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Genetic diversity of the critically endangered *Verbascum davidoffii* Murb. (Scrophulariaceae) and implications for conservation

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***Abstract*:**

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Verbascum davidoffii Murb. (Scrophulariaceae), one of the rarest plant species in Bulgarian flora, is a local endemic, protected by the National Biodiversity Act, included in the Red List of vascular plants, as well as in the Red Data Book of Bulgaria with conservation status "Critically Endangered". Its distribution is limited due to anthropogenic pressure, specific ecological requirements and low reproductive capability. In this study, we aimed to measure the genetic diversity level in the unique single world population of *Verbascum davidoffii* located in Pirin National Park, Bulgaria. We found high genetic diversity in the excitant population of the species. The present study indicates that the primary objective in conservation of *Verbascum davidoffii* is to preserve as much as possible of its evolutionary potential.

Key words: *Verbascum davidoffii*, endemic species, genetic diversity, conservation

***Apstrakt*:**

Petrova, G., Petrov, S., Bancheva, S.: ***Genetički diverzitet kritično ugrožene vrste Verbascum davidoffii Murb. (Scrophulariaceae i implikacije za konzervaciju. Biologica Nyssana, 7 (2), Decembar 2016: 101-106.***

Verbascum davidoffii Murb. (Scrophulariaceae), jedna je od najređih biljaka flore Bugarske i lokalni endemit koji je zaštićen nacionalnim Zakonom o biodiverzitetu, uključen u Crvenu listu vaskularnih biljaka, kao i u Crvenu knjigu Bugarske sa konzervacionim statusom "kritično ugrožena". Njeno rasprostranjenje je ograničeno zbog antropogenog pritiska, specifičnih ekoloških prohteva i niske reproduktivne sposobnosti. U ovom istraživanju, naš cilj bio je da merimo nivo genetičkog diverziteta u jedinstvenoj populaciji *Verbascum davidoffii* na svetu, lociranoj u Nacionalnom parku Pirin, Bugarska, gde je utvrđen visok genetički diverzitet.

Ovo istraživanje je ukazalo na to da je primarni cilj u konzervaciji vrste *Verbascum davidoffii* da se njen evolucioni potencijal sačuva u što većoj meri.

Ključne reči: *Verbascum davidoffii*, endemična vrsta, genetički diverzitet, konzervacija

Introduction

Bulgarian vascular flora consists of more than 4000 species. This high level of plant diversity is determined by various factors and the geographical position of the country is a main factor, allowing the distribution of diverse floristic elements on a relatively restricted territory. The richest in endemic species are the genera *Centaurea* (cornflower), *Verbascum* (mullein), *Anthemis* (dog fennel), *Silene* (catchfly), etc. (Biserkov et al., 2015).

The genus *Verbascum* comprises of about 360 species of flowering plants in the Scrophulariaceae family (Firat, 2015). *Verbascum davidoffii* Murb. (Scrophulariaceae), one of the rarest plant species in Bulgarian flora, is a local endemic, protected by the national Biodiversity Act, included in the Red List of vascular plants and in the Red Data Book of Bulgaria with conservation status "Critically Endangered". The unique single world population of the species is located between the valleys of river Bunderitsa and Razlozhki Suhodol, Pirin National Park, Bulgaria (**Fig. 1**). Its distribution is limited due to anthropogenic pressure, specific ecological requirements and low reproductive capability (Assyov & Denchev, 2015).

Genetic diversity is of fundamental importance in the continuity of a species as it provides the necessary adaptation to the prevailing biotic and abiotic factors, and enables changes in the genetic composition to cope with environmental changes (Rao & Hodgkin, 2002). Understanding of the genetic variation within and between populations is essential for the establishment of efficient conservation practices for rare plants. Small populations are expected to demonstrate lower genetic diversities than larger populations and *vice versa* (Willi et al., 2006; Li et al., 2012). Because of the small size of *V. davidoffi* population, we hypothesized that the genetic diversity of the species will be low.

The loss of genetic diversity is one of the key aspects in many conservation programmes of threatened plant species (Hamrick & Godt, 1996). Thus, the present study was designed as a first attempt towards investigating the genetic diversity of *V. davidoffii*. The increasing availability of PCR-based molecular markers allows the detailed analyses and evaluation of genetic diversity in plants (Idrees & Irshad, 2014). Currently, there is no an universal method for all applications and each technique has its own advantages and limitations. Among the various types of available molecular

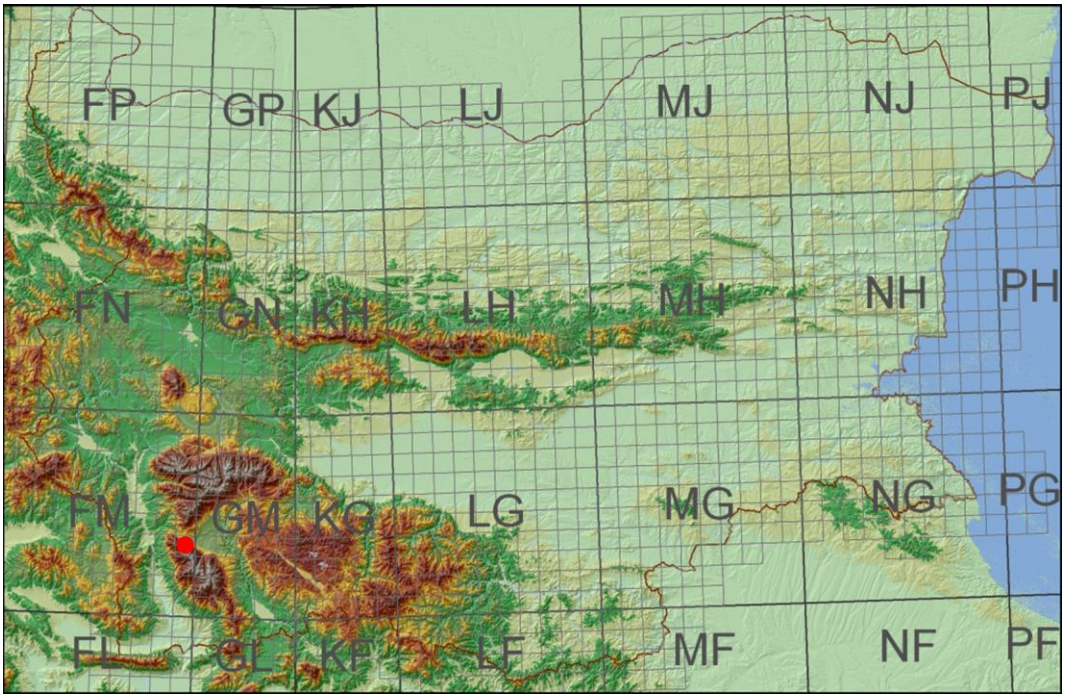

Fig. 1. Geographic location of *Verbascum davidoffii* population in Bulgaria.

markers, Inter-simple sequence repeats (ISSRs) do not need prior knowledge of the genome to be analyzed (Zietkiewicz et al., 1994). The ISSR analysis requires comparatively low amount of DNA, the utilization of long primers allows more stringent annealing temperatures and reveals more polymorphic fragments (Fang & Roose, 1997; Wolfe & Liston, 1998; Camacho & Liston, 2001). Considering these advantages of ISSR primers, we attempted to use this marker system in order to analyze the pattern of genetic diversity of *V. davidoffii*, given that such knowledge will be important for more efficient planning of the strategies for conservation of this endangered plant species.

Material and methods

Species analyzed

Morphology and biology. Biennial herbaceous plant. Stem up to 80 cm high, usually unbranched. Basal leaves obovate, densely hairy. Flowers in groups of 2–4 in the upper part of the stem. Calyx 5–merous, with black glandular hairs. Corolla 5-merous, golden yellow, up to 3.5 cm in diameter. Fruit spherical to ovate capsule. Fl. VI–VII, fr. VII–IX. Reproduction by seeds (Assyov & Denchev, 2015).

Distribution in Bulgaria and description of species' habitat. The population of *V. davidoffii* consists of very low number of individuals distributed between the valleys of River Bunderitsa and Razlozhki Suhodol, Pirin National Park. The park encompasses over 40 000 ha of unique nature, at an altitude between 1008 and 2914 m in the Pirin Mountains, southwest Bulgaria. Pirin National Park comprises diverse limestone mountain landscapes with glacial lakes, waterfalls, caves and predominantly coniferous forests. *V. davidoffii* inhabits grassy openings and light, rocky places in forests of *Pinus heldreichii* on calcareous soils (Assyov & Denchev, 2015).

Plant material

Fourteen individuals were sampled during the flowering stage from the *V. davidoffii* population in Pirin National Park (Latitude (N): 41°76'82"; Longitude (E): 23°42'63").

DNA extraction

Genomic DNA was extracted from young leaves following the modified CTAB - procedure of Doyle & Doyle (1987).

ISSR analysis

ISSR primers (Microsynth, Balgach, Switzerland) in the initial screening that had a high level of polymorphism, repeatability, and the best scorability were selected, after the screening of 35 primers on the subset of collected samples (**Tab. 1**). Polymerase chain reactions were performed in a volume of 25 µl, containing a final concentration of 1 × PCR buffer (Fermentas, Vilnius, Lithuania), 1 U Taq DNA polymerase (Fermentas, Vilnius, Lithuania), 100 µM of each dNTP, 1 µM of each primer and 50 ng of extracted DNA. PCR cycling conditions were as follows: 5 min. initial denaturation at 95 °C, 35 cycles of amplification [45 s at 94 °C, 1 min. at the annealing temperature (T_a), 2 min. elongation at 72 °C] and a final elongation step of 5 min. at 72 °C. PCR experiments were performed with a TC-5000 gradient thermal cycler (Techne, Staffordshire, UK). To determine the optimal annealing temperature for each primer, an interval of 10 °C around the melting temperature (T_m) was tested. The temperatures leading to clear patterns were then repeated until the optimal T_a was selected for each primer for routine ISSR fingerprinting. The reproducibility of the

Table 1. Sequences, annealing temperature, total number and number of polymorphic bands for selected ISSR primers in the present study of *Verbascum davidoffii*.

Primer sequence (5'→3')	Total number of bands	Number of polymorphic bands	Percent of polymorphic loci (%)	Annealing temperature (ºC)
$(GA)_8T$	12	12	100	60
$(GA)_8A$	13	13	100	60
$(CA)_8G$	11	11	100	60
$(AC)_8T$	17	17	100	60
$(AC)_8C$	12	12	100	60
$(AC)_8G$	9	9	100	60
$(AG)_8YT$	9	9	100	55
$(AG)_8YC$	5	5	100	55
$(GA)_8YG$	5	5	100	55
$(AC)_8YT$	10	10	100	55
	103	**103**	**100**	

technique was tested by replicating each amplification reaction twice. To further ensure the quality, PCR reactions were performed with one positive and one negative control. The PCR products were analyzed on 2% agarose gels (Fermentas, Vilnius, Lithuania) in 0.5 × TBE buffer. A 100-bp plus DNA ladder size standard (Fermentas, Vilnius, Lithuania) was used to estimate the length of PCR products. The gels were stained by incorporating 1.5 µl of ethidium bromide (0.5 mg/ml) in 100 ml agarose. Electrophoresis was run for 1.5 h at 150 V, the ISSR profiles were visualized with a UV transilluminator (TFP-M/WL, Vilber Lourmat, Eberhardzell, Germany) and further analyzed with a video image analyzer.

Software data analysis

The ISSR band profiles were treated as dominant markers and each locus was considered as a bi-allelic locus with one amplifiable and one null allele. The well resolved and consistently reproducible amplified DNA fragments as bands were scored with regards to their presence (1) or absence (0). The assignment of ISSR bands to genetic loci was performed semi-automatically using the GelAnalyzer 2010a image analysis software (http://www.gelanalyzer.com). The genetic diversity was measured using GenAlEx v.6.5 (Peakall & Smouse, 2012).

Results and discussion

Genetic diversity

The low level of genetic diversity is considered to be a common feature of endemic plant species and it is generally attributable to the small size of their populations (Huang et al., 2009; Zhu et al., 2009; Petrova et al., 2014). However, in contrast to our expectations, the genetic pattern of *V. davidoffii* revealed in this study was characterized with a high level of genetic diversity.

Ten of 35 tested ISSR primers resulted in polymorphic ISSR profiles including 4 poly (AC), 3 poly (GA), 2 poly (AG) and 1 poly (CA) di-nucleotide primers (**Tab. 1**). The number of bands produced by the used primers ranged between 5 (primers $(AG)_8$ YC and $(GA)_8$YG) and 17 for the primer $(AC)_8$T. The total number of alleles produced by the ten primers is 103. A 100% polymorphism was scored for all primers. Examples of photographs illustrating the ISSR fingerprinting by selected primers are shown in **Fig. 2**. These include ISSR fingerprinting revealed by primers $(CA)_8$G (**Fig. 2A)** and $(GA)_8$YG (**Fig. 2B)**.

The data from the ISSR-analysis were combined in a single matrix and the genetic coefficients (Shannon's information index, *SI*; Nei's genetic diversity, *h* and Unbiased diversity, h_u) were calculated to describe the genetic pattern of the excitant population of *V. davidoffii*. The Shannon's information index (*SI*) was estimated to be 0.406 and the ISSR derived unbiased diversity (h_u = 0.266) from the present study was close to the average value of narrow and outcrossing plant species according to the Nybom statistic, where estimates derived by the dominantly inherited markers are very similar and may be directly comparable (Nybom, 2004). In summary, our study indicated that the critically endangered Bulgarian endemic *V. davidoffii* is able to maintain high level of genetic diversity even its small population size.

The observed maintenance of genetic diversity in *V. davidoffii* is surprising, considering the limited area of distribution of the species, as well as its "Critically endangered" status. In recent years, a lot of studies revealed high levels of genetic variability in rare or narrow endemic plants (Helenurm, 2001; Zawko et al., 2001; Xue et al., 2004; Jia et al., 2016; Turchetto et al., 2016). Among the various factors, the breeding system is an important factor that may be invoked to explain high levels of genetic diversity in rare plants (Hamrick & Godt, 1996). Previous studies on the mating system of *V. davidoffii* suggested that the species is predominantly outcrossing (Stefanova-Gateva, 1995). The outcrossing and long-lived

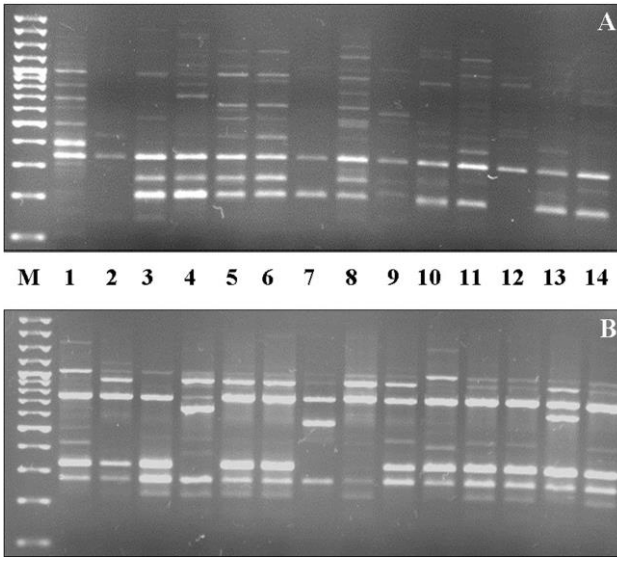

Figure 2. Examples of photographs illustrating ISSR fingerprints of *Verbascum davidoffii* by ISSR primers $(CA)_8$G (**A)** and $(GA)_8$YG (**B)**. Lanes 1 ÷ 14 stand for individual samples from the population of the species. M: 100 bp Plus DNA Ladder.

plants commonly have higher levels of genetic diversity than selfing plants (Nybom, 2004)). Therefore, we assume that the high level of genetic load maintained in *V. davidoffii* is probably due to its reproductive system and may be contributed to the outcrossing behavior of the species.

Generally, mountain plants are among the species most vulnerable to global warming, because of their isolation and narrow geographic distribution. Altitudinal gradients in mountains modify environmental conditions and put populations under different selective pressures that can easily be linked to ongoing climate changes (Walther et al., 2005; Gonzalo-Turpin & Hazard, 2009). *Verbascum davidoffii* is distributed in a single high altitude population (Assyov & Denchev, 2015). In the present study, we did not observe a correlation between the ISSR derived genetic diversity of the species and the size of its population despite the theoretical prediction that small populations might lose genetic variation. Thus, we hypothesize that the genetic diversity pattern of *V. davidoffii* may be a result from recently dispersed individuals from different population sources, and it may not respond immediately to the reduction in population size.

Conservation implications

The high genetic diversity of *V. davidoffii* indicates that the major factors that threaten the persistence of its population are ecological factors rather than genetic. The main risk is that this species is found in only one population and no other natural populations were found. In order to protect it against extinction and loss of its available genetic resources, we recommend collection of seeds from as many individuals as possible and storage in seed banks and living collections. We suggest that conservation programs should be improved to protect its natural habitat. Pirin National Park was established in 1962 and it was added to the World Heritage List in 1983. Although, it has long been a subject to tourism pressure, largely caused by the development of ski facilities. Such activities may affect the values and integrity of the property and therefore require rigorous control. The development of sustainable forms of tourism would guarantee preservation of ecosystems in the park. We recommend the establishment of variety of educational programs that directly present conservation and biodiversity issues. The key outcomes include enhanced visitor experience, administrative support, volunteer activities and ecological restoration.

Conclusion

The high level of genetic diversity enables *V. davidoffi* to adapt to changing environments. The maintenance of genetic diversity in the excitant population of the species is an indicator that its current endangered status is not caused by genetic factors. The results reported herein suggest that the high genetic diversity of the species may derive from an ancestral population. However, the ecological requirements of the species, as well as its origin and relationship with other related species should be further elucidated.

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