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GENETIC RELATIONSHIPS AMONG DIFFERENT BROOMRAPE RACES FROM THE BLACK SEA BASIN

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Abstract

Microsatellite loci represent prevalent molecular markers for assessing genetic diversity, phylogenetic and evolutionary relationships between different in origin broomrape taxon. In the present study, the genetic differences and relationships among 3 broomrape races from Bulgaria, Turkey, Moldova and Romania were identified by ISSR markers. The obtained results on genetic diversity parameters showed that race H is more variable than races G and E. However, the gene diversity within populations was the greatest in race G, followed by race H and E. The limited gene flow for races H and G determined significant genetic differentiation of populations. Conversely, a comparatively moderate gene flow for race E pointed to a little differentiation among populations. These findings were also corroborated by AMOVA, demonstrating high genetic diferences within (89%) and lower (11%) among races. The investigation also revealed the existence evolutionary differences between all races. This situation proves the action of evolutive molecular mechanisms which offered, in time, discrete genetic particularities at broomrape, indicating that the existing races are unstable over time and they are constantly adapting to the new more sunflower resistance hybrids.

Key words*: Orobanche cumana, ISSR markers, genetic relationships, broomrape races.*

INTRODUCTION

The current situation related to progression of broomrape in modern agriculture, and therefore reducing of the world's total production of sunflower seed and oil is worrisome. Moreover, it should be kept in mind that, depending on the agro-ecological conditions, the different factors induce imbalances in the coexistence degrees between the parasite and its host, resulting in significant harvest losses, for example, the small number of intensive autochthonous varieties and hybrids (Strelnikov, 2017), the application of large doses of fertilizers (Pinashkin, 2012), non-compliance with rotation periods (Lukomets & Antonova, 2015), failure to comply of some technological procedures (Pimakhin et al., 2000; Pinashkin, 2012).

One of the most effective and environmentally safe ways to combat this parasitic plant is the development of continuous breeding programs, aimed at creating of sunflower hybrids resistant to new highly virulent broomrape biotypes.

To do this, it is important to know the racial structure of parasite populations, the characteristics of new virulent biotypes and their relationship with sunflower. Finding and creating of resistance sources to the new most virulent broomrape races is becoming increasingly relevant.

For about 200 years, eight broomrape races (A, B, C, D, E, F, G and H) were recorded (Molinero-Ruiz et al., 2015). Over the last 10-12 years, new more aggressive biotypes, like E, F, G and H are considered as the most virulent (Melero-Vara et al., 2000; Kaya et al., 2004), which they are rapidly spreading and taking over new areas across the globe (Fernández-Martínez, 2012). At present, races H+ and I were registered (Duca et al., 2017). The problem of highly virulent broomrape races is very important for countries geographically located in the Black Sea region, including Bulgaria, Turkey, Romania and Republic of Moldova (Kaya et al., 2004; Duca, 2014, 2019).

In Bulgaria, infections of sunflower by races A and B of *O. cumana* were reported for the first time in 1935. Then in 1966, it was found the sort Peredovik has been the most severely affected by the race C (Petrov, 1968). At the beginning of the 1990s, the new physiological races of the parasite D and E massively

attacked the area under crops of sunflower in many regions of the country (Shindrova et al., 1998). In the year 2000, it was found a very aggressive population of broomrape in the northeastern part of Bulgaria identified as race H which was not reported later (Shindrova, 2006; 2012). In the first years of the 21st century race E of *O. cumana* remained predominant in Bulgaria until race F was widely spread (Shindrova, 2006). Nowadays, the prevailing presence of races E (35%), G (80%) and a decline in the presence of race F (16%) was determined in the sunflower growing areas of Bulgaria (Batchvarova, 2014; Encheva, 2018).

The expanded of the parasite across sunflower cultivation areas in the Trakya region of Turkey was first reported in the second half of 1940s (Demirci & Kaya, 2009). The period of immunity until the early 80s ensured by the introduction of sunflower resistant sorts, in the 1990s gave way to explosion of virulence of new race F in the Trakya region (Demirci & Kaya, 2009). Nowadays, race F is widely distributed in Trakya region, which accounts for more than half of the total sunflower areas in the country (Kaya et al., 2004; Semerci et al., 2010). Moreover, the existence of more virulent race G was confirmed in several locations of the Trakya region (Molinero-Ruiz et al., 2013; Kaya, 2014). In the past few years, the infections of sunflower crops by *O. cumana* have also become frequent in other important production areas such as Cukurova, Middle Anatolia and Black Sea regions, but the race/s of the parasite have not been determined yet (Evci et al., 2011; Kaya, 2014).

In Romania, the broomrape has been observed since the 1940s, which began to rapidly forming races with high virulence (Vranceanu et al., 1980; Pacureanu, 2014). In the period of 1960–1963, two broomrape races were identified as A and B, in the years 1975-1980, three other races (C, D and E), after 16-19 years (in 1996-1999) appeared race F, and recently, after a period of only 8-10 years, a new population of the parasite that overcomes the genes of resistance to race F, called race G (Pacureanu, 2014). As occurred in other countries, races A to E in Romania were effectively controlled through genetic resistance for several decades, until race F was identified (Pacureanu, 2014). In recent years, especially in the area of Tulcea and Brăila, the presence even more virulent form of the parasite (race H) was found (Rîşnoveanu et al., 2016).

The close commercial ties of Moldova with many states of the East and West, mild climate, 76% fertile agricultural soils and isolated private farms with numerous difficulties in management contributed to the creation of favourable conditions for the rapid emergence of new local broomrape races. The first-ever report of broomrape (race B) to the sunflower growing areas in Moldova dates back to 1937 (Arhiva Naţională a Republicii Moldova. Fond 3, inv. 2, d. 376). Several decades later (in the 1960s), a new broomrape biotype in different regions of the country, named the Moldovan race or race C, has been identified (Sharova, 1969). The replacing of the old sunflower susceptible cultivars with the new resistant ones allowed to forget about the problem until the 1990's. In the early 2000s, broomrape again manifested itself in its highly aggressive new races E and F, and, recently, more-virulent races G and H have been recorded in sunflower growing areas of Moldova (Gisca et al., 2013; Duca, 2014; 2019).

Identification of broomrape races is useful for breeding studies and analysis about genetic diversity and characterization of *O. cumana* populations by molecular markers. These are effective possible tools of gaining knowledge about the genetic structure of race and their evolution progress.

Although numerous molecular studies have been carried out, regarding the genetic structure and variability of the sunflower broomrape in various countries, the mechanisms of races appearance and their evolution are not yet fully understood.

The main purpose of our study was to investigate the genetic diversity and genetic differentiation of 23 *O. cumana* populations from the Black Sea basin, belonging to 3 races, using ISSR (Inter Simple Sequence Repeats) molecular markers. This technique is based on the amplification of DNA segments between two microsatellite repeated regions by means of unanchored or anchored at 3` or 5` end with 1 to 4 degenerate bases extended into the flanking sequences. For the first time this

method was proposed and implemented on the different animals and plants by Zietkiewicz and Gupta in 1994 (Zietkiewicz et al., 1994; Gupta et al., 1994). Thus, the information from this study allows a better understanding of parasite diversity and may be useful in different breeding programs on the creation of sunflower hybrids resistant to broomrape.

MATERIALS AND METHODS

Plant material. In the frame of this study, 23 sunflower broomrape populations from 4 countries (Bulgaria, Turkey, Republic of Moldova and Romania), located in Black Sea region were used. In order to establish race composition of them, greenhouse experiment was realised. The germination of *O. cumana* seeds was realized in pots, using four Romanian differential lines and hybrids of sunflower for the races E, G and H, as well as susceptible control Performer hybrid, that does not contain specific resistance genes (Or) (Clapco, 2021). A set of sunflower differential lines for broomrape races from A to E, each line carrying a single dominant gene (Or1 through Or5, respectively) were kindly provided by National Agricultural Research and Development Institute (Fundulea, Romania). Two broomrape populations were classified as race E, five identified as race G and the other 16 belonged to race H (Table 1).

Table 1. Origin and race status of 23 studied *O. cumana* populations

Nr.	Population code	Population/ Origin	Number of accessions
Race E			
1.	RM12	Căzănești/Telenești/Moldova	11
2.	RM13	Congaz/Comrat/Moldova	12
Race G			
3.	RM10	Popeasca/Stefan Voda/Moldova	21
4.	RM11	Chisinau/ Moldova	22
5.	B1	Debovo/Pleven/ Nikopol/Bulgaria	12
6.	B2	Selanovtsi/Vratsa/ Oryahovo/Bulgaria	12
7.	T5	Trakia/Turkey	11
Race H			
8.	RM1	Izbiște/Criuleni/Moldova	12
9.	RM2	Svetlii/Comrat/Moldova	12
10.	RM3	Taraclia/Moldova	11
11.	RM4	Soroca/Moldova	12
12.	RM5	Alexander field/Cahul/Moldova	8
13.	RM6	Bălți I/Moldova	12
14.	RM7	Bălți II/Moldova	11
15.	RM8	Prepelița/Sîngerei/Moldova	12
16.	RM9	Grigorievca/Căușeni/Moldova	12
17.	B3	Radnevo/Stara Zagora/Radnevo/Bulgaria	12
18.	B4	Rosenova/Dobrichka/Dobrich/Bulgaria	12
19.	T1	Keşan/Edirne/Thrace/Turkey	9
20.	T2	Adana/Turkey	7
21.	T3	Merkez/Edirne/Thrace/Turkey	7
22.	T4	Lüleburgaz/Kirklareli/Thrace/Turkey	7
23.	R1	Brăila/Romania	12

Fresh tissue samples from each population were collected and stored at –70°C until DNA extraction. In total 269 broomrape accessions representing 23 populations were included in a comparative study on genetic diversity as well as genetic structure analysis of populations.

DNA extraction. Frozen plants were used for total DNA extraction by means of Thermo Scientific GeneJET Plant Genomic DNA Purification Mini Kit #K0791 according to the manufacturer's protocol (Thermo Fisher Scientific, USA). Quality and quantity of isolated DNA were determined by spectrophotometer (model T60 UV-VIS, PG Instruments Limited, England) and, also checked by 0.8% agarose gel electrophoresis in 1xTAE buffer (40 mM Tris-acetat, pH 8.0; 1mM EDTA) at 2.5 V/cm (Sambrook & Russell, 2001).

ISSR analysis. Thirteen most effective ISSR primers were used for PCR amplification, which were selected out of readily available 14 primers (Table 2). This set included those primers reported by Benharat et al. (2002) in analyse of genetic diversity among broomrape populations.

Table 2. Inter simple sequence repeat (ISSR) primers used in this study

Nr.	Primer code	Sequence (5′→ 3′)	NBN	GC, %
1.	BC 807	AGAGAGAGAGAGAGAGT	17	47
2	BC 810	GAGAGAGAGAGAGAGAT	17	47
3.	BC 835	AGAGAGAGAGAGAGAGYC	18	56
4.	BC 841	GAGAGAGAGAGAGAGAYC	18	56
5.	BC 857	ACACACACACACACACYG	18	56
6.	$(CAA)_5$	CAACAACAACAACAA	15	33
7.	$(GACA)_4$	GACAGACAGACAGACA	16	50
8.	$(CA)_8RG$	CACACACACACARG	14	57
9.	$(CTC)_4RC$	CTCCTCCTCCTCRC	14	71
10.	$(CAG)_5$	CAGCAGCAGCAGCAG	15	67
11.	$(CT)_8TC$	CTCTCTCTCTCTCTCTTC	18	50
12.	$(CA)_6AC$	CACACACACACAAC	14	50
13.	$(AG)_8YA$	AGAGAGAGAGAGAGAGYA	18	50

R = (A, G); Y=(C, T); NBN = nitrogenous bases number; GC, % = percentage content of cytosine (C) and guanine (G) nucleotides in primer.

The PCR solution (15 µl) contained: 60 ng ADN; 200 µM dNTP mixture (dATP, dCTP, dGTP, dTTP); 0.4 µM concentration of each primer; 1 U/µL DreamTaq Green DNA polymerase in buffer solution (1x); ultrapure water; 2.5 mM $MgCl_2$ (Thermo Scientific, USA). The amplification was carried out in the thermocycler Genset 9700 (Applied Biosystems, USA) according to the standard steps of polymerase chain reaction (Sambrook & Russell, 2001). PCR reactions were

performed under the following conditions: 5 min. at 95°C for initial denaturation, 35 cycles of 30 s at 95°C (denaturation), 45 s at 45°C (annealing), and 2 min at 72°C (extension), followed by a final extension of 5 min at 72°C. Amplified products were separated by electrophoresis according to their molecular weight on 2% agarose gels submerged in 1xTAE buffer, and then stained with ethidium-bromide solution (final concentration of 0.5 μg/ml) for 20 min. The molecular size of amplicons was estimated by running GeneRuler Express DNA Ladder ready-to-use SM1553 in the gel as standard size marker (Thermo Fisher Scientific, USA). The amplified DNAs visualized on a UV transilluminator (wave length λ = 305 nm) and documented using the Gel documentation system Doc-Print VX2, model SXT-F20.M (Vilber Lourmat, France).

Data analysis. The DNA amplified fragment analysis was carried out with the Photo-Capt V.15.02 program. The data generated by the program were compiled into a binary data matrix, namely (1) as presence and (0) absence of ISSR fragments for each primer.

The polymorphism information content (PIC) of each dominant ISSR marker or its discriminatory ability, that depends on the number of alleles detected and their distribution frequency, was computed according to Roldán-Ruiz (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.

Resolving power (*Rp*) is a parameter used to characterize the ability of the primer/s to detect the differences in allele's distribution between a large number of the sampled genotypes and divide it's into a group (Prevost & Wilkinson, 1999):

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

The Simpson's coefficient (h_j) or discrimination potential of each primer was calculated by formula (Lüdtke et al., 2010):

$h_j = \sum \left(\frac{1 - \sum p_i^2}{n} \right)$, where p_i is the frequency of the i-th allele and n is corresponds to the number of loci detected by each primer. A value of 1 indicates that the primer is able to discriminate between all samples, and a value of 0 indicates that all samples are identical.

The marker index (*MI*) is used to evaluate the efficiency of a marker system and calculated as the product of the effective multiplex ratio (*EMR*) and the polymorphism information content (*PIC*) (or average expected heterozygosity (*He*)) (Powell et all, 1996): $MI = PIC \times EMR$, where EMR is defined as the product of the total number of loci per primer (n) and the fraction of polymorphic loci (n_p) by formula: $EMR = n_p \left(\frac{n_p}{n} \right)$.

The observed number of alleles (Na), effective number of alleles (Ne), Nei's gene diversity (H), Shannon's Information index (I), number of polymorphic loci (NPL), percentage of polymorphic loci (PPL), total genetic diversity (Ht), genetic diversity within populations (Hs), coefficient of genetic differentiation (Gst) and gene flow among populations (Nm) were calculated using POPGENE V.1.32 software. XLSTAT 2014 V.2014.5.03 and also POPGENE V.1.32 software packages were used to calculate the Nei's and Euclidean genetic distances as well as to construct dendrograms by UPGMA and Ward's methods. AMOVA (Analysis of molecular variance) test as implemented in GenAlex 6.501 was used to analyse of the genetic diversity and genetic structure of broomrape populations.

RESULTS AND DISCUSSIONS

In the present study, the different origin broomrape populations from the Black Sea basin were selected in order to acquire a deeper insight into their population structure and genetic variability at the race level. Within physiological races, there are exchanges of genes/polygenes (introgression) that deviate from the normal distribution of the rare segregating recessive forms, and also the rare heterozygous forms, which later are eliminated by natural selection (Louarn et al., 2016). These continuous variations can only be maintained under mutation pressure, thus contributing to the appearance of new characters (Pineda-Martos et al., 2013), such as

the level of aggression of the sunflower broomrape races (Rodríguez-Ojeda et al., 2013).

For these reasons, it is of scientific and practical interest to investigate the broomrape races discrimination using molecular markers (i), evaluate their comparative genetic diversity and detect the different levels of variability within race E, G and H (ii) and to ascertain whether the genetic relationships findings based on ISSR markers confirm or conflict the physiological data (iii).

Out of 14 ISSR primers tested, 13 primers have proved a high reproducible and informative in estimating broomrape genepool available. Investigation of the genetic diversity among 23 *O. cumana* populations belonging to 3 races based on molecular ISSR-markers has allowed for revealing both the significant differences and similarities depending on the analysed race or type of primer. The polymorphism level in *O. cumana* races (E-86.64%, G-90.80%, H-94.48%) is high enough (Table 3).

Table 3. The marker polymorphism and amplification profile of 13 ISSR primers used to analyze the genetic diversity of *O. cumana*

Primer	Fragment sizes, bp				Percentage of polymorphism, %			
	O. cumana	race E	race G	race H	*O. cumana*	race E	race G	race H
BC807	300-4358	340-2200	340-4358	300-3000	89.66	75.00	80.77	91.3
BC810	430-5353	430-2500	430-4749	430-5333	92.86	83.33	89.47	92.59
BC835	444-4577	490-2536	490-3899	444-4577	100.00	88.89	95.00	90.48
BC841	280-5000	280-1660	320-2680	280-5000	96.55	81.82	95.00	96.30
BC857	385-3000	420-2340	385-2429	420-3000	95.65	100.00	94.44	100.00
$(CA)_6AC$	395-3759	950-2558	395-3759	744-2558	91.30	100.00	94.12	90.00
$(CA)_6RG$	454-2476	454-1402	454-2476	454-1749	93.75	70.00	91.67	85.71
$(CT)_8TC$	689-5333	900-2520	689-5333	840-4867	96.30	90.91	100.00	95.24
$(AG)_8YA$	230-4699	536-1320	230-4699	230-4699	97.22	100.00	100.00	91.67
(CAA)5	429-4254	660-2200	429-4254	660-3348	87.50	90.00	80.95	95.00
(CAG)5	620-5000	620-2575	620-5000	620-4838	96.43	100.00	95.65	100.00
$(CTC)_4RC$	418-5353	500-3898	418-5133	500-5353	96.43	71.43	86.36	100.00
(GACA)4	388-3902	1117-2134	388-3902	816-3118	86.67	75.00	76.92	100.00
Total/Mean	230-5353	280-3898	230-5333	230-5353	93.87	86.64	90.80	94.48

For race E, 13 ISSR primers generated a total of 129 amplicons in different size ranging from 280 to 3898 bp out of which 113 (87.60%) products were polymorphic, 15 (11.63%) were specific and one (0.78%) amplicon in 700 bp, generated by the primer $(CTC)_4RC$ was monomorphic (Figure 1).

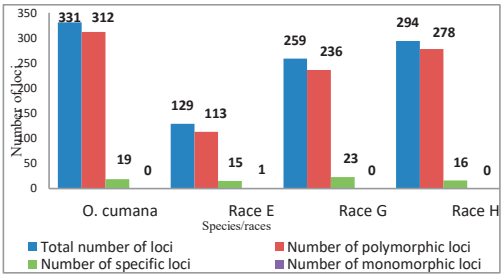


Figure 1. Marker polymorphism of 13 ISSR primers used to analyze the genetic diversity of *O. cumana*

It should be noted that for race G these same primers produced a total of 259 amplified bands, which size ranged from 230 to 5333 bp out of which 236 (91.12%) products were polymorphic, 23 (8.88%) were specific (Figure 1).

However, for race H, the highest number of loci (a total 294) was found, which range from 230 to 5353 bp, out of which 278 (94.56%) products were polymorphic and 16 (5.44%) were specific (Figure 1).

From our data, the specific loci were detected in all races, namely for race E-11.63% or 15 out of the total 129 studied loci, at race G-8.88% or 23 loci out of 259, race H- 5.44% or 16 loci out of 294 (Figure 1). The monomorphic band (700 bp with $(CTC)_4RC$) was observed only in the race E. These data clearly shows that the race E contains the largest percentage of specific loci that distance it evolutionarily from the race G and to a greater extent from the race H.

According to the frequency distribution analysis (331 alleles for 13 loci at 3 races), the number of abundant alleles are with roughly equal in all 3 races. However, it is worth noting that the rare alleles are 15 at race E, but races G and H each has 47 and 48, from which we conclude that the genome of the race E is more stable than the races G and H genomes. Regarding the common alleles in the genome of

race E was revealed only 58, while in the genomes of races G and H - there are 169 and 201, respectively (Figure 2).

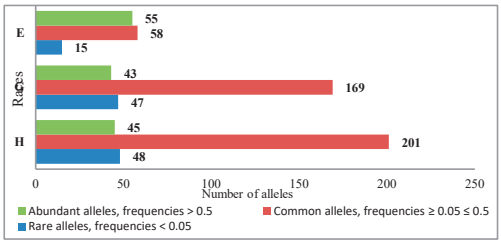


Figure 2. Frequency distribution
of all 331 alleles at 13 loci for 3 races

From these results we can conclude that races G and H are more similar to each other than to race E, which confirms again that race E is more distant of races G and H in evolutionary time. Also the fact should be emphasized that for the first time race E was mentioned in 1980 in Spain (Melero-Vara et al., 1989), rase G – in 2006 in Bulgaria (Shindrova & Penchev, 2012) and race H – in 2014 in Romania (Păcureanu et al., 2014).

However, we received more information about population structure of *O. cumana* races while analysing the ISSR bands distribution on 7 groups of allele frequencies (0.0-0.2, 0.2-0.3, 0.3-0.4, 0.4-0.6, 0.6-0.7, 0.7-0.9, 0.9-1.0) (Figure 3). These results showed the lowest diversity according to different allele frequency at race E in comparison with the races G and H. It just confirms once again the conclusions on the availability of immigration activity or gene passive transfer into populations of race E through gene flow, that over time violates the interpopulation isolation, equalizes gene background and serves as a deterrent regarding speciation. It was found, that the increase genetic diversity for the different allele frequency in races G and H is due to the activating the genetic drift process. Simultaneously, against this background, there was observed the dominant number of rare alleles which could lead to further formative mutations.

The adaptive challenges that matter most in the survival of species in environmental changes are what lead to evolutionary changes in the populations that form this species. Assessment of the level of genetic variation in broomrape populations and also within races to which they relate, it is determined largely by the percentage of polymorphism. Moreover, understanding the mechanisms and selective forces of gene flow between the populations may not only contribute to knowledge of parasitic species evolutionary and phylogeny, but it is also critical to these processes management.

Evaluation of the genetic diversity of broomrape populations by the statistical parameters revealed that the high proportion of diversity among the different broomrape races was accounted for race H (Na=1.89, Ne=1.37, H=0.23, I=0.36, NPL=294, PPL=88.82) and lower values of these index - for races G and E. In addition, the total gene diversity (Ht) and coefficient of gene differentiation among populations (Gst) of race H, were the highest (0.25 and 0.62, respectively), which cannot be said about races G (0.21 and 0.52, respectively) and especially E (0.12 and 0.30, respectively) (Table 4).

However, the gene diversity within populations was the greatest in race G (Hs=0.10), followed by race H (Hs=0.09) and E (Hs=0.08). Limited gene flow for races H and G (0.30 and 0.45,

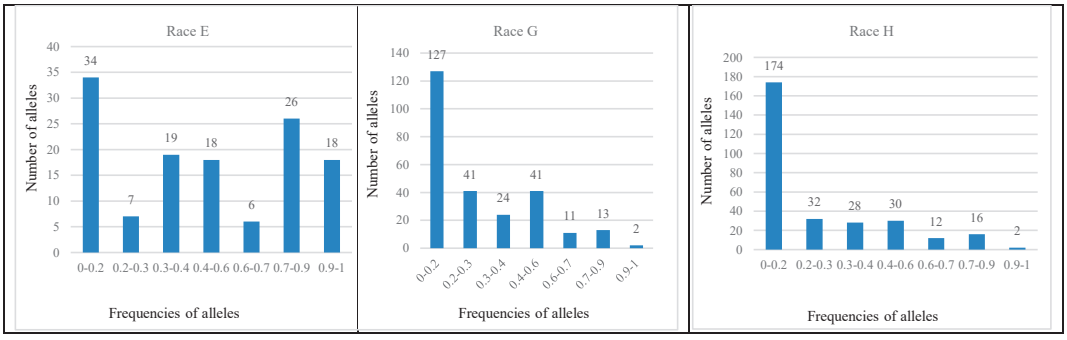


Figure 3. Distribution of the ISSR fragments with different frequencies in 3 *O. cumana* races from the Black Sea Basin

respectively) showed a significant level of genetic differentiation of populations. Conversely, a comparatively moderate level of gene flow was found to be for race E (Nm=1.19), which points to a little differentiation among populations (Table 4). The genetic diversity parameters of the races G (H=0.22, I=0.34, NPL=259, PPL=78.25) and H (H=0.23, I=0.36, NPL=294, PPL=88.82) were higher than at race E (H=0.12, I=0.18, NPL=128, PPL=38.67). This is explained by the moderate level of gene flow for the race E (Nm=1.19), which prevents the differentiation their populations (Gst=0.30) and the limited gene flow for races H and G (Nm=0.30 and Nm=0.45, respectively), that favors the growth of the significant level of genetic differentiation of populations these races (Gst=0.62 and Gst=0.52, respectively).

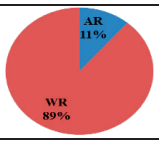
Table 4. Genetic diversity parameters and genetic differentiation of *O. cumana* species/races using 13 ISSR markers

Species/ races		Number of analysed samples	Na	Ne	H	I	Ht	Hs	Gst	Nm (Gst)	NPL	PPL
O. cumana	Mean	269	2.00	1.37	0.23	0.37	0.24	0.18	0.28	1.30	331	100.00
	SD		0.00	0.32	0.17	0.22	0.03	0.02				
E	Mean	23	1.39	1.19	0.12	0.18	0.12	0.08	0.30	1.19	128	38.67
	SD		0.49	0.31	0.17	0.25	0.03	0.02				
G	Mean	78	1.78	1.37	0.22	0.34	0.21	0.10	0.52	0.45	259	78.25
	SD		0.41	0.36	0.19	0.26	0.04	0.01				
H	Mean	168	1.89	1.37	0.23	0.36	0.25	0.09	0.62	0.30	294	88.82
	SD		0.32	0.33	0.17	0.23	0.03	0.01				

Na=observed number of alleles; Ne=effective number of alleles; H=Nei's gene diversity; I=Shannon's Information index; Ht=total gene diversity; Hs=gene diversity within populations; Gst=(Ht-Hs)/Ht, coefficient of gene differentiation among populations; Nm=gene flow among populations from Gst; NPL=number of polymorphic loci; PPL=percentage of polymorphic loci; SD=standard deviation.

AMOVA analysis was carried out on the distance matrix, demonstrating significant genetic differences between and within races. Of the total genetic diversity, 11% was due to differences among races and 89% was contributed to differences within races ($P<0.001$) (Table 5). Out of this analysis, it follows that the highest genetic diversity of broomrape occurred within races (89%).

Table 5. AMOVA analysis of *O. cumana* different origin populations grouped by race status based on ISSR data

Source of variation	Degree of freedom, df	Sum of squares, SS	Mean squares, MS	Estimate of variance component, Est. Var.	Differentiation, (Phi), P<0.001	Molecular dispersion distribution diagram
Among races	2	718.427	359.214	4.627	PhiPT=0.112	
Within races	266	9713.335	36.516	36.516		
TOTAL	268	10431.76	-	41.143	P<0.001	

Our results confirm that the races at the first stage of divergent evolution appear and develop within populations in the geographically grouped regions, being influenced under the same environmental factors. For these reasons, the process of natural selection has a more or less uniform character, so that most races possess a common pool of genes (Duca & Martea, 2020) and common phenotypic characters (Clapco et al., 2020). The pairwise race PhiPT values revealed minimum variation between races H and G (0.096), similar to H and E (0.104). Maximum variation between races G and E (0.231) was established (Figure 4).

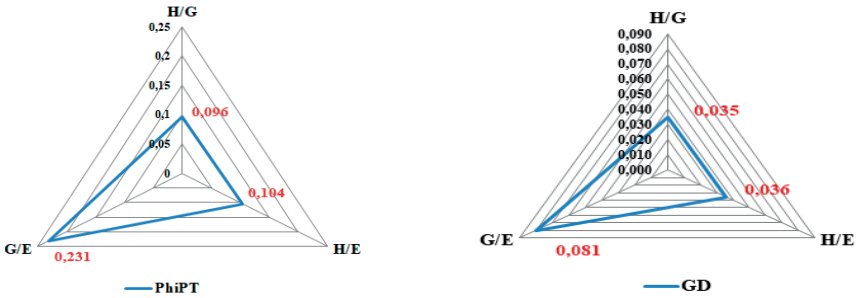


Figure 4. Pairwise comparisons between races on based PhiPT values (on the left) and Nei's unbiased measures of genetic distance (on the right)

Nei's unbiased measures of genetic distance (GD) between broomrape races were calculated with POPGENE (version 1.32) using ISSR data. Based on the results for pairwise comparisons of GD values between 3 races was identified a closer genetic similarity between races G and H (0.035), more genetic distant were races E and H (0.036) and the most genetically distant were found to be races E and G (0.081) (Figure 4).

Relationships between races were further illustrated by dendrograms with the UPGMA method based on Nei's genetic distance and Ward's method by means of Euclidean distance. In both cases, the broomrape populations on race category were classified into 2 groups: the first group combined populations belonging to race G and H, whereas populations of race E fell into separate second group (Figure 5).

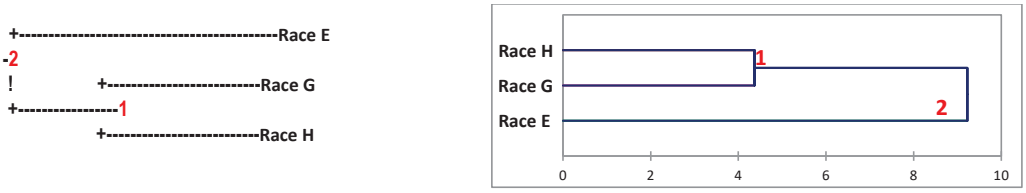


Figure 5. Dendrogram generated using UPGMA method with Nei's genetic distance (on the left) and Ward's method by means of Euclidean distance (on the right), showing relationships between 3 broomrape races, using ISSR data

CONCLUSIONS

The presented data are intended to fill the gaps in knowledge of genetic structure of the broomrape populations at the race level that are characteristic of the Black Sea basin.

1. ISSR analysis of 23 broomrape populations from Black Sea region revealed quite high levels of DNA polymorphism (93.87%). All 13 primers demonstrated a good capacity for race differentiation that is already found at the level of percentage of polymorphism index (races E-86.64%, G-90.80% and H-94.48%). The discrimination power of ISSR markers at race level was observed on most of the molecular-genetic indices and was graphically identified by the cluster analysis.

2. The comparative evaluation of genetic diversity between races E, G and H detected the different levels of variability at them. Monomorphic loci were observed only in the race E, while the specific loci were detected in all races (E-11.63%, G-8.88% and H - 5.44%). The highest percentage of specific loci was found for race E that it is more distant from races G and H in evolutionary time. The frequency distribution analysis confirms this conclusion and reveals that the races G and H are more similar to each other.

3. PhiPT coefficient values and genetic distance data have led to similar conclusions about evolutionary relationships between three broomrape races: the race E has a bigger molecular difference to that of the race G than from H, the last two races being related

genetically most closely. Clustering of populations revealed the pattern of genetic and evolutionary subdivision into 2 groups: the most virulent new races G and H were comprised in the first and less virulent race E in the second, thus being confirmed by the physiological data and genetic relationships detected by using ISSR markers.

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