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CONTRIBUTION TO MOLECULAR GENETIC CHARACTERIZATION OF *HELLEBORUS MULTIFIDUS* Vis. IN BOSNIA AND HERZEGOVINA

Doprinos molekularno genetičkoj karakterizaciji *Helleborus multifidus* Vis. u Bosni i Hercegovini

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Abstract

Helleborus multifidus Vis. is endemic Illyric-Adriatic species with distribution range in Italy, Slovenia, Croatia, Bosnia and Herzegovina, Montenegro and Albania. Although few studies reported different taxonomic categories for *H. multifidus*, this one is the first using molecular-genetic markers (*trnL* region and *mat*K of chloroplast DNA and nuclear *ITS*1 and *ITS*2 region) for genetic characterization of *H. multifidus* presented at three localites in Bosnia and Herzegovina. The results revealed that PCR-RFLP on *trn*L intron was not informative for testing inter- or intrapopulation diversity. Contrary, analysis of *mat*K, *ITS*1 and *ITS*2 sequences showed differences between populations from Trebinje region and Kupreško polje, pointing to the need to include additional analyses in order to confirm these findings.

Key words: *Helleborus multifidus*, *trn*L, *mat*K, *ITS*1, *ITS*2

INTRODUCTION – *Uvod*

The genus *Helleborus* L. is a small genus of the family Ranunculaceae and includes perennial herbaceous species with rhizomes or erect, rather woody stems and digitate or pedate leaves. The inflorescences are different type of cymes with large flowers composed of five perianth segments (TUTIN, 1996). Species of the genus are widespread in Europe and western Asia, with the disjunction of *H. thibetanus,* distributed in East Asia (ZONNEVELD, 2001). The Balkans is considered the centre of the genus diversification with the highest number of species in the peninsula (SUN ET AL., 2001).

The genus is traditionally divided into two subgenera, *Helleborus* and *Helleborastrum* Spach., with six sections. Albeit, molecular phylogenetic investigations support the existence of six monophyletic sections, the subgeneric division is not recognized (SUN ET AL., 2001). The classification scheme is based on morphology, pollen and seed morphology, hybridization capability and nuclear DNA content (ZONNEVELD, 2001; MEINERS ET AL., 2011). The taxonomic resolution of many

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species is still the matter of debate among taxonomists. All species having distinct morphology are also distinctive in terms of molecular divergence (SUN ET AL., 2001). However, the relationships between the poorly differentiated species in section *Helleborastrum* are still not resolved (SUN ET AL., 2001).

The number of species and subspecies varies from 11 species (TUTIN, 1996), 15 species and 10 subspecies (MATHEW, 1989; ZONNEVELD, 2001) to 22 species based on recent taxonomic reviews (MEINERS ET AL., 2011). All these classifications were based on different methods and require further taxonomic clarification.

Helleborus multifidus Vis. is endemic Illyric-Adriatic species with distribution range in Italy, Slovenia, Croatia, Bosnia and Herzegovina, Montenegro and Albania (MARTINIS, 1973). The Catalogue of life recognizes three subspecies (*H. multifidus* ssp. *hercegovinus* (Martinis) B. Mathew, *H. multifidus* ssp. *laxus* (Host) Martinis and *H. multifidus* ssp. *multifidus* within the complex of *H. multifidus* (ROSKOV ET AL., 2017). However the *H. multifidus* taxon shows an extreme polymorphy of morphological characters and includes many other 'microspecies' (ZONNEVELD, 2001; MEINERS ET AL., 2011). It mostly grows on calcareous and dolomite soils, having a wide altitudinal range, from coastal to inland higher mountain habitats. It is a typical element of thermophyllous forests from the *Quercetalia pubescentis*, *Erico-Pinetalia* orders, and various other shrub formations, rocky meadows (ŠILIĆ, 1990).

The aim of this study was genetic characterization of *H. multifidus* collected at three localities in Bosnia and Herzegovina using molecular variation of *trn*L region of chloroplast DNA, *mat*K, *ITS*1 and *ITS*2 markers.

MATERIAL AND METHODS – *Materijal i metode*

Sampling of plant material – Uzorkovanje biljnog materijala

Plant material of 110 hellebore specimens was collected during the plant flowering phase (April-June 2015) from three different localities in Trebinje region (locality 1 - 43°09'79" N, 18°24'52" E and locality 2 - 43°11'57" N, 18°24'07" E) and Kupreško polje (locality 3 - 43°94'99" N, 17°20'32" E) (Figure 1). Fresh leaves were separated immediately into paper bags and stored at -20˚C.

Picture 1. Geographical display of sampling regions
Slika 1. Geografski prikaz lokaliteta na kojima je izvršeno uzorkovanje

To avoid putative interrelationships between the specimens, the distance between sampled individuals was 30m. Vouchers of sampled individuals were deposited in the Institute of Genetic Engineering and Biotechnology, University of Sarajevo (HM_0515, HM_3515, HM_1715).

DNA extraction- Izolacija DNK

Frozen and fresh plant tissue was used for DNA extraction (ca. 10-20 mg per sample). Leaves were grounded using mortar and pestle, and transferred to 1.5ml tubes. Total plant DNA was extracted using modified CTAB procedure (PADMALATHA ET AL., 2008). After extraction, genomic DNA was analyzed by electrophoresis on a 1.5 % (w/v) agarose gel in 1x SB (Sodium borate) buffer, pH8 (BRODY ET KERN, 2005) and visualized under UV light after staining with Midori green (Nippon Genetics Europe).

Molecular markers – Molekularni markeri

For the purpose of genetic characterization *trn*L region of chloroplast DNA, *mat*K, *ITS*1 and *ITS*2 were observed.

Amplification of *trnL* (UAA) intron was performed using primers decribed by TABERLET ET AL. (1991) in 15 µl reactions consisting of 1 µl of template DNA, 2 mM Tris-HCl (pH 8.0), 10 mM KCl, 0.2 µM of each primer, 0.2 mM dNTPs, 2.5 mM $MgCl_2$ and 1 unit of Taq DNA polymerase (Gdansk). PCR amplification was carried out in 30 cycles, 45s denaturation at 95°C, 30s annealing at 51°C, 45s extension at 72°C and 10 min final extension at 72°C. Afterwards, amplified *trnL* (UAA) intron was digested with the restriction enzymes (*Taq I, HpyF31, Hinf I, Hind III, Hind II, Rsa I, EcoR I, Ava II, Ban I* and *Alu I*), in order to create enzymatic profiles.

The plastid region of maturase K gene was amplified using 390F and 1440R primers according to FIOR ET AL. (2006). Amplification was performed in 35 µl reactions consisting of 1 µl of template DNA, 2 mM Tris-HCl (pH 8.0), 10 mM KCl, 0.2 µM of each primer, 0.2 mM dNTPs, 2.5 mM $MgCl_2$ and 1 unit of Taq DNA polymerase (Gdansk). PCR parameters were 1 min denaturation at 94°C, 30s annealing at 48°C, 1 min extension at 72°C, 8 min final extension at 72°C with the total of 26 cycles.

The nuclear regions *ITS1* and *ITS2* (internal transcribed spacers 1 and 2) fragments were amplified in separate reactions using primers described by WHITE ET AL. (1990). PCR reactions were performed under the same chemical regime as for the *matK*. PCR amplification was carried out in 30 cycles, 30s denaturation at 95°C, 30s annealing at 50°C, 1 min extension at 72°C and 10 min final extension at the same temperature.

PCR products were sequenced by Macrogen Inc. Europe as part of their regular capillary DNA sequencing services. *MatK*, *ITS1* and *ITS2* amplicons were sequenced only in forward direction.

Plastidial matK and ITS Sequence Assembly, Alignment and Annotation – Uređivanje, poravnanje i anotacija hloroplastne matK i ITS sekvenci

Plastidial *matK* and nrDNA sequence identification analyses from *Helleborus multifidus* were performed against the FASTA program (PEARSON, 1994). BLAST network service (BENSON ET AL., 2003) in GenBank at NCBI was used for final sequence identification, searching for the best identity and similarity scores in local databases. Sequencing reads were assembled using DNASTAR's Lasergene software EditSeq (BURLAND, 2000) and examined manually by electropherograms for sequencing errors. Multiple sequence alignment analyses for two *matK* and four nrDNA sequences (*ITS1* and *ITS2*) were performed using ClustalW Ver.1.6 (THOMPSON ET AL., 2011) under default parameters. MSA analyzed sequences and outputs were optimized using Jalview 2.9.0b2 (WATERHOUSE ET AL., 2009) and edited by Bioedit v5.09 (HALL, 1999). Two partial *matK* and four nrDNA consensus

sequences were deposited in the GenBank database (GenBankID's: KY908380, KY908381, KY908382, KY908383, KY908384, KY908385).

RESULTS AND DISCUSSION – *Rezultati i diskusija*

High quality DNA was successfully yielded from all 110 collected samples. The *trnL* (UAA) intron was also successfully amplified and digested with ten different enzymes. Six of them (i.e. *Taq I, Hinf I, Hind II, AvaII, Rsa I, Alu I*) showed restriction activity. However, based on the fragment size data, it was noticed that samples in all of the populations have the same enzymatic profile for all six applied enzymes (Figure 2). Consequently, no intra- or interpopulation polymorphism was detected. This may be due to the inadequacy of applied markers, which are probably not sufficiently sensitive for the detection of genetic diversity within this taxon.

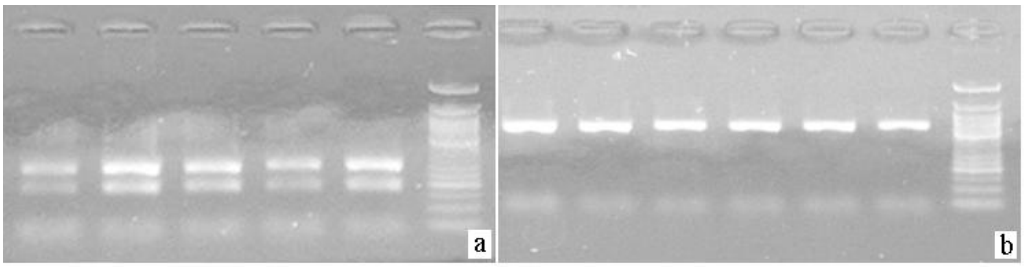

Figure 2. Enzymatic profiles generated by *Rsa I* (a) and *Ava II* (b) enzymes
Slika 2. Enzimatski profili generirani primjenom Rsa I (a) i Ava II (b) enzima

Maturase K sequences (average length 870bp) from five *Helleborus multifidus* samples (from three localities) were successfully sequenced and analyzed for discriminatory power. It was found that two out of five *matK* sequences of the samples were not identical (Figure 3). Estimation of nucleotide diversity showed two polymorphic sites within *matK* with an average p value of 0.001 (Table 1).

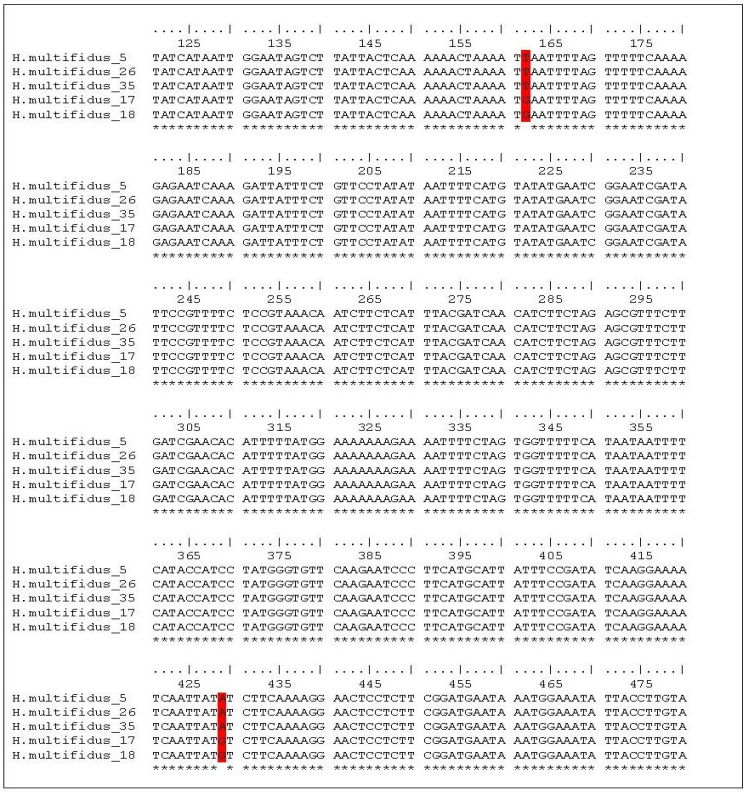

Figure 3. *MatK* sequence region (120bp-480bp) with two highlited nucleotide polymorphisms
Slika 3. Region sekvence matK (120bp-480bp). Dva polimorfna nukleotidna mjesta su naglašena

Table 1. Estimates of nucleotide distance (p) between *matK s*equences of five hellebore samples
Tabela 1. Procjena nukleotidne distance (p) matK sekvenci za pet uzoraka

H.multifidus_5				
H.multifidus_26	0,000			
H.multifidus_35	0,000	0,000		
H.multifidus_17	0,002	0,002	0,002	
H.multifidus_18	0,002	0,002	0,002	0,000

Four sample sequences for internal transcribed spacer 1 (*ITS1*, average length 260bp), and three sequences for internal transcribed spacer 2 (*ITS2*, average length 310bp) were successfully sequenced and annotated. Discriminatory power analyses were performed in order to reveal the resolution of this molecular marker. Two polymorphic sites were found within *ITS1* sequence (Figure 4), while only one

nucleotide polymorphism was found within *ITS2* sequence (Figure 5). Estimation of *ITS1* and *ITS2* nucleotide diversity showed non-identical sequences with an average p value of 0.007 (Table 2) and 0.002 (Table 3), respectively.

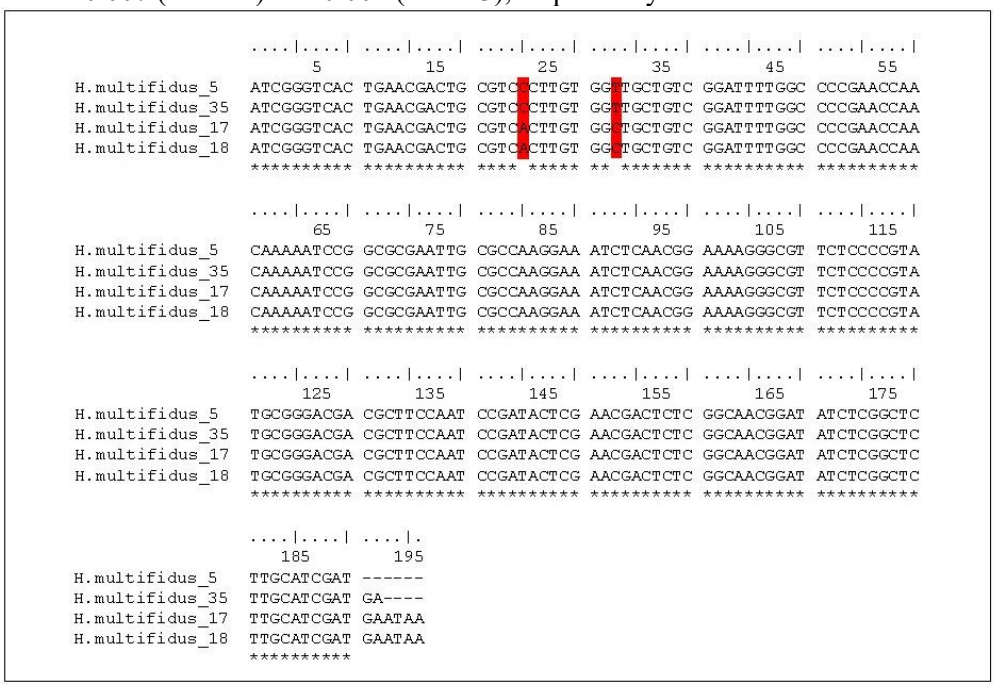

Figure 4. Complete *ITS1* sequence region with two highlited nucleotide polymorphisms
Slika 4. Kompletan region sekvence ITS1. Dva polimorfna nukleotidna mjesta su naglašena

Table 2. Estimates of nucleotide distance (p) between *ITS1 s*equences of four hellebore samples
Tabela 2. Procjena nukleotidne distance (p) ITS1 sekvenci za četiri uzorka

H.multifidus_17			
H.multifidus_18	0,000		
H.multifidus_5	0,011	0,011	
H.multifidus_35	0,011	0,011	0,000

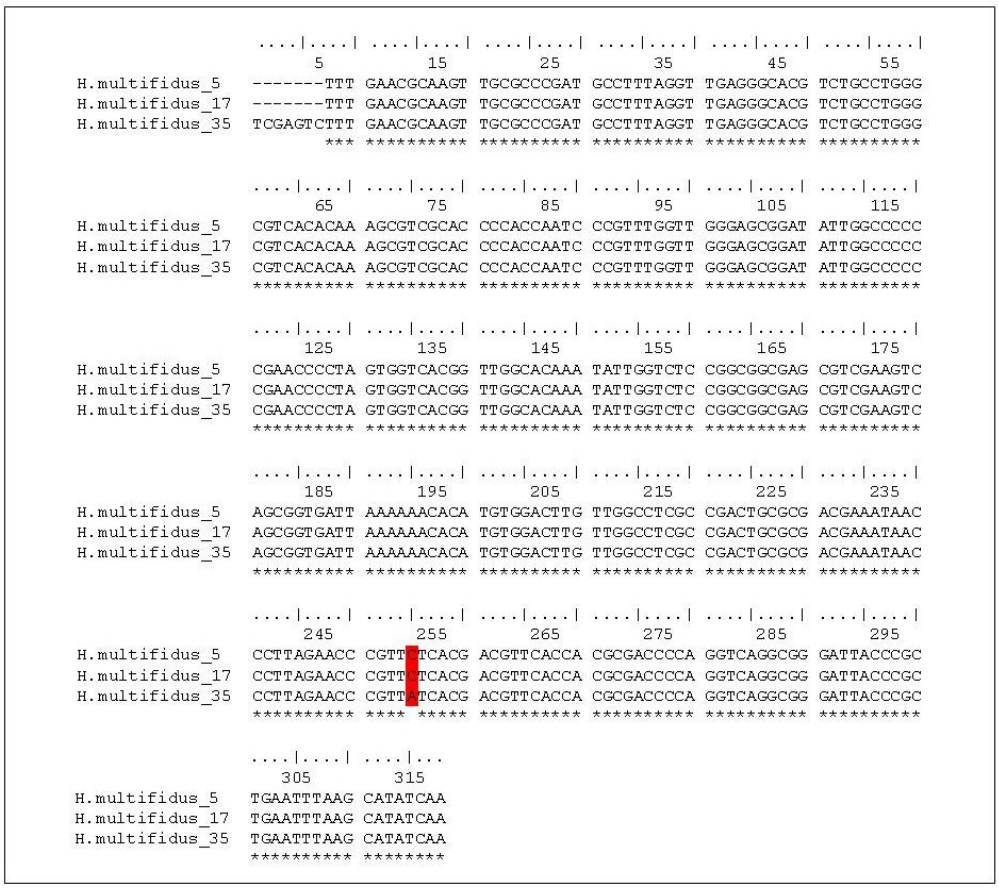

Figure 5. Complete *ITS2* sequence region with a single highlited nucleotide polymorphism
Slika 5. Kompletan region ITS2 sekvence sa jednim naglašenim nukleotidnim polimorfizmom

Table 3. Estimates of nucleotide distance (p) between *ITS2 s*equences of three hellebore samples
Tabela 3. Procjena nukleotidne distance (p) ITS2 sekvenci za tri uzorka

H.multifidus_5		
H.multifidus_35	0,000	
H.multifidus_17	0,003	0,003

Clearly, the samples from locality 3 (H.multifidus_17 and H.multifidus_18) form a cluster according to *matK* and *ITS1* molecular markers (not a single nucleotide polymorphism was found), while the samples from locality 1 (H.multifidus_5) and locality 2 (H.multifidus_35 and H.multifidus_26) are grouped into the other cluster

according to *matK* sequences. A third cluster is formed by H.multifidus_5 and H.multifidus_35 based on nrDNA sequences. These results indicate evident interpopulation differences. This could be attributed to the fact that populations from Trebinje region do not belong to the same taxon in order to the samples from Kupreško polje. Also, the geographic distance and spatial isolation between the studied populations could be the result of nucleotide variation. It is important to emphasize that localities 1 and 2 were previously described by MARTINIS (1793) as a *locus classicus* for *Helleborus hercegovinus*. Also, ZONNEVELD (2001) allocated *H. hercegovinus* as a subspecies of *H. multifidus*, based on the genome size analyses (C-value). Most recent studies conducted by MEINERS ET AL. (2011) singled it out as a separate species (*H. hercegovinus*) based on the amount of nuclear DNA and the multi-locus AFLP data. On the other hand, the Catalogue of life (ROSKOV ET AL., 2017) does not recognize *H. hercegovinus* as a distinct species, but rather as a subspecies.

Evidently many uncertainties are present in taxonomic status of *H. multifidus*. Although analyses of *ITS1*, *ITS2* and *matK* molecular markers conducted in this study were not informative for clarifying taxonomic status of species, our results indicate the differences that require further research.

CONCLUSIONS – *Zaključci*

The aim of the present study was to provide an insight in molecular-genetic characterization of *Helleborus multifidus* Vis. in Bosnia and Herzegovina. The results revealed that PCR-RFLP on *trnL* intron was not informative for testing interpopulation or intrapopulation diversity within analyzed taxon. However, *matK*, *ITS1* and *ITS2* sequences pointed out the existence of differences between tested samples, indicating the differences between the populations from Trebinje region and Kupreško polje.

Further molecular-genetic analyses and designed sampling are necessary for molecular-genetic characterization of hellebore species in Bosnia and Herzegovina and adjacent regions to resolve their relationships.

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SAŽETAK

H. multifidus je endemična ilirsko-jadranska vrsta rasprostranjena u Italiji, Sloveniji, Hrvatskoj, Bosni i Hercegovini, Crnoj Gori i Albaniji. Iako nekoliko studija navodi različite taksonomske kategorije za *H. multifidus*, prvi put su korišteni molekularno-genetički markeri (trnL region hloroplastne DNA, matK, ITS 1 i ITS 2) u genetičkoj karakterizaciji populacija *H. multifidus* sa tri lokaliteta u Bosni i Hercegovini. Rezultati provedenog istraživanja su pokazali da PCR-RFLP metoda *trnL* introna nije informativna u utvrđivanju interpopulacijskog i intrapopulacijskog diverziteta analiziranog taksona. Međutim, sekvence *mat*K, *ITS*1 i *ITS*2 ukazuju na postojanje razlika između populacija kukurjeka iz okoline Trebinja i populacija sa Kupreškog polja, ukazujući na nužnost dodatnih analiza.

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