EFFICIENCY OF MICROSATELLITE MARKERS IN GENOTYPING OF *OROBANCHE CUMANA* POPULATIONS

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Abstract

Microsatellite (SSR) markers have been accepted and employed as useful tools for measuring genetic diversity and divergence within and among populations. In this study, the utility of 15 SSR markers in discrimination of 33 *Orobanche cumana* (broomrape) populations from different geographical locations (Moldova, Romania, Bulgaria, Serbia, Turkey, China) was assessed. A total of 279 *O. cumana* plants were genotyped and 110 alleles identified. The level of genetic polymorphism of SSR markers was evaluated by calculating the *effective number of alleles per locus* (Ne), which demonstrated an average of 5.243, *Polymorphic Information Content* index (PIC: 0.745), *Nei's genetic diversity* (H: 0.782) and, *Resolving power* (Rp: 5.794). Most markers showed PIC values higher than 0.5, indicating a big genetic polymorphism in *O. cumana* populations. Based on the Rp index and PIC values, genetic diversity in the broomrape populations from Turkey (Rp: 4.774, PIC: 0.722) and Moldova (Rp: 4.394, PIC: 0.716) was higer than in other populations. However, the studied microsatellite markers system characterized very well the genetic structure of all *O. cumana* populations included in this study. Seven SSR markers (Ocum-052, Ocum-059, Ocum-074, Ocum-081, Ocum-087, Ocum-196, Ocum-197) were selected based on the statistical analysis as the most informative and efficient markers for measuring genetic diversity in *O. cumana*.

Key words: *SSR markers, genetic polymorphism, genotyping, population, Orobanche Cumana*

Microsatellite markers also known as *simple sequence repeats* (*SSR*) are DNA sequences that consist of short, tandemly repeated motifs (5-50-fold repetitions) of one to six base pairs in length (Vieira M.L.C. *et al*, 2016). They are widely dispersed across the genome, especially in the euchromatin, coding and non-coding nuclear and organellar DNA, have a known location and can be easily identified by methods of molecular biology (Kalia R.K. *et al*, 2010; Phumichai C. *et al*, 2015).

Over the last years, advances in molecular genetics methodology have led to widespread use of codominant molecular markers, mainly SSR, because of their desirable attributes, such as high variability and informativeness, multiallelic nature, specific chromosomal location, and experimentally reproducible among related species (Mason A.S., 2015; Zeni Neto H. *et al*, 2020).

Thus, SSR sequences are greatly useful in studies of genetic diversity among species, populations and individuals, population structure analysis, conservation and restoration of biodiversity, constructing genetic linkage maps, taxonomy, phylogenetic features of biological species and evolutionary processes in plants (Sheriff O., Alemayehu K., 2018; Vieira M.L.C. *et al*, 2016; Kalia R.K. *et al*, 2010). Microsatellite or SSRs are especially used to analyze genetic diversity, relationship and population structure in different plant species such as cotton (Ditta A. *et al*, 2018), sugarcane (Zeni Neto H. *et al*, 2020), rice (Becerra V. *et al*, 2017), mung bean (Kaur G., *et al*, 2018), peanut (Bosamia T.C. *et al*, 2015), sorghum (Billot C. *et al*, 2013), palm (Kpatènon M.J. *et al*, 2020), onion (Ricciardi L. *et al*, 2020), medicinal plants (El-Bakatoushi R., Ahmed D.G.A., 2017; Zhong A. *et al*, 2019; Stavridou E. *et al*, 2021) etc.

More recently, molecular researches employing SSR markers was focused on the *Orobanche cumana* species (broomrape) a root parasitic plant, specific to sunflower crop, which causes severe yield and quality losses in sunflower production. Thus, some data regarding genetic diversity and population structure of broomrape populations from different countries like Romania, Russia (Guchetl S. *et al*, 2014), Turkey (Bilgen B.B. *et al*, 2019), Bulgaria (Pineda-Martos R. *et al*, 2014), Republic of Moldova (Duca M. *et al*, 2017), Spain (Pineda-Martos R. *et al*, 2013; Martín-Sanz A. *et al*, 2016) etc., have been reported.

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Considering the fact that knowledge of the genetic variability in the broomrape populations is important to develop resistance-breeding strategies for the sunflower crop against parasite, the aim of the current study was to analyze genetic diversity and to estimate the efficiency of the SSR markers in discrimination of broomrape populations.

MATERIALS AND METHODS

The plant material used in this study included a total of 33 broomrape (*Orobanche cumana* Wallr.) populations from different geographical locations: 11 populations from Republic of Moldova (MD), one population from Romania (RO), 4 – Bulgaria (BG), 7 – Serbia (SB), 7 – Turkey (TR) and 3 from China (CN). Seeds of these populations were collected from naturally infested sunflower fields and then used in the greenhouse experiment (Duca M., *et al,* 2021) with sunflower susceptible genotype Performer offered by NARDI (National Agricultural Research and Development Institute) Fundulea. Aerial shoots were frozen in liquid nitrogen and stored at -80 °C until genetic analysis.

Genomic DNA extraction (279 individuals) was performed according to the instructions of the used kit (K0791, *Thermo Scientific*). The quantification of isolated DNA samples was performed by methods described in the literature (Sambrook J., Russell D., 2001).

SSR-PCR amplification reaction was performed in a total volume of 15 µL including 60 ng of DNA, 200 µM each type of dNTP, 0.4 µM SSR primer; 1 U/DreamTaq Green DNA Polymerase in buffer (1x) and 2.5 mM MgCl2 (Thermo Scientific). Amplification program (Veriti-96Well Thermal Cycler, Applied Biosystem): 3 min at 95 °C; 35 cycles: 30 s at 95 °C, 45 s at 57 °C, 1 min at 72 °C; 5 min at 72 °C. PCR products were separated in 8% polyacrylamide gel, TBE buffer (1x), vertical electrophoresis at 230V intensity (Sambrook J., Russell D., 2001). GeneRuler Low Range DNA Ladder SM1191 (Thermo Scientific) was used as the molecular marker. The primers proposed by Pineda-Martos R. (Pineda-Martos R. *et al*, 2013) were used.

Statistical analysis was performed using the following programs: Photo-Capt (version 15.02), GenAlEx 6.5 (Peakall R., Smouse P.E., 2012) and Microsoft Excel Office 2010. *Polymorphic Information Content* index (PIC) and *Resolving power* (Rp) were calculated according to the formula proposed by Botstein D. (Botstein D. *et al*, 1980) and Prevost A. (Prevost A., Wilkinson M.J., 1999), respectively.

RESULTS AND DISCUSSION

According to the obtained results, 14 of 15 studied microsatellite markers are polymorphic loci. Only Ocum-122 is a monomorphic locus (generated one allele: 244 bp), which was excluded from the subsequent analysis.

The polymorphism of the studied microsatellite markers was evaluated using the statistical indices like: N (*total number of alleles/ locus*), Ne (*effective number of alleles/locus*), PIC (*Polymorphic Information Content*), H (*Nei's genetic diversity*) and Rp (*Resolving power*) (*table 1*).

A total of 110 alleles with a length between 76-343 bp were identified. The analysis of the obtained amplification products showed differences in number and size, depending on the marker. Thus, the *total number of alleles* (N) ranged from 3 to 16 alleles per locus. The highest number of alleles was identified for Ocum-197 (16 alleles), Ocum-052 (11), Ocum-087 (10), followed by the Ocum-059, Ocum-074, Ocum-081 and Ocum-196 markers, each of them identifying 8 alleles. The lowest number of alleles was observed in the case of Ocum-174 with 3 alleles (*table 1*).

Table 1

Characteristics of investigated microsatellite markers system

Marker name	Allele size range (*bp*)	N	Ne	PIC	H	Rp
Ocum-052	111-192	11	5.879	0.807	0.830	9.910
Ocum-059	85-136	8	6.285	0.822	0.841	7.890
Ocum-070	100-145	7	3.915	0.704	0.745	5.550
Ocum-074	101-146	8	6.277	0.820	0.841	6.270
Ocum-075	98-147	7	5.023	0.769	0.801	0.020
Ocum-081	76-131	8	5.051	0.774	0.802	6.130
Ocum-087	109-200	10	5.211	0.780	0.808	6.400
Ocum-108	143-196	7	4.305	0.736	0.768	5.160
Ocum-141	192-226	4	3.289	0.634	0.696	4.040
Ocum-160	128-177	7	5.051	0.771	0.802	5.440
Ocum-174	190-211	3	2.999	0.593	0.667	3.860
Ocum-196	187-343	8	7.220	0.846	0.862	7.930
Ocum-197	96-190	16	10.515	0.897	0.905	8.350
Ocum-206	118-164	6	2.375	0.490	0.579	4.170
Mean (±SEM)	**76-343**	**7.857 (±1.796)**	**5.243 (±1.178)**	**0.745 (±0.063)**	**0.782 (±0.050)**	**5.794 (±1.401)**

Note: bp – base pairs; N – total number of alleles/locus; Ne – effective number of alleles/locus; PIC – Polymorphic Information Content; H – Nei's genetic diversity; Rp – Resolving power; ± standard error of the mean in parentheses; highly informative PIC > 0.5; reasonably informative 0.25< PIC <0.5; less informative PIC <0.25 (Botstein D. et al, 1980).

The effective number of alleles per locus (Ne) revealed an average of 5.243 for the entire system of studied microsatellite loci, indicating a relatively high level of genetic variation within *O. cumana* populations. The lowest effective number of alleles was identified by Ocum-206 (2.375) and Ocum-174 (2.999). The primers that had the highest Ne index (Ocum-197: 10.515; Ocum-196: 7.220; Ocum-059: 6.285; Ocum-074: 6.277; Ocum-052: 5.879; Ocum-087: 5.211) were different from those listed above, showing a difference between indices N and Ne (Table 1). The effective number of alleles, calculated based on the relative frequency of alleles, indicates the alleles that are significant for characterizing a marker. Thus, the differences between the N and Ne reveal a diversity of molecular profiles obtained by each marker, as well as the presence of a large number of alleles with relatively low frequencies.

Polymorphic information content index (PIC) ranged between 0.490 (Ocum-206) and 0.897 (Ocum-197) with an average of 0.745 (Table 1). Based on the PIC index, a measure of polymorphism introduced by Botstein et al (Botstein D. *et al*, 1980), a high degree of genetic variability was attested for all analyzed markers. Thus, the PIC values were higher than 0.50 (Botstein D. *et al*, 1980) in the majority of cases, excepting Ocum-206 (0.490). Microsatellites with many alleles and a PIC value near 1.00 are most recommended for genetic variability studies. Overall, the PIC values from this study are similar to those reported by Ziadi et al (2018) for the *O. cumana* populations from Turkey (Ziadi S. *et al*, 2018).

At the same time, the values of the *Nei's genetic diversity* index (H or expected heterozygosity) calculated for the microsatellite sequences confirmed the high level of polymorphism detected by the previously analyzed indices. The values of the coefficient H varied from 0.579 (Ocum-206) to 0.905 (Ocum-197) with an average of 0.782 (*table 1*) and highlighted a high proportion of heterozygous plants within investigated populations. Markers that were remarked in the case of PIC and H indices showed maxime values for Ocum-197, Ocum-196, Ocum-074, Ocum-059, Ocum-052, Ocum-087 loci (Table 1). Also, the comparative analysis of the obtained results through the previously calculated statistical parameters showed that the same markers were distinguished by the highest number of alleles (N) and effective number of alleles (Ne). This finding is determined by the multialelic nature and the ability of microsatellite markers to highlight monolocus polymorphism (Mason A.S., 2015).

The *Resolving power* (Rp) values of the 14 SSR markers varied from 0.020 (Ocum-075) to 9.910 (Ocum-052) with a mean of 5.794 (Table 1). The Rp index depends on the distribution of alleles within individuals and characterizes the ability of each marker to detect the level of allelic variation between genotypes. Four loci showed the highest Rp values, namely Ocum-052 (9.910), Ocum-197 (8.350), Ocum-196 (7.930) and Ocum-059 (7.890), being able to distinguish all 279 individual broomrape plants included in this study.

Efficacity of these SSR markers were also analyzed in relation to the origin of the investigated populations (Table 2).

The most of SSR markers had PIC values higher than 0.50 in all broomrape populations, indicating on the genetic variability at the intrapopulational level.

The highest PIC values were found in the case of: Ocum-052, Ocum-059, Ocum-075, Ocum-087, Ocum-196, Ocum-197 (Table 2). The lowest values were identified by Ocum-160 (0.076) for the populations from Serbia, indicating a very low level of intrapopulational polymorphism, followed by Ocum-206 (0.375) for the populations from Moldova, Romania, Bulgaria, Turkey, Ocum-059 for the samples from China, and Ocum-074 (0.405) for those from Serbia that suggest a moderate level of polymorphism within populations (*table 2*).

Table 2

The level of genetic diversity and differentiation of *O. cumana* populations from different countries estimated by microsatellite markers

Marker name	PIC (*Polymorphic Information Content*)						Rp (*Resolving power*)					
	MD	RO	BG	SB	TR	CN	MD	RO	BG	SB	TR	CN
Ocum-052	0.806	0.768	0.768	0.768	0.839	0.811	9.983	0.000	0.000	0.000	9.641	5.556
Ocum-059	0.812	0.788	0.819	0.806	0.802	0.375	7.983	6.364	9.364	5.673	5.846	0.000
Ocum-070	0.700	0.619	0.645	0.587	0.566	0.715	3.390	2.000	2.318	2.980	4.667	5.778
Ocum-074	0.756	0.782	0.843	0.405	0.798	0.703	7.153	6.909	6.409	1.959	5.282	0.000
Ocum-075	0.768	0.768	0.768	0.768	0.768	0.780	0.000	0.000	0.000	0.000	0.000	0.333
Ocum-081	0.667	0.593	0.694	0.593	0.850	0.744	5.915	0.000	5.273	0.000	7.077	6.000
Ocum-087	0.703	0.785	0.793	0.786	0.846	0.703	0.000	6.545	4.727	4.000	7.897	0.000
Ocum-108	0.724	0.593	0.574	0.695	0.763	0.652	5.220	0.000	3.364	2.000	5.897	2.778
Ocum-141	0.613	0.593	0.593	0.676	0.593	0.592	2.000	0.000	0.000	2.286	0.000	3.778

Ocum-160	0.760	0.748	0.750	0.076	0.664	0.788	6.424	6.364	7.318	2.041	4.615	6.512
Ocum-174	0.593	0.593	0.593	0.591	0.593	0.586	0.000	0.000	0.000	3.429	0.000	5.222
Ocum-196	0.838	0.746	0.846	0.703	0.787	0.771	7.254	5.455	9.091	0.000	4.615	5.333
Ocum-197	0.900	0.872	0.878	0.751	0.864	0.859	6.203	8.909	7.091	8.490	11.282	9.556
Ocum-206	0.375	0.375	0.375	0.526	0.375	0.774	0.000	0.000	0.000	4.000	0.000	6.667

Note: MD – Moldova; RO – Romania; BG – Bulgaria; SB – Serbia; TR – Turkey; CN – China; highly informative PIC > 0.5; reasonably informative 0.25< PIC <0.5; less informative PIC <0.25 (Botstein D. et al, 1980).

This finding completes the previously formulated conclusion regarding the parameters N and Ne, which in the case of Ocum-075 generated the same number of alleles for all analyzed samples (excepting China), indicating the similarity of molecular profiles of these genotypes. Similar results were found inclusive for sequences Ocum-174 and Ocum-206, which were characterized by a zero contribution in the differentiation of the populations from the Republic of Moldova, Romania, Bulgaria and Turkey. Some molecular markers demonstrate no capacity to differentiate the individuals of more than one broomrape population, as for example: Ocum-052 (for populations from Romania, Bulgaria, Serbia), Ocum-141 (Romania, Bulgaria, Turkey), Ocum-081 (Romania, Serbia), and Ocum-087 (Moldova, China) (Table 2). However, SSR markers with a high capacity to discriminate genotypes were identified. Thus, Ocum-197, Ocum-059, Ocum-074, Ocum-196 and Ocum-087 showed the highest values for the majority groups of populations. It is also important to note that the marker Ocum-052, which highlighted a zero efficacy in differentiation of broomrape genotypes from Romania, Bulgaria and Serbia, showed the highest values of Rp index for other populations, originating from the Republic of Moldova (9.983), Turkey (9.641) and even from China (5.556). Thus, there was determined a high level of discrimination of broomrape populations by the markers: Ocum-197 (Rp: 6.203-11.282), Ocum-059 (5.673-9.364), Ocum-074 (1.959-7.153), Ocum-052 (5.556-9.983), Ocum-196 (4.615-9.091) and Ocum-087 (4.000-7.897).

To characterize the SSR marker system used in the genotyping of different populations of *O. cumana*, their efficacy and informativeness were evaluated based on the average values of the PIC and Rp parameters calculated per group of populations. According to the data obtained for PIC, the set of markers used in this study reveals large amount of genetic variations within and among populations. The PIC and Rp indices showed maximum average values for the populations from Turkey (0.722 and, respectively, 4.774), Republic of Moldova (0.716 and 4.394), Bulgaria (0.710 and 3.925) and China (0,703 and 4.109) (*figure 1*).

According to the obtained results, the analyzed marker system is more informative and useful for evaluation of diversity and genetic structure of *O. cumana* populations belonging from Bulgaria, Turkey, Republic of Moldova and China.

The efficiency of this set of SSR markers for genetic diversity studies in *O. cumana* has been previously confirmed for populations of sunflower broomrape from Spain (Pineda-Martos R. *et al*, 2013), Romania, Russia (Guchetl S. *et al*, 2014), Bulgaria (Pineda-Martos R. *et al*, 2014), Republic of Moldova (Duca M. *et al*, 2017, 2020a, 2020b), Turkey (Ziadi S. *et al*, 2018; Bilgen B.B. *et al*, 2019) etc. However, scientific researches about the genetic diversity of *O. cumana* populations with various molecular markers are limited.

In the present study, all microsatellites (SSR) were polymorphic, excepting Ocum-122 (monomorphic locus). Similar results, were reported by Pineda-Martos et al (2013), which evaluated the genetic structure of 50 populations from Spain with the same 15 SSR markers and determined that all loci are polymorphic, including Ocum-122.

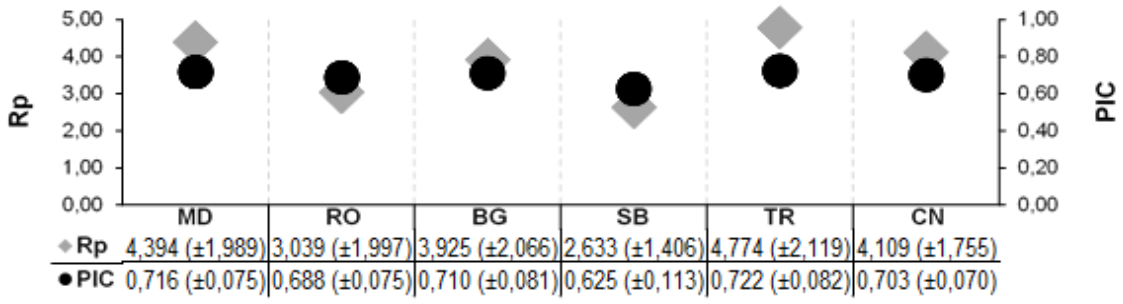

Figure 1 **The mean of polymorphism information content (PIC) and resolving power (Rp) values for loci revealed by SSR markers in *O. cumana* populations from different countries**

Note: PIC – *Polymorphic Information Content*; Rp – *Resolving power*; MD – Moldova; RO – Romania; BG – Bulgaria; SB – Serbia; TR – Turkey; CN – China; ± standard error of the mean in parentheses.

Bilgen *et al* (2019) reported that 8 SSR loci (investigated in our study as well) were polymorphic in 5 populations of *O. cumana* from Turkey. Contrary to previous data, the mean number of alleles per locus (7.857) detected in the current study among 279 *O. cumana* individuals was higher than the mean number of alleles per locus (2.2) reported by Pineda-Martos et al (2014) for 244 individual broomrape plants from Bulgaria. This difference might be attributed to the diverse *O. cumana* populations investigated in this study, which included populations from different geographical origins. The same set of markers were also recently used by Belay et al (2020) to study the genetic diversity of *O. crenata* populations collected from Ethiopia. According to those results 11 SSR markers from 30 were effective to study the diversity of *O. crenata* populations. The average number of alleles (9.6), gene diversity (0.82) and polymorphic informational content (0.80) values for SSR loci were similar with the results obtained by us at *O. cumana*.

PIC is a significant parameter of a marker, which indicate its potential to differentiate various individuals. In our study, PIC showed great average value for all investigated broomrape populations (0.745, with range 0.625-0.722) (Figure 1). These results are in agreement with the values reported by Meszaros et al (2007), which analyzing the genetic diversity in barley by using 26 SSR markers, established a high mean PIC value (0.72, with a range of 0.14-0.93). Romero et al (2019) studying the genetic identity of 26 varieties of quinoa based on 20 SSR markers revealed an effective number of alleles of 5.36, mean heterozygosity of 0.80, and a mean polymorphic information content of 0.81. A recent study performed by Guzmán et al (2020) using a set of 21 microsatellite markers for 42 *Capsicum* genotypes (representing 11 species) have also showed an average PIC value of 0.78.

Several studies have suggested that the greatest genetic variability is observed when different species were genotyped using SSR markers. In conclusion, SSR markers give a large amount of polymorphic information and they are ideal for distinguishing between genotypes that are genetically similar.

CONCLUSIONS

The effectiveness of microsatellite markers in the genotyping of different *O. cumana* populations was evaluated using different statistical parameters and a high level of genetic diversity (N: 7.857; Ne: 5.243; PIC: 0.745; H: 0.782; Rp: 5.794) was revealed.

Based on the analysis of allelic polymorphism, the markers (Ocum-052, Ocum-059, Ocum-074, Ocum-081, Ocum-087, Ocum-196, Ocum-197), which presented the highest values of calculated statistical parameters (N: 8-16 alleles; Ne: 5.051-10.515; PIC: 0.774-0.897; H: 0.802-0.905; Rp: 6.130-9.910) were highlighted. These markers are the most informative and can be used to study genetic diversity of *O. cumana* populations. The less informative markers were: Ocum-141, Ocum-174 and, Ocum-206.

Genetic diversity of Turkish (Rp: 4.774; PIC: 0,722) and Moldavian (Rp: 4.394; PIC: 0,716) *Orobanche cumana* populations was higher than in other populations. However, the studied microsatellite (SSR) markers system characterized very well the genetic structure of all *O. cumana* populations included in this study.

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