ASSESSMENT OF GENETIC DIVERSITY OF HABERLEA RHODOPENSIS FRIV. BY ITS1 MARKERS

E. Daskalova, S. Dontcheva, I. Kadieva, M. Gevezova, V. Toneva, I. Minkov, I. Denev
University of Plovdiv, Plant Physiology and Molecular Biology Department, Plovdiv, Bulgaria
Correspondence to: Iliya D. Denev
E-mail: iliden@uni-plovdiv.bg

ABSTRACT
Haberlea rhodopensis Friv. is a Balkan endemic plant and a tertiary relic with highly fragmented habitat. Its populations are isolated and often inhabit different environmental conditions. We found significant variations in many morphology and phenology traits between and even within H. rhodopensis populations. It is interesting from evolutionary, genomic and ecological point of view to assess whether the variations can be attributed only to the plant adaptability or it could be attributed also to differences on genetic level. This initial study included three geographically isolated populations from Rhodopi and Stara Planina mountains. We assess at DNA level the polymorphism between these populations by internal transcribed spacer 1 (ITS1) sequences. As a donor of plant material we used plants from the in vitro gene bank for Gesneriaceae family established at the Dept. of Plant physiology and Molecular biology.

Initial results from our analyses showed that the population of H. rhodopensis for the region of Chervenata stena possess two single nucleotide substitutions (transistions from thymine to cytosine) at relative positions 37 and 94. The other two populations showed high similarity of ITS1 sequences regardless of the geographic isolation. The obtained results demonstrated that the ITS1 regions is suitable as molecular marker for studying genetic polymorphism between H. rhodopensis populations but it should be combined with other marker genes that are represented in a single copy in the plant genome as suggested by (3). Our initial results indicate that we could expect unusual patterns of polymorphisms distribution between Haberlea rhodopensis populations.

Keywords: Haberlea rhodopensis Friv., population polymorphism, molecular markers, ITS

Introduction
Haberlea rhodopensis Friv. (Rhodope silivryak) and Ramonda serbica Panč. (Serbian ramonda) are the only representatives of the tropical family Gesneriaceae in Bulgaria. The most prominent feature of these beautiful and endangered plants is the “resurrection” - a feature quite rare among angiosperms. Resurrection plants can enter into anabiotic and survive after long existence in this state (up to 31 months for Haberlea rhodopensis) (9, 15). Haberlea and Ramonda plants are widely used as model systems for studying the photosynthetic and metabolic processes in extreme drought conditions (for example 11, 18).

Recently, our research group has established a national in vitro gene bank for Gesneriaceae family at the University of Plovdiv. It contains over 30 origins of Haberlea and Ramonda in vitro plants representing natural populations in Bulgaria. As a donor of plant material, the in vitro gene bank has many advantages: year-round access to plants of various source populations, possibility to generate genetically identical in vitro plants (clones), possibility for selection of plants with desired constant characteristics (drought resistance, antioxidant potential, antimicrobial activity) etc. The selected plants can be multiplied and continuously propagated to maintain numbers necessary for analyses, so there is no need to collect specimens from natural habitats. This is especially important for protected species as Haberlea rhodopensis Friv.

Another interesting feature of Balkan resurrection plants is that they are Tertiary relics, very interesting research objects from evolutionary point of view. As other relic species, Haberlea rhodopensis Friv. has patchy distribution (highly fragmented habitat). Most of its localities are isolated with more than 10 km from each other, so the exchange of genetic information between them is impeded.

On the other hand, our recent studies reveal unexpected ecological plasticity of Haberlea rhodopensis Friv. (7) In relation to the different light and water conditions, two types of Haberlea habitats can be distinguished: “classic” habitats (on overshadowed rocks facing the North, with high air humidity and altitude in most cases above 500 m. a.s.l.) and arid habitats – on rocks directly exposed to the sunlight, out of the forest coverage, with low air humidity and high light exposure. Almost all morphological characteristics, phenological traits, as well as the presence and duration of the anabiotic stage vary significantly between the two types of habitats. Arid habitats are characterized with bigger plant density, much smaller individual plants and paler color, which correspond to less chlorophyll content. The flowering and seed ripening generally occur 1-2 months earlier than in the classic habitats when located approximately at the same altitude. The plants from arid habitats also enter anabiotic stage 1-3 months earlier than these in classic habitats, and the anabiosis duration is usually longer,
while the plants from some of the classic habitats may not enter anabiosis at all.

It is interesting to study whether and to what extent the phenotype variations among different populations are due to the plant adaptability or the geographic isolation has caused also some differences on DNA level. Therefore in this study, we focus on another important aspect of resurrection plants evolution and ecology – the genetic variability of the species. For this purpose in vitro plants from three remote populations with significant morphology and phenology variations were used to analyze polymorphism in the internal transcribed spacer 1 (ITS1) region.

The ITS1 region is one of the most popular markers for phylogenetic inference at the generic and infrageneric levels in plants and animal species. It is part of the 18S–5.8S–26S nuclear ribosomal cistron. The nuclear rDNA genes are represented in multiple copies in plant genomes (Alvarez and Wendel, 2003). The structure of the rRNA encoding region is the following (in 5′–3′ direction: external transcribed spacer (ETS) – 18S rRNA gene – first internal transcribed spacer (ITS1) - 5.8S rRNA gene – second internal transcribed spacer (ITS2) – 26S rRNA gene. ITS sequence data are widely used in taxonomy and phylogeny research since 1994 and have provided insights into phylogenetic history, polyploid ancestry, genome relationships, historical introgression, and other evolutionary questions (3, 19).

The differences in environmental conditions and the isolation are prerequisites for polymorphism, and the population polymorphism is at the base of divergence, evolution of new traits and speciation (12). In this respect, it is interesting from evolutionary, genomic and ecological point of view, to study the level of DNA the polymorphism between *H. rhodopensis* natural populations. The current study represents an initial assessment of the polymorphism between three distant populations of *Haberlea rhodopensis* Friv. using internal transcribed spacer 1 (ITS1) sequences as a molecular marker.

### Materials and Methods

#### Plant sources

The plants in this study represent three natural Bulgarian populations of *Haberlea rhodopensis*. In order to be sure that they represent different populations, the localities are chosen to be remote and with different level of isolation. The distance between them is about 100 km air distance (Fig. 3). The environmental conditions of the localities are also very different (Table 1).

**Table 1**

<table>
<thead>
<tr>
<th>Locality</th>
<th>Population/Subpopulation</th>
<th>Coordinates</th>
<th>Altitude ±10 m</th>
<th>Type of habitat</th>
</tr>
</thead>
<tbody>
<tr>
<td>Madzharovo</td>
<td>East Rhodopean</td>
<td>N41º38.510 E025º52.760</td>
<td>135</td>
<td>Arid</td>
</tr>
<tr>
<td>Chervenata stena, near Bachkovo</td>
<td>Central Rhodopean</td>
<td>N 41º55.840’ E 024º52.036’</td>
<td>640</td>
<td>“Classic”</td>
</tr>
<tr>
<td>Biala reka, near Kalofer</td>
<td>South Balkan</td>
<td>N 42º39.778’ E 024º57.318’</td>
<td>610</td>
<td>“Classic”</td>
</tr>
</tbody>
</table>

### In vitro micropropagation

The Gesneriaceae gene bank contains plants generated from seeds as starting material. The seeds were collected from source localities and their viability was tested.

- **Seeds collection:** The seeds were collected in July during expeditions to the habitats of interest. For better conservation, the seeds were collected together with seed capsules. Then they were stored at dry and airy place at temperature near 25°C. The aim was to collect seeds from as much plants as possible, covering the entire reachable area of the habitat. This approach allows maximum of the habitat biodiversity to be included into the in vitro gene bank. Simultaneously, in order to not hamper the natural reproduction of plants, not more than 1-2 capsules from each plant (on average) were collected.

- **Germination test.** The seed capsules were removed, and the very fine seeds (under 0.5 mm in diameter) were passed through fine sieves. In order to test the viability of the seeds in vivo, they were put on Petri dishes with moist filter paper and stored at 23°C until a plantlet with two green leaves appears.
The genetic bank is maintained using original in vitro technologies (14, 23). We use the direct organogenesis method, modified and adapted for Haberlea and Ramonda by our research group (8).

The direct organogenesis method has the following stages:

- **Dry Sterilization.** The seeds were put into Eppendorf tubes and were sterilized in an excicator on the fume of sodium hypochlorite and HCl.

- **In vitro germination.** Sterilized seeds were put in vitro in vials with nutrient medium WPM, without sugar and hormones. At the end of this stage, a normal organogenesis of individual microplants was observed.

- **Micropropagation.** The Haberlea rhodopensis microplants from the previous stage were transferred on nutrient media WPM with phytohormones BAP and IAA (14, 23). The medium was supplemented with an antioxidant - 200 μg/ml filter sterilized glutathione and the pH was stabilized with K-phosphate buffer.

- **Rooting.** At the end of this stage, the microplants formed a stable root system.

- **Adaptation.** The rooted plants were planted in vivo in a turf-perlite mixture 1:1 (v/v) and they were grown under controlled air humidity and temperature in a growth camera for a period of three weeks. At the stages 2, 3 and 4, the microplants were grown in vitro in growth cameras with controlled temperature 21°C±1°C, at light regime 16 h light (day)/8 h darkness (night).

**Study of ITS polymorphisms**

**DNA extraction:** Fresh leaves from the in vitro cultivated plants were frozen in pre-cooled with liquid nitrogen mortar and pestle and ground 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 concentrations of the isolated DNA samples, while the ratios A260/A280 and A260/A230 to determine 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:** We used two ITS1 primers from the primer Set Ribosomal primers (University of British Columbia, Nucleic Acid-Protein Service Unit, NAPS Unit, www.michaelsmith.ubc.ca/services/NAPS/Primer_Sets/) namely: ITS fw: 5’-TCC TCC GCT TAT TGA TAT GC-3’ and ITS Rev 5’-GGA AGT AAA AGT CGT AAC AAG G-3’. Because the production of this primers set 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 10 mmol final concentration.

**PCR reactions:** Approximately 150 ng DNA template was taken for each sample and mixed in 250 μL PCR tube with 1 μL of each primer (10 mmol.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 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°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 6,5 mL of loading dye (Fermentas #R0611), loaded onto 1% agarose gel containing 0,5 mg/ml ethidium bromide (final concentration) covered with 0,5X TBE buffer and separated by applying 7 volts per cm electrical currency. The size of the products was determined by comparison with DNA by comparison with a DNA ladder (Fermentas GeneRuler#SM0311). The PCR products were visualized by UV light.

**PCR product isolation, cloning and sequencing:** The PCR products were isolated from the agarose by QIAquick Gel Extraction Kit (Qiagen, cat No 28704) following the original protocol. The concentration of the PCR products was determined spectro-photometrically and 2-4 μL of them was used for A/T cloning. QIAGEN PCR Cloning Kit (cat No 231124) was used to clone the PCR products according to the original protocol. The ligation reactions were mixes with 250 μL freshly prepared competent bacterial (E. coli-TOP 10 - Invitrogen) cells. The plasmids containing PCR products were isolated by QIAprep Spin Miniprep Kit (Cat No 27104) following the original protocol. The isolated plasmids were dissolved in 50 μL buffer (10 mM Tris-Cl, pH 8.5) and sent for sequencing to MWG – Biotech AG, Frankfurt, Germany.

**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 multiple alignments
Results and discussion

The suitability of the primers was initially tested using several different PCR conditions. In order to achieve optimal amplification we did vary the amounts of DNA template from 50 to 300 ng and annealing temperatures from 48 to 57°C. The optimal amplification of the ITS1 region was achieved with 150 ng (2 µl) DNA template and annealing temperature 55°C (Fig. 1). The amplified PCR products were with the expected size (about 450 bp). They were isolated from the gel, cloned in pDrive vectors as described in materials and methods and sent for sequencing.

The online nblast (2) in the NCBI database demonstrated high similarity of the isolated ITS1 sequences with the annotated ITS1-5.8S-ITS2 sequence of *H. rhodopansis* Friv. (20).

The multiple alignments of the isolated by us sequences with the one annotated in NCBI is presented on Fig. 2.

The results demonstrated the very high sequence similarity between the populations from the three distinct geographic locations. Only the samples from the Central Rhodopi region (Chervenata Stena) displayed two transitions from thymine (T) to cytosine (C) at relative positions 37 and 94 in ITS1 spacer. The positions of the transitions were determined according the the annotated in NCBI ITS1 sequence (20). Since the studies five samples from each population and the products were independently cloned and sequences in both directions we consider these replacements true.

The ITS region of the ribosomal cistron is one of the most popular sequences for phylogenetic analyses at the generic and infrageneric levels in plants. The main feature that makes this region so widely used in molecular phylogeny and evolution studies is the combination of highly conservative (rRNA genes) and highly variable (ETS, ITS) regions. Other advantages of the use of ITS sequences are:

- The ITS region is relatively short (500-800 nt) (21)
It is easily amplified by PCR using universal primers complementary to the conservative ITS-flanking regions of rRNA genes (6, 16)

ITS regions are easily amplified even from diluted or degraded DNA samples (10)

ITS regions are highly variable – they could accumulate differences between closely related species and even between populations within a species (1).

As the ITS regions are not functional, the evolution of these sequences seem to occur according to the neutral model (Kimura, Li…), in which the genetic drift is the major driving force. Natural selection cannot operate on non-functional sequences because they don’t have adaptive meaning for the organism, so the changes occurring in such sequences are random and accumulate mutations uniformly with the time (molecular clock) (12, 22). This makes neutral sequences useful markers for phylogeny analyses because the mutation rate reflects the divergence time between populations/species. (5, 22)

In our experiment, the high sequence similarity could mean that the isolation of the populations is a recent event, or that the populations are not completely isolated.

Perhaps the most interesting fact is that the mutation rate doesn’t seem to be related to the level of isolation. Although the ear distance between the three analyzed localities is almost equal and is about 100 km air distance, in Rhodopizes there are more intermediate localities, while there are no such localities between Balkan and Rhodopean Haberlea populations (Fig. 3). This is why we expected to find more differences between the Rhodopean and Balkan populations than between the two Rhodopean ones. The two unexpected C-T substitutions in the Chervenata stena population could be a sign for local increase in the mutation rate. It is possible that these changes reflect rapid and local molecular events in the ITS region, randomly fixed in the Chervenata stena population. These initial results show that we could expect unusual patterns of distribution of polymorphisms between Haberlea populations.

On the other hand, various molecular processes impact ITS sequences. Among the most prevalent complications is the existence in many plant genomes of extensive sequence variation, arising from ancient or recent array duplication events, genomic harboring of pseudogenes in various states of decay, and/or incomplete intra- or interarray homogenization (3). This is the reason in most recent studies ITS polymorphism analyses to be accompanied with other independent methods.

Fig. 3. The geographic positions of analyzed (red dots) and other (orange dots) H. rhodopensis localities and the populations they represent: 1) East Rhodopean (Madjarovo); 2) Central Rhodopean (Chervenata Stena); 3) South Balkan (Biala reka)

In their extensive review (3), Alvarez and Wendel recommend together with ITS sequences other single-copy nuclear genes to be used. Another reason to use genes along with ITS sequences would be the possibility to correlate the observed changes with certain phenotype traits. In this respect in our future research, we plan to assess the levels of polymorphism between stress-related single-copy nuclear genes of H. rhodopensis.

Acknowledgments
This research is supported by the National Science Fund, at the Ministry of Education and Science, Bulgaria, grants № DO 02/236 and IFS-B-606. The authors Evelina Daskalova and Iliya Denev have equal contribution to the article.

REFERENCES