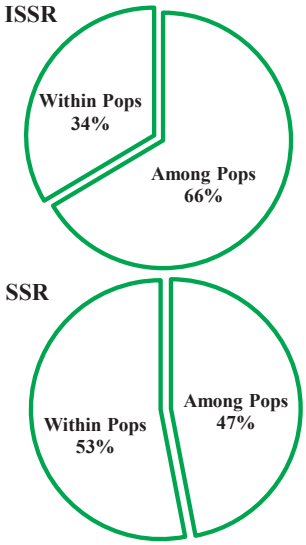

Figure 1. Analysis of molecular variance (AMOVA)

The high polymorphism at interpopulation level, as well as the high power of discrimination identified by the investigated ISSR markers are due to their biallelic and dominant nature.

Also, a significant correlation (r =0.48) was found between ISSR and SSR binary data set by the Mantel test. This correlation reveals the microsatellite nature of both systems of markers and the high level of genetic polymorphism identified by them. The both microsatellite marker sets used have proved a high reproducibility and specificity.

These results showed a high degree of genetic variations within genotypes of *O. cumana* from Serbia which depends directly on environment and the planted lines of sunflower. At the same time, a greater genetic variability determines the evolution of more virulent physiological races of broomrape.

CONCLUSIONS

In the present study the ISSR and SSR microsatellite markers were compared for their efficiency in analyze the genetic diversity of Serbian broomrape populations.

The SSR markers have been more informative in revealing a high intrapopulation genetic variability based for the specific genetic diversity coefficients (H =0.63; PIC =0.58) than ISSR (PIC =0.33). But, the ISSR markers were found more effective in identifying a high interpopulation genetic variability as evidenced by the higher discriminatory potential (Rp =13.85) compared to the SSR (Rp =2.46).

These results were also confirmed by analysis of molecular variance (AMOVA) which showed that for SSR markers 53% of the total genetic variability was due to differences within the populations, and for ISSR markers 66% was attributed to differences among the populations.

Based on the calculated statistical parameters, ISSR markers as: BC807, BC835, BC841, BC857, $(AG)_8YA$, $(CTC)_4RC$, $(CAA)_5$, $(CAG)_5$, $(CT)_8TC$ together with SSR: Ocum-052, Ocum-059, Ocum-075, Ocum-087, Ocum-108, Ocum-196, Ocum-197, Ocum-206 were the most efficient markers and can be successfully utilized in estimating genetic diversity of *O. cumana* populations.

Findings from this research showed evidence for high degree of genetic variations among and within populations of *O. cumana* from Serbia which may lead to change in virulence of parasite and appear more virulent physiological races of broomrape in the future.

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