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BIODIVERSITY AND ECOSYSTEMS

# IDENTIFICATION OF ISSR MARKERS FOR STUDYING THE BIODIVERSITY OF BULGARIAN REPRESENTATIVES OF GENUS *OROBANCHE* SUBSECTION *MINORES*

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## ABSTRACT
*The family Orobanchaceae consists of parasitic species. One group of taxonomically problematic species is subsection Minores (Beck) Teryokhin genus Orobanche. The species in this subsection (except O. crenata) have been often assigned to aggregate O. minor because of their great morphological similarity. The aim of this study was to find useful molecular makers for characterization of the biodiversity of subsection Minores, Orobanche in Bulgaria and their phylogenetic relationships.*
*Six species of subsection Minores were examined: O. minor Sm.; O. amethystea Thuill.; O. esulae Pančić var. bulgarica T. Georgiev, O. pubescens d'Urv. O. loricata Rchb. and O. crenata Forssk. Genomic DNA was isolated from flowering stems and used as a template for ISSR PCR performed with ten different ISSR primers. The ISSR products were separated on agarose gel and visualized by UV-light. The molecular masses of the polymorphic bands were determined and used to fill Boolean matrices that were subjected to cluster analysis. The suitability of each primer for distinguishing each of the studied species was discussed. The resulting cladogram, based on the average Euclidean distances, displayed grouping by species and by geographic populations. This study confirmed that the assignment of the small-flowered species to agg. O. minor has only practical value and is not genetically justified. The study confirmed the independent taxonomic status of the Balkan endemic species O. esulae.*

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**Keywords:** Broomrapes, ISSR markers, *Minores*, molecular taxonomy, *Orobanchaceae*, *Orobanche*, phylogeny

## Introduction
The broomrapes (*Orobanchaceae*) are chlorophyll-lacking obligate root holoparasites (33). The taxa in this group have lost many morphological characteristics useful for species identification and delimitation and possess only a few features suitable for taxonomic purposes. The variability within the species is high and hampers the attempts to create proper determination keys.

Particularly problematic are the species within the genus *Orobanche* subsection *Minores* (Beck-Mannagetta) Teryokhin (28). This group is characterised by small-flower species with an exceptionally large range of angiosperm hosts from at least 16 orders (25) and probably includes many cryptic taxa (10). According to Stoyanov (27) it is represented by six species in the Bulgarian flora. Because of their high morphological similarity, five of the species (*Orobanche minor* Sm, *O. loricata* Rchb., *O. amethystea* Thuill., *O. esulae* Pančić, *O. pubescens* d'Urv.) were grouped in the aggregate *O. minor* (7, 9, 12). According to Musselman (17, 18) this aggregate consists of only one species, which displays wide morphological variability caused by the host plant and is poorly resolved even by broad-scale molecular phylogenetic analyses (16, 23, 26).

Among them the Balkan endemic species *O. esulae* was described by Pancic (22) for the region of Pirot. It is represented in Bulgaria by a variety – *O. esulae* var. *bulgarica* T. Georgiev (11), which has a high morphological similarity to *O. minor*. The taxonomic position of *O. loricata* is problematic as well – according to some authors it is one species: *O. loricata* (1, 2, 3), while others described it as *O. picridis-hieracioides* Scultz (14), *O. picridis* Schultz (1, 5, 12), *O. artemisiae-campestris* Vauch. (10, 34).

On the other hand, *O. crenata* Forssk. was assigned as a member of a separate subsection according to the classic scheme of Beck (2). The phylogenic investigations based on ITS sequences (26) resulted in a revision that assigned *O. crenata* to subsect. *Minores*. Recently *O. serbica* Beck & Petrovic was incorporated in the *Minores* as a result of the taxonomical revision proposed by Carlón et al. (6).

During the last two decades a number of molecular methods have been applied for taxonomic and phylogenetic analyses of different organisms including broomrapes. Several authors used plastid sequences (4, 8, 21), nuclear 18S r DNA (4, 20), as well as ITS sequences (4, 31) to study the phylogeny of this challenging group. Based on the plastid genome sequences there were detected and confirmed the relations between four species of genus *Orobanche* (32) and were identified four species in subsect. *Minores* (3).

The inter-simple sequence repeats (ISSR) are semi-arbitrary 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 (35). This technique of amplification does not require genome sequence information and yields multilocus and highly polymorphous patterns (19, 35). Each band corresponds to a DNA sequence delimited by two inverted microsatellites (5). The ISSR method is highly informative and combines the speed of RAPD and the reliability of the SSR. The high reliability and simplicity of the method, compared to RFLP and RAPD, made it the preferred choice in the taxonomical works. These features, combined with the higher productivity, have made the method attractive in the investigations of genetic variation within closely related species (19), and in investigations of subspecies and populations (5). By the ISSR method successful taxonomical division of *O. hederae, O. amethystea, O. cernua* and *O. cumana* was done using five different primers (3). The molecular diversity in and between the populations of *O. crenata* is another confirmation that the ISSR markers can be a precise method for identification (24).

One of the representatives of subsect. *Minores* – *O. minor*, was recently subjected to extensive study in the UK. Using ISSR and sequence-characterized amplified region (SCAR) markers, Thorogood et al. (29, 30) recently demonstrated that in Britain, *O. minor* comprises of genetically divergent populations associated with different hosts.

In our previous study we tested one hundred ISSR primers (University of British Columbia Nucleic Acid-Protein Service Unit, UBC Primer Set #9) for their suitability for studying the biodiversity of Bulgarian broomrape species. We found that 13 ISSR primers produce polymorphic bands suitable to distinguish the known sections and genera. Other three primers can distinguish the genera and probably higher taxonomy ranks. The obtained results provided a good opportunity to study broomrape's biodiversity in Bulgaria (15).

The aim of this study was to find selective ISSR markers that will allow us to distinguish the Bulgarian representatives of subsect. *Minores* on the level of species and infraspecific taxa and to study their phylogenic relations.

## Materials and Methods

Six species of *Orobanche* subsect. *Minores* were investigated: *O. minor* Sm., *O. loricata* Rchb. *O. amethystea* Thuill. *O. esulae* Pančić var. *bulgarica* T. Georgiev, *O. pubescens* d'Urv., and *O. crenata* Forssk. The samples were collected from different host plants in different southern Bulgaria regions during 2006-2008. The voucher specimens are deposited in the Herbarium of Agricultural University - Plovdiv SOA (**Table 1**).

### DNA preparation

Fresh flowering stems from the collected plants were frozen in mortar and pestle pre-cooled with liquid nitrogen and grinded to fine powder, of which 100 mg was transferred immediately into a pre-cooled microcentrifuge tube for DNA extraction by DNeasy plant mini kit (Qiagen cat. No 69104) following the original protocol. The absorption at 260 nm was used to determine the concentrations of the isolated DNA samples, while the ratios A260/A280 and A260/A230, to determine the presence of contaminations like proteins, polyphenolic compounds, sugars and lipids. The average amounts of isolated DNA were 250-300 ng and the above counted contaminations were present in negligible amounts.

### Primers

The sixteen ISSR primers preselected by Hristova et al. (16) from primer Set #9 (University of British Columbia, Nucleic Acid-Protein Service Unit, NAPS Unit, http://www.michaelsmith.ubc.ca/services/NAPS/ Primer_Sets/) were tested in this study. 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.

### ISSR-PCR reaction conditions

Approximately 150 ng of DNA template was taken for each sample and mixed in a 200 μL PCR tube with 1 μL primer (100 $mmol \cdot L^{-1}$ concentration), 25 μL PCR master mix (Fermentas, Cat No K0171) and 22 μL DNase-free water (supplied with the master mix kit). The PCR tubes were placed in a TC-512 THERMAL CYCLER (Techne) PCR apparatus and the PCR amplification was carried out by using the following program: initial DNA melting at 94 °C – 5 min; next 35 cycles of 94 °C – 1 min; 55 °C – 1 min 30 s; 72 °C – 2 min 30 s, and final extension at 72 °C for 6 min. The PCR products were mixed with 7.5 mL of loading dye (Fermentas #R0611), loaded onto 1.5% agarose gel containing 0.5 $mg \cdot mL^{-1}$ ethidium bromide (final concentration) covered with 1X TAE buffer and separated by applying 7 volts per cm electrical currency. The size of the products was determined by comparison with a DNA ladder (Fermentas GeneRuler#SM0311). The PCR products were visualized by UV light.

### Data analysis

The gel images were captured by BIO-VISION+3026.WL system (Vilber Lourmat) using four different exposition times and processed by accompanying software. The unambiguous amplified bands were scored by molecular masses using the program GelPro Analyzer. Next they were manually allocated into classes of molecular weights for completion of Boolean matrices for the presence/absence (0/1) of bands with the results for each primer.

The binary data were used to construct rectangular matrices using the PAST ver. 1.89 computer program (13) from the data for each gel exposition. The distances obtained from all images were recalculated to average distances for each primer. All the average matrices were summarized to a resulting distance matrix. The results based on genetic distances of the studied species were used to construct a resulting unrooted tree by the T-Rex 3.0a1 software (Vladimir Makarenkov, University of Quebec in Monreal), using the Unweighted Neighbor-Joining method. The dendrogram was plotted by the PhyloDraw software ver. 0.82.

## Results and Discussion

The suitability of the primers from primer set#9 for molecular taxonomy studies of broomrapes was tested previously (15). This study allowed to preselect sixteen ISSR primers. For

**TABLE 1**

List of voucher specimens – location (floristic regions, UTM coordinates, nearest toponym and altitude), hosts, collection dates, collector, sample and voucher specimen number

| Location and host plant | Date and collector | Sample number | Voucher number |
|---|---|---|---|
| ***1. Orobanche minor* Sm:** | | | |
| **Slavyanka:** | | | |
| 34TGL29. Paril, 833 m, pl.n. *Viola arvensis* Murray | 2008-06-12 (K. Stoyanov) | **2008.016** | SOA s.n. |
| **Rhodope Mts. (West)**: | | | |
| 34TGM13. Eleshnitsa, 756 m, pl.n. *Vicia hirsuta* (L.) Gray | 2008-06-12 (K. Stoyanov) | **2008.015**<br>**2008.019** | SOA s.n.<br>SOA 059363; |
| 34TGM40. Blatska, 535 m, pl.n. *Medicago* sp. | 2008-06-13 (K. Stoyanov) | **2008.018** | SOA 059359; |
| ***2. Orobanche loricata* Rchb.** | | | |
| **Tracian plain:** | | | |
| 35TLG15. Byaga, 400 m, pl.n. ? *Achillea* sp./*Anthemis* sp. | 2008-05-25 (K. Stoyanov) | **2008.007** | SOA 059365 |
| ***3. Orobanche amethystea* Thuill.:** | | | |
| **Rhodope Mts. (Middle):** | | | |
| 35TLG05. Markovo, 438-475 m, pl.n. *Eryngium campestre* L. | 2007-05-27 (K. Stoyanov) | **2007.063** | SOA s.n. |
| | 2008-05-31 (K. Stoyanov) | **2008.004.** | SOA 059334 |
| ***4. Orobanche esulae* Pančić var. *bulgarica* T. Georgiev** | | | |
| **Rhodope Mts. (Middle):** | | | |
| 35TLG13. Yugovo, 729 m, pl.n. *Euphorbia esuloides* Velen. | 2007-05-26 (K. Stoyanov) | **2007.065** | SOA 059456 |
| 35TLG25. Asenovgrad, 309 m, pl.n. *E. nicaeensis* All. | 2007-05-09 (K. Stoyanov) | **2007.051**<br>**2007.053** | SOA s.n.<br>SOA 059424 |
| 35TLG25 Lyaskovo, 826 m, pl.n. *E. nicaeensis* All. | 2007-05-06 (K. Stoyanov) | **2007.026 2007.028** | SOA s.n.<br>SOA 059507. |
| ***5. Orobanche pubescens* d'Urv.** | | | |
| **Rhodope Mts. (Middle):** | | | |
| 35TLG26. Asenovgrad, 252-260 m, pl.n. *Orlaya grandiflora* (L.) Hoffm. | 2006-05-07 (K. Stoyanov) | **2006.021** | SOA s.n. |
| | 2007-04-22 (K. Stoyanov) | **2007.005** | SOA s.n. |
| **Thracian Plain**: | | | |
| 35TKG86. Bessapara Hills, 413-417 m, pl.n. *Orlaya grandiflora* (L.) Hoffm. | 2008-05-25 (K. Stoyanov) | **2008.006**<br>**2008.009** | SOA s.n.<br>SOA s.n. |
| ***6. Orobanche crenata Forssk.*** | | | |
| **Rhodope Mts. (Middle)** | | | |
| 35TLG25. Assenovgrad, 240 m, pl.n. *Astragalus* sp. | 2006-05-07 (K. Stoyanov) | **2006.022** | SOA 059405 |

the needs of this study we tested these sixteen primers and found that ten of them successfully amplified polymorphic PCR products with the DNA from the studied species. The pattern of the polymorphic products was suitable for molecular taxonomy purposes (**Fig. 1**) and demonstrated clustering of the studied samples in groups corresponding to the known species and populations. Three of the primers were with single 3'-arbitrary nucleotide: p809, p817 and p826; six, with two 3'-arbitrary nucleotides: p836, p840, p841, p851, p855 and p857; and one, with three 5'-arbitrary nucleotides – p891, as presented in **Table 2**.

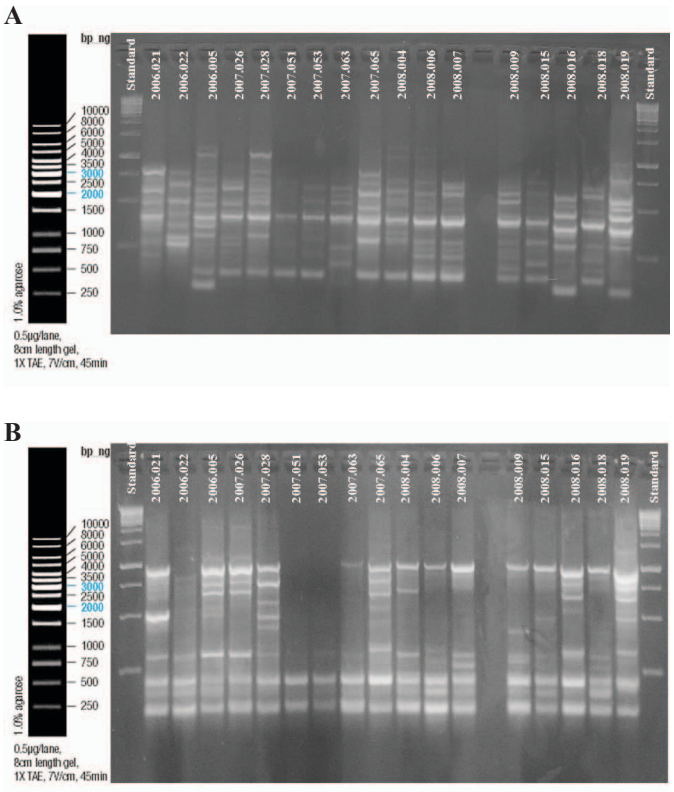



**Fig. 1.** ISSR products amplified by primers 840 (**A**) and 891 (**B**). The samples from left to right are listed in **Table 1**.

The molecular masses of the PCR products were manually allocated into classes (**Table 3**) and used to fill Boolean matrices for the presence/absence (0/1) of bands for each primer. The cluster analyses of the obtained binary data by PAST software allowed us to build rectangular cladograms based on Euclidian distances between different samples (**Fig. 2**).

The analyses of the distribution of the polymorphic ISSR products obtained with primers p836, p851, p855 separated *O. minor* in a single cluster (**Fig. 2D, G, H**). To some extent similar grouping was observed with products from p817, p826, p840, p841 and p891 (**Fig. 2B, C, E, F, J**), while primer p857 divided the *O. minor* cluster into populations by the geographic origin (**Fig. 2I**).

The ISSR products of primers p817, p826 and p836 differentiated *O. esulae* in separate single clusters (**Fig. 2B, C, D**), whereas the data from primers p809 and p841 (**Fig. 2A, F**) can distinguish between different geographic populations of *O. esulae*.

**TABLE 2**

Sequences of the ISSR primers used in this study*

| Primer | Sequence |
|---|---|
| p809 | $(AG)_8G$ |
| p817 | $(CA)_8A$ |
| p826 | $(AC)_8C$ |
| p836 | $(AG)_8YA$ |
| p840 | $(GA)_8YT$ |
| p841 | $(GA)_8YC$ |
| p851 | $(GT)_8YG$ |
| p855 | $(AC)_8YT$ |
| p857 | $(AC)_8YC$ |
| p891 | $HVH\ (TG)_7$ |

* Y – (C, T); H – (A,C,T); V – (A,C,G)

**TABLE 3**

Grouping of the polymorphyc products by number and size of the of bands

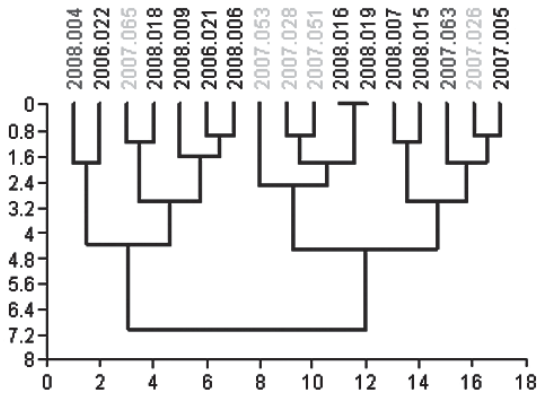
| Primer | Number of polymorphic bands | Range of the sizes of the amplified bands, (min-max size in bp) |
|---|---|---|
| p809 | 16 | 62 – 984 |
| p817 | 16 | 204 – 1202 |
| p826 | 14 | 207 – 984 |
| p836 | 25 | 49 – 1394 |
| p840 | 17 | 35 – 1234 |
| p841 | 19 | 82 – 1074 |
| p851 | 16 | 137 – 1368 |
| p855 | 18 | 145 – 1279 |
| p857 | 22 | 158 – 1094 |
| p891 | 21 | 88 - 1000 |

The samples of *O. pubescens* were grouped in separated single clusters by primers p817, p826 and p836 (**Fig. 2B, C, D**). Primers p841, p851 and p855 can be used to distinguish between geographic populations of *O. pubescens*.

*Orobanche amethystea* formed a separated single cluster only when analyzed by primer p826 (**Fig. 2C**).

Unlike the above species, *O. pubescens* clustered either with *O. minor* when analyzed by the products of primers p826, p836, p840, p841 and p857 (**Fig. 2C, D, E, F, I**) or with *O. esulae* according to primers p817, p851, p855 and p891 (**Fig. 2B, G, H, J**). Similarly, *O. loricata* usually clustered with *O. minor* by primers p817, p826, p836, p840, p857 and p891 (**Fig. 2B, C, D, E, I, J**), and in a few cases with *O. esulae* by primers p841 and p855 (**Fig. 2F, H**).

We had a single sample of *O. crenata* and, when tested by primers p817, p836, p840, p855, p857, p891, it clustered with *O. esulae* (**Fig. 2B, D, E, H, I, J**). Only according to primer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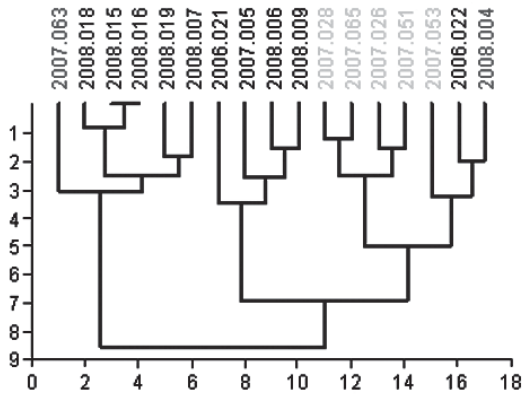
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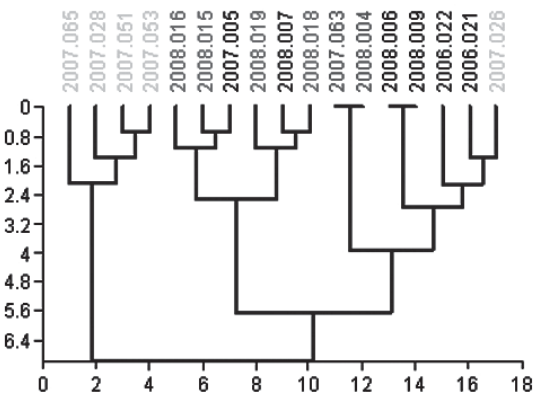
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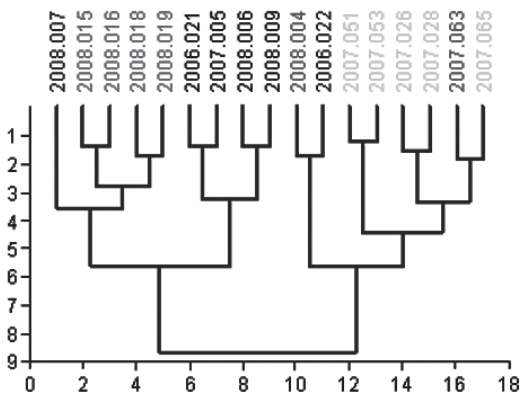
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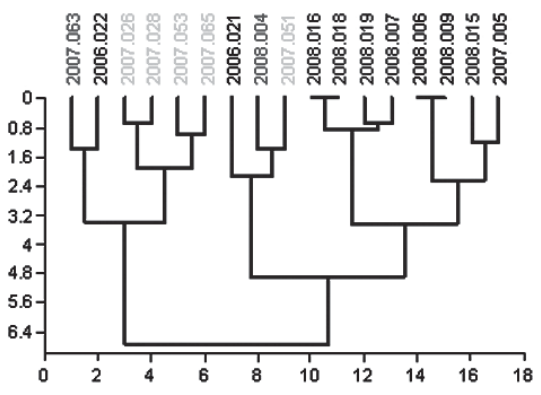
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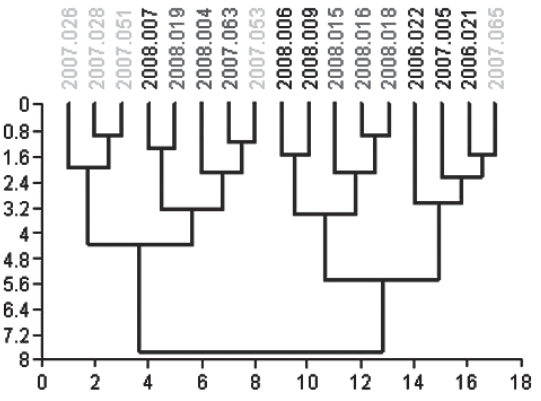
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F

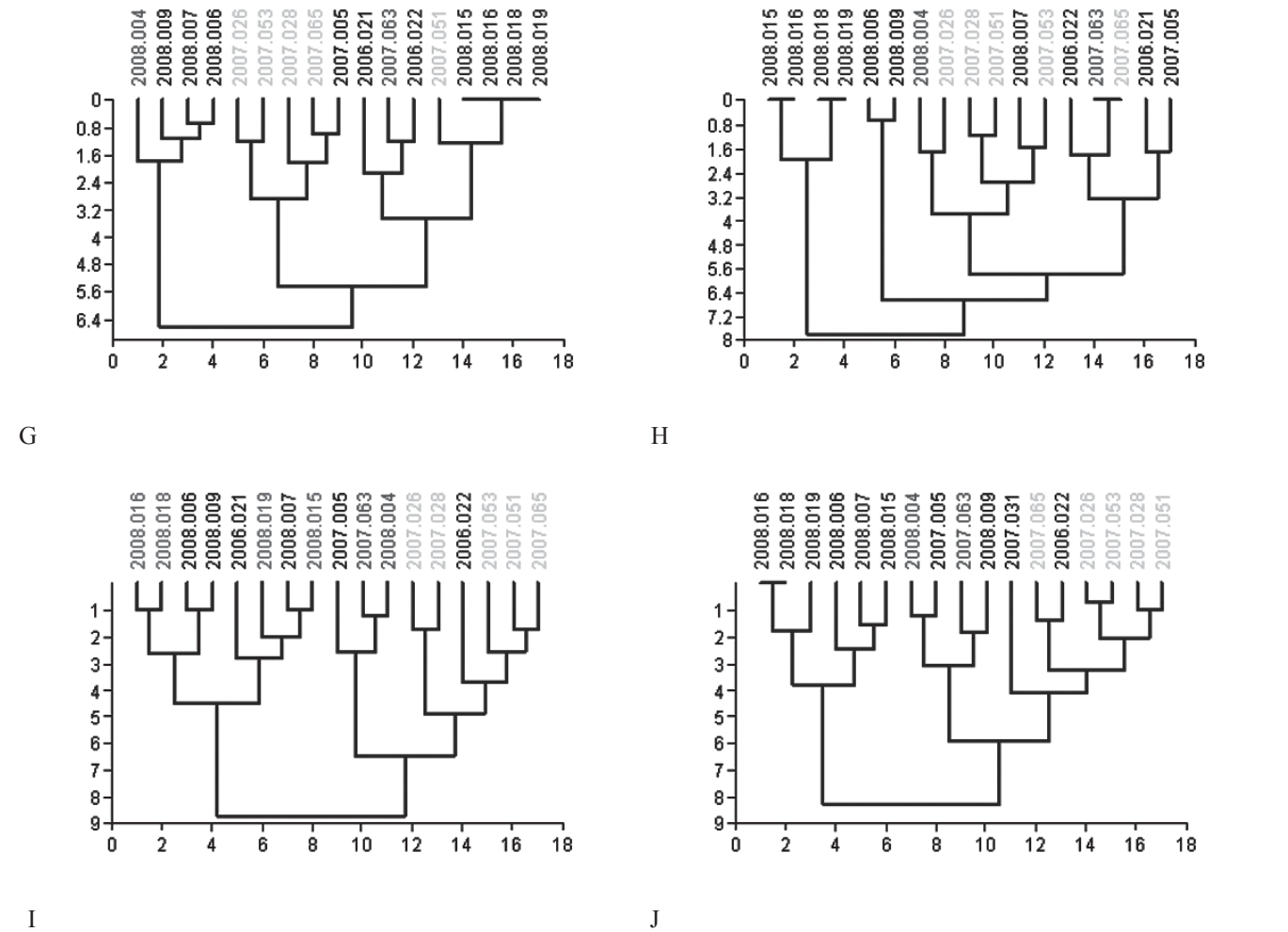


**Fig. 2.** Cladograms built with the data about distribution of the ISSR products amplified by the primers p809 (**A**), p817 (**B**), p826 (**C**), p836 (**D**), p840 (**E**), p841 (**F**), p851 (**G**), p855 (**H**), p857 (**I**), and p891 (**J**). The sample numbers correspond to: *O. minor* Sm. – 2008.015, 2008.016, 2008.018, 2008.019; *O. loricata* Rchb. – 2007.063, 2008.004, 2008.007; *O. esulae* Pančić – 2007.026, 2007028, 2007.051, 2007.053, 2007.065; *O. pubescens* d'Urv. – 2006.021, 2007.005, 2008.006, 2008.009; *O. crenata* Forssl. – 2006.022. More details are provided in **Table 1**.

p826 did the sample form a joint cluster with *O. minor* (**Fig. 2C**). None of the tested ten primers was able to separate *O. crenata* in an independent group.

*O. amethystea*, based on primers p836, p840, p841, p855, p857, p891, demonstrated similarity to *O. esulae* (**Fig. 2D, E, F, H, I, J**). Only according to primer p826 did the species form a joint cluster with *O. pubescens* and *O.minor* (**Fig. 2C**).

In general, although some primers can cluster well three of the studied species (*O. minores*, *O. pubescens* and *O. amethystea*) and can even show differences between geographically isolated populations of *O. minores* and *O. pubescens*, no single primer can separate all the species. Therefore we did not give separate weight to the selected primers and preferred to use the Adansonian principle – without weighing the results by conservatism, but instead treating all the collected data as equal and using different methods of calculating the phylogenetic scheme. As a result of this approach we constructed a resulting unrooted tree by T-Rex 3.0a1 software, using the unweighted neighbor-joining method.

The resulting cladogram is presented in **Fig. 3**. The grouping of the results suggested that *O. esulae*, in spite of the morphological similarity, is closer to *O. pubescens* and *O. loricata* than to *O. minor*. On the other hand *O. loricata* and *O. amethystea* showed closer relationships with *O. esulae*. The primer that produced the closest results to this presumption was p836, followed by p857, which can also distinguish geographically isolated populations of *O. minor.* A specific marker for *O. esulae* could be p826, while for *O. minor* the best one is p855.

The resulting cladogram demonstrated grouping not only by species but also, in some cases, by geographically isolated population. This was shown for the samples of *O. pubescens*.

According to the final resulting cladogram it is obvious that in spite of the high morphological similarity, the members of agg. *O. minor* are a single species. The only case of high similarity was detected between *O. minor* and *O. loricata*.

This study confirmed the status of the Balkan endemic species *O. esulae*, because it formed a separate clade from *O. minor*, regardless of the similar morphology.

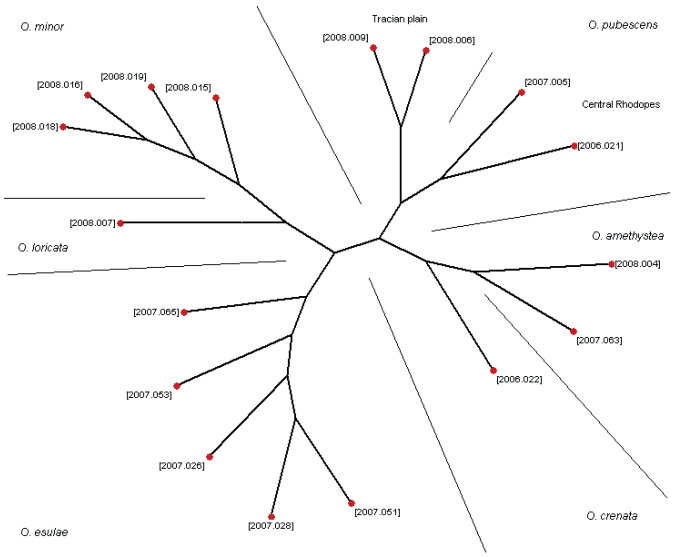


**Fig. 3.** The resulting cladogram based on genetic distances of the studied species using the Unweighted Neighbor-Joining method. The sample numbers correspond to: *O. minor* Sm. – 2008.015, 2008.016, 2008.018, 2008.019; *O. loricata* Rchb. – 2007.063, 2008.004, 2008.007; *O. esulae* Pančić – 2007.026, 2007.028, 2007.051, 2007.053, 2007.065; *O. pubescens* d'Urv. – 2006.021, 2007.005, 2008.006, 2008.009; *O. crenata* Forssl. – 2006.022. More details are provided in **Table 1**.

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