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APPLICATION OF ISSR METHODS IN STUDYING BROOMRAPE'S (*OROBANCHACEAE*) BIODIVERSITY IN BULGARIA

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ABSTRACT

In Bulgaria the holoparasitic family of broomrapes (Orobanchaceae) is represented by 25 species from two genera. The local diversity of the broomrapes in Bulgaria is still grossly under-studied using modern approaches. This not only leads to unclear status of taxonomically problematic species and their local intraspecific taxa, but also hampers the assessment of their relevance as actual or potential treats for agricultural. The aim of this study was to select ISSR primers that amplify polymorphic microsatellite loci suitable both for taxonomic and population studies. One hundred ISSR primers (University of British Columbia Nucleic Acid-Protein Service Unit, UBC Primer Set #9) were tested. Specimens from five different species (Phelipanche ramosa, P. mutelii, P. purpurea, Orobanche alba and O. minor), collected from different locations were used to isolate genomic DNA. The PCR reactions were carried out in a TC-512 THERMAL CYCLER (Techne). The amplified unambiguous bands were scored to compile a presence/absence matrix. Cluster analyses were performed with PAST software. We found that 13 ISSR primers produce polymorphic bands suitable to distinguish the known sections and genera. Other 3 primers can distinguish the genera and probably higher taxonomy ranks. The obtained results open a good opportunity to study broomrape's biodiversity in Bulgaria.

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Introduction

Non-photosynthetic flowering species that have lost their autotrophic properties of plants in favor of a parasitic lifestyle have evolved in at least 11 independent angiosperm lineages and account for approximately 1% of all angiosperm species (2, 9). Among them broomrapes (*Orobanchaceae*) demonstrate a higher level of adaptation because they are chlorophyll-lacking obligate root holoparasites that depend entirely on their hosts thereby depleting them of nutrients, minerals and water. The family has a worldwide distribution, but the main centers of distribution are the Mediterranean, Northern Africa, and western North America (15, 30)

The phylogenic origin of these plants and their taxonomy is often subject of debates. They possess only a few morphological features suitable for taxonomy purposes and yet even they are quite changeable. The variability within the species is too high and hampers the attempts to create proper determination keys.

The phylogeny of *Orobanchaceae* among other flowering plants is uncertain. Many authoros accept that *Orobanchaceae* is independent family (10, 11, 28, 32). Some authors however propose that *Orobanchaceae* is part of *Scrophulariaceae* (3, 7, 13, 27), while other (20) argue that substantial part of *Scrophulariaceae* belong actually to *Orobanchaceae*.

The recent investigations supported by molecular phylogenetic analyses have resulted in re-definition of *Scrophulariaceae* and related families in the order Lamiales (1, 5, 20, 21). Hemiparasitic species, formerly placed in *Scrophulariaceae* subfamily *Rhinanthoideae*, and *Orobanchaceae* were shown to comprise a monophyletic group. Parasitism is believed to have evolved once in the group, followed by multiple independent origins of holoparasitism from hemiparasitic ancestors (31). Based on these molecular analyses Young et al. (31) proposed that *Orobanchaceae* is a morphologically diverse family of predominantly herbaceous, parasitic plants. The majority of species are facultative or obligate root parasites, which may be photosynthetic (hemiparasites) or totally dependent on the host plant (holoparasites). According to this classification *Orobanchaceae* consists of 89 genera, containing ca. 2061 species (18).

In Bulgaria the family *Orobanchaceae* is represented by 2 genera known in the Bulgarian literature as *Orobanche* s.l. (8, 10, 12, 25): 1) *Orobanche* includes 19 species, two of which are reported as endemic for the Balkan peninsula: *O. serbica* and *O. esulae*. 2) *Phelipanche* comprises of 6 species (26). In the both genera are reported many intraspecific taxa, based on morphological criteria. However, only a few recent studies based on modern methods have included materials from Balkan countries - Greece and Croatia (22, 29). Our current knowledge about broomrapes diversity and distribution in Bulgaria is based on morphological data and floristic records. So, the local biodiversity is still grossly under-studied. This not only leads to unclear status of species of *Orobanchaceae* and their habitats, but also hampers the assessment of their relevance as actual or potential agricultural pest species.

Since 1994, a new molecular marker technique called inter simple sequence repeat (ISSR) has been available (33). ISSRs are semiarbitrary markers amplified by PCR in the presence of one primer complementary to a target microsatellite. The primers are 16-18 bp long composed of a repeated sequence and could be flanked at the 3' or 5' end by 2-4 arbitrary nucleotides – anchored primers (33). Such amplification does not require genome sequence information and leads to multilocus and highly polymorphous patterns (17, 33). Each band corresponds to a DNA sequence delimited by two inverted microsatellites. The applicability of the ISSR for taxonomic studies of Orobanchaceae was recently demonstrated by Benharrat et al. (4). The authors studied *Orobanche hederae*, *O. amethystea*, *O. cernua* and *O. cumana* by five different ISSR primers and obtained taxonomically significant results.

A study based on combination of classical taxonomic approaches with modern molecular techniques (i.e. ISSR) can greatly enhance our understanding of broomrape diversity in the region including the description and resurrection of new and neglected taxa with local or regional distribution.

The aim of this investigation was to find selective markers which would allow to distinguish species at different taxonomy levels. Therefore we examined five of the widely spread in Bulgaria species from the both genera – *Orobanche* and *Phelipanche*. The *Orobanche alba* and *O. minor* were incorporated in the study as members of the two subsections *Minores* and *Glandulosae* from genus *Orobanche,* while *Phelipanche ramosa*, *P. mutelii* and *P. purpurea* as members of the two sections from genus *Phelipanche*. *P. ramosa* and *P. mutelii* are closely related species, *P. purpurea* belongs to subsection *Areanerie*.

Materials and Methods

Collection of samples and specimen

Specimens from five different species (*Phelipanche ramosa, P. mutelii, P. purpurea, Orobanche alba* and *O. minor*), were deposited in the herbarium of Agricultural University (SOA), while samples from flowering tissue were be used in molecular taxonomy studies. The voucher specimens are represented in **Table 1** as follows: species (in bold), region (underlined), UTM coordinates (MGRS squares 10x10 km), nearest toponym, altitude, name of the proved host of the specimen (in italic), date, collector's name, herbarium number and numbers of the used samples (marked with "#").

Isolation of genomic DNA

Samples from flowering tissues of the collected broomrapes were frozen with liquid nitrogen and grinded to fine powder in pre-cooled mortar and pestle. About 100 mg of the powder was placed in pre-cooled microcentrifuge tube and used isolated with kit for DNA extraction (AnalytikJena Cat # 845-KS-1060050) following the standard protocol.

Primers

100 ISSR primers were tested (University of British Columbia Nucleic Acid-Protein Service Unit, UBC Primer Set #9 – NAPS Unit. http://www.michaelsmith.ubc.ca/services/NAPS/Primer_Sets/) Because the production of Primer Set #9 was discontinued by UBC –NAPS Unit the primers were ordered from Metabion International AG, Martinsried, Germany and upon arrival were dissolved in DNase-free water to 100 mmol final concentration.

PCR reaction conditions

PCR reaction mix –to obtain uniformity in PCR reactions we used PCR master mix (Fermentas, Cat No K0171). 25 μL of it were mixed in 250 μL PCR tubes with 2 μL DNA template; 1 μL of ISSR primer (100 mmol.L^{-1} concentration); and 22 μL DNase-free water (supplied with the master mix kit). The PCR tubes were places TC-512 THERMAL CYCLER (Techne) PCR apparatus and the PCR amplification was carried-out by using the following program: initial DNA melting at 94^0 C – 5 min; next 35 cycles of 94^0 C – 1 min; 31/63°C – 1 min 30 s (annealing temperatures we chosen to be 2^0 C below the melting temperatures of primers); 72^0 C – 2 min 30 s. The PCR products were mixed with 7,5 μL of loading dye (Fermentas #R0611), loaded onto 1,5% agarose gel containing 0.5 μg/ml ethidium bromide (final concentration) covered with 1X TAE buffer and separated by applying 3,5 volts per cm electrical currency. The size of the products was determined by comparison with DNA

TABLE 1

Voucher specimens

***Phelipanche purpurea* (Jacq.) Sojak** Tracian plain: 35TKG86, Trivoditsi, 201 m, *Achillea* cf. *millefolium*, 22.05.2006 (K.Stoyanov) SOA 059200 (#2006.051)
***Phelipanche ramosa* (L.) Pomel** Rhodopi Mts. (W): 34TGM15, Yurukovo, 840 m, *Nicotiana tabacum*, 14.09.2006 (K.Stoyanov) SOA 059213 (#2006.170)
***Phelipanche mutelii* (F.W.Schultz) Pomel** Rhodopi Mts. (E): 35TLG52, Gabrovo, 650 m, *Nicotiana tabacum*, 10.08.2006 (K.Stoyanov) SOA 059208 (#2006.151, #2006.154)
***Orobanche alba* Steph. f. communis Beck** Rhodopi Mts. (M): 35TLG25, Asenovgrad, 258 m, *Thymus* cf. *pulegioides*, 22.04.2007 (K.Stoyanov) SOA s.n. (#2007.003)
***Orobanche minor* Sm.** Rhodopi Mts. (W): 34TGM13, Eleshnitsa, 756 m, *Vicia hirsuta*, 12.06.2008 (K.Stoyanov) SOA s.n. (#2008.015)

ladder (Fermentas GeneRuler#SM0311). The PCR products were visualized by UV light.

Data analyses

Images were captured by BIO-VISION+3026.WL system (Vilber Lourmat) and processed by accompanying software. Cluster analyses were performed with PAST software.

Results and Discussion

DNA isolation

The isolation of high quality DNA from broomrapes is relatively difficult because of they accumulate high amounts of starch and polyphenols. Therefore we used flowers and upper parts of the flowering stem for genomic DNA isolation. About 200 mg of frozen tissue from each species was used.

The absorption of samples at 260 nm was used to determine concentrations of the isolated DNA samples, while the ratios A260/A280 и A260/A230 to determine presence of contaminations like proteins, polyphenolic compounds, sugars and lipids. According to the observed data the average amounts of isolated DNA were 250-300 ng and the above counted contaminations were present in negligible amounts.

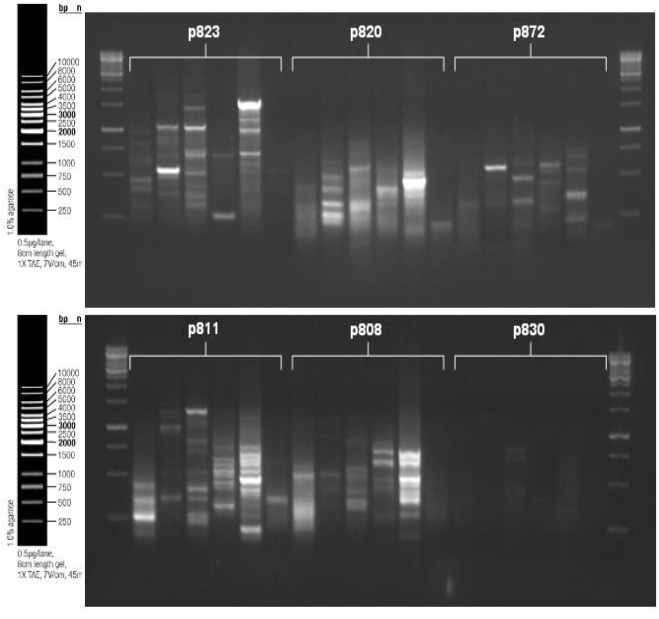

Fig. 1. Testing of different ISSR primers. The PCR products were mixed with 7,5 µl of loading dye loaded onto 1,5% agarose gel containing 0.5 µg/ml ethidium bromide (final concentration) covered with 0,5X TBE buffer and separated by applying 3,5 volts per cm electrical currency. The size of the products was determined by comparison with DNA ladder (Fermentas GeneRuler#SM0311). The picture presents PCR products visualized by UV light. For each primer the samples has the following pattern (from left to right): *P. purpurea*, *P. ramosa*; *P. mutelii*; *O. alba*; *O. minor* and *Negative control*

Selection of ISSR primers

Initially we tested the ability of each primer to produce polymorphic bands suitable for distinguishing sections and genera with template DNA from the five different species: *P. ramosa* and *P. mutelii* (*Phelipanche* sect. *Phelipanche*), *P. purpurea* (*Phelipanche* sect. *Arenarie*), *O. alba* (*Orobanche* subsect. *Glandulosae*) and *O. minor* (*Orobanche* subsect. *Minores*). The primers were grouped by their melting temperature because it varied from 35^0 C to 63^0 C. The PCR reactions with each primer were carried out at annealing temperature with 2^0 C lower than the melting temperature calculated by the manufacturer. The amplified PCR products were separated on 1,5% agarose gel (**Fig. 1**).

Optimization of the ISSR - PCR conditions: In order to optimize the annealing temperatures of the selected ISSR primers we used gradient from 48^0 C to 60^0 C. The obtained results (**Fig. 2**) indicated that the optimal temperatures are between 54^0 C and 56^0 C.

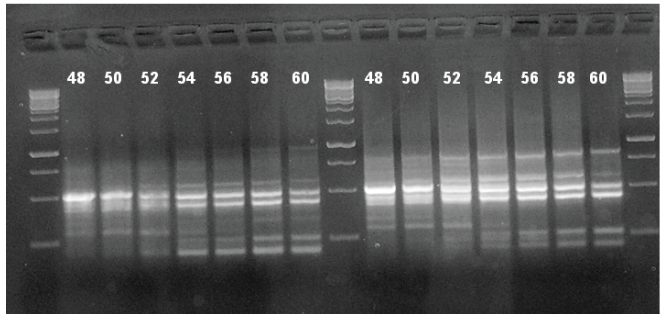

Fig. 2. Optimization of annealing temperatures for the selected ISSR primers. Different annealing temperatures are indicated. The PCR products were mixed with 7,5 µl of loading dye loaded onto 1,5% agarose gel containing 0.5 µg/ml ethidium bromide (final concentration) covered with 0,5X TBE buffer and separated by applying 3,5 volts per cm electrical currency. The size of the products was determined by comparison with DNA ladder (Fermentas GeneRuler#SM0311)

Data analyzes

Amplified unambiguous bands were manually scored to compile a presence/absence matrix that was processed with PAST software in order to build clusters. We found that 13 ISSR primers produce polymorphic bands suitable for distinguishing sections and genera. Their products showed clustering that coincides with the accepted taxonomic scheme (**Fig. 3** a, b, c, f). We also found other 3 primers that can distinguish higher taxonomy ranks but not between the sections (**Fig. 3** d). Most of the primers produced bands with unique pattern for each specie but dendrograms showed unknown grouping (**Fig. 3**e). Probably they could be used on different levels for instance to differentiate intraspecific taxa or populations. The results obtained for each tested primer were summarized in **Table 2**.

The microsatellite regions are hyper variable, short stretches of DNA, characterized by mono-, di- or trinucleotide repeats that are the targets for the ISSR primers (17, 30). DNA polymorphisms elucidated by ISSR PCR makes possible to identify differences between closely related species and the populations in single species. The use of ISSRs in studying broomrape diversity is relatively recent approach. Such studies enabled scientists in this field to characterize several *Orobanche* species (4), and to distinguish populations of O. crenata and *P. ramosa* (4,6,23).

TABLE 2.

Grouping of the ISSR primers according to their applicability for molecular taxonomy of Orobanchaceae family

№	Sequence	№	Sequence
No PCR amplification		**Unknown grouping**	
		856	$(AC)_8$ YA
801	$(AT)_8$ T	858	$(TG)_8$ RT
802	$(AT)_8$ G	859	$(TG)_8$ RC
803	$(AT)_8$ C	861	$(ACC)_6$
804	$(TA)_8$ A	862	$(AGC)_6$
805	$(TA)_8$ C	866	$(CTC)_6$
806	$(TA)_8$ G	868	$(GAA)_6$
822	$(TC)_8$ A	869	$(GTT)_6$
824	$(TC)_8$ G	874	$(CCCT)_4$
825	$(AC)_8$ T	875	$(CTAG)_4$
831	$(AT)_8$ YA	876	$(GATA)_2(GACA)_2$
832	$(AT)_8$ YC	877	$(TGCA)_4$
833	$(AT)_8$ YG	878	$(GGAT)_4$
837	$(TA)_8$ RT	879	$(CTTCA)_3$
838	$(TA)_8$ RC	880	$(GGAGA)_3$
860	$(TG)_8$ RA	881	$(GGGTG)_3$
864	$(ATG)_6$	884	HBH $(AG)_7$
871	$(TAT)_6$	886	VDV $(CT)_7$
882	VBV $(AT)_7$	888	BDB $(CA)_7$
883	BVB $(TA)_7$	891	HVH $(TG)_7$
885	BHB $(GA)_7$	895	AGAGTTGGTAGCTCTTGATC
887	DVD $(TC)_7$	896	AGGTCGCGGCCGCNNNNNNATG
889	DBD $(AC)_7$	897	CGACTCGAGNNNNNNATGTGG
892	TAGATCTGATATCTGAATTCCC	898	GATCAAGCTTNNNNNNATGTGG
894	TGGTAGCTCTTGATCANNNNN	900*	ACTTCCCCACAGGTTAACACA
899	CATGGTGTTGGTCATTGTTCCA	**Grouping by genera**	
Unknown grouping		846	$(CA)_8$ RT
807	$(AG)_8$ T	854*	$(TC)_8$ RG
808	$(AG)_8$ C	857	$(AC)_8$ YG
809	$(AG)_8$ G	**Grouping by genera and sections**	
810	$(GA)_8$ T	811	$(GA)_8$ C
817	$(CA)_8$ A	812	$(GA)_8$ A
818	$(CA)_8$ G	813	$(CT)_8$ T
819	$(GT)_8$ A	814	$(CT)_8$ A
820	$(GT)_8$ C	815	$(CT)_8$ G
823	$(TC)_8$ C	816	$(CA)_8$ T
827	$(AC)_8$ G	826	$(AC)_8$ C
829	$(TG)_8$ C	840	$(GA)_8$ YT
830	$(TG)_8$ G	841	$(GA)_8$ YC
834	$(AG)_8$ YT	851	$(GT)_8$ YG
835	$(AG)_8$ YC	855	$(AC)_8$ YT
838	$(TA)_8$ RC	872	$(GATA)_4$
842	$(GA)_8$ YG	873	$(GACA)_4$
843	$(CT)_8$ RA	**Grouping by sections**	
844	$(CT)_8$ RC	828	$(TG)_8$ A
845	$(CT)_8$ RG	836	$(AG)_8$ YA
847	$(CA)_8$ RC	**Producing smear**	
848	$(CA)_8$ RG	863	$(AGT)_6$
849	$(GT)_8$ YA	865	$(CCG)_6$
852	$(TC)_8$ RA	867	$(GGC)_6$
853	$(TC)_8$ RT	870	$(TGC)_6$

The primers that produced only a set of faint products are marked with asterisk (*).

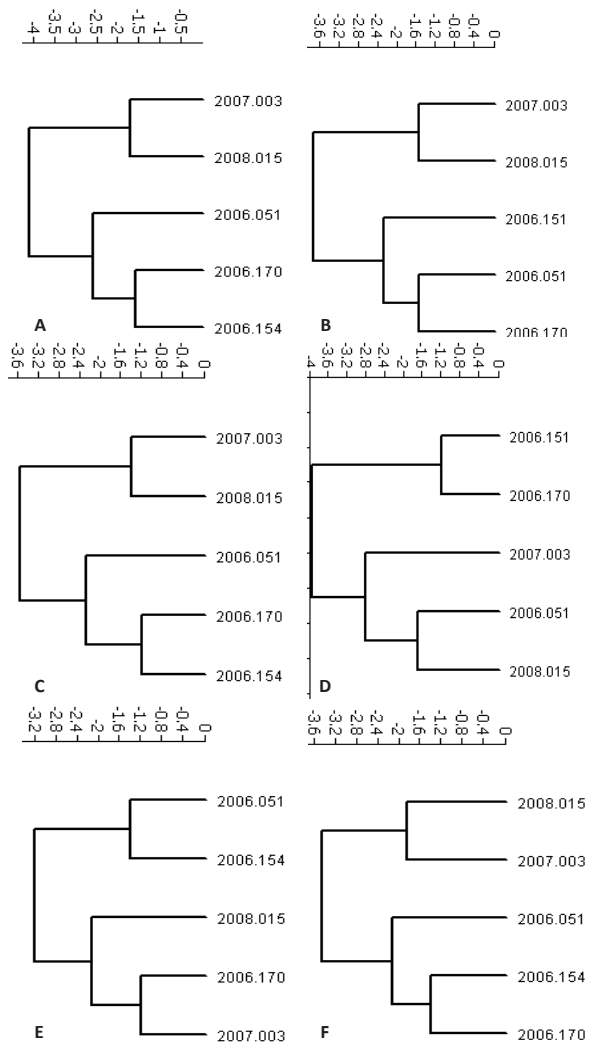

Fig. 3. Dendrograms produced with PAST software after processing of presence/absence matrix of ISSR products. The numbers on the axe represent the range of similarity between the evaluated samples. Result were obtained with the following primers: $(AC)_8$[Y]T – **a**; $(GA)_8$[Y]C – **b** ; $(GACA)_4$ – **c**; $(AG)_8$[Y]A – **d**; $(AG)_8$C – **e;** $(CT)_8$ T – **f.** The sample 2006.051 is *P. purpurea*; 2006.170 – *P. ramosa*; 2006.154 – *P. mutelii*; 2007.003 – *O. alba* and 2008.015 – *O. minor*

The aim of this study was to identify more universal set of primers that can discriminate not only between closely related species and populations, but also between species form the two genera represented in Bulgaria. The primers used by the other authors did not produce PCR products in several occasions when we used DNA templates from genera *Orobanche* subsect. *Glandulosae,* and subsect. *Minores*. Therefore similarly to Roman et al., (23) we used the primer Set #9 of the University of British Columbia, Nucleic Acid-Protein Service Unit, but we tested the whole collection of one hundred primers.

In general, comparison of the sequences of the seven selected ISSR primers revealed that they are based on **$(AC)_8$** or **$(CA)_8$** motive. Such primers can distinguish very well the tested broomrape species. This finding could mean that in broomrape genome the distribution of microsatellite motives like **$(TG)_n$** or **$(GT)_n$** is specific and has taxonomic significance.

The best results were obtained with 3'-anchored **$(AC)_8$YY** primers. They worked well with the arbitrary sequences of T and C nucleotides (**Fig. 3** a, b, c, f). Primers **$(AC)_8$NN** with combinations of A of G in 3'- arbitrary sequences usually did not produce PCR products or in isolated cases (3'- [Y]G) the clustering can not distinguish the sections in the genera. Primers based on **$(AG)_8$** or **$(GA)_8$** motives distinguished genera and sections (**Fig. 3**d). The same was true for unanchored primers based on **(GATA)n** and **(GACA)n** motives. These results are in very good agreement with the observations of other authors. For instance Benharrat et al., (4) used **$(GATA)_4$** primer to detected polymorphism between the two closely related species *O. cernua* and *O. cumana*. The same primer allowed Buschmann et al.(6) to detect polymorphism between populations of *P. ramosa*, collected from different regions. The primers selected by Roman et al., (23) to study variability among *O. crenata* populations also were based on $(GA)_8$, $(AG)_8$ and $(AC)_8$.

Primers with motives like e **(AGT)n**, **(CCG)n**, **(GGC)n** and **(TGC)n** produce too many bands when unanchored, while anchored did not amplified PCR products at all.

Primers containing **(ATG)n**, **(TA)n** and **(AT)n** motives did not produce PCR products. It could be due either to the absence of such microsatellite motives in broomrapes genome or because such primers have too low melting temperature – usually bellow 40^0 C.

In general the grouping of species in dendrograms constructed with the results obtained by the seven **$(AC)_8$YY** primers correspond to the accepted taxonomic schemes of the known species. The study also demonstrated the high reproducibility of the results obtained by ISSR method. These findings open good opportunity for ISSR – based molecular taxonomy study of biodiversity and taxonomy of broomrape species in Bulgaria.

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