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ORIGINAL ARTICLE

Morphological variation, genetic diversity and genome size of critically endangered *Haberlea* (Gesneriaceae) populations in Bulgaria do not support the recognition of two different species

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Abstract *Haberlea* is one of the few Gesneriaceae genera that has entered Europe. It is a highly endangered genus and red-listed in Bulgaria. Two species, *H. rhodopensis* and *H. ferdinandi-coburgii*, have been described to occur in Bulgaria, but this has never been addressed systematically. Here, we used molecular ISSR markers, morphological and nuclear DNA content to investigate the taxonomic and genetic status of *Haberlea* in Bulgaria. We found low levels of genetic diversity but significant genetic differentiation among the 12 investigated populations, with a strong separation between Balkan Mountain populations in the north and Rhodope Mountain populations in the south. However, the multivariate morphological analyses did not support such a division. The population from near Lovech, the type locality of the putative species *H. ferdinandi-coburgii*, did not differ in ploidy level from *H. rhodopensis* and did not form a separate entity in neither of the analyses and the existence of this species is therefore not supported.

Keywords Genome size · *Haberlea ferdinandi-coburgii* · *Haberlea rhodopensis* · ISSRs · Principle component analysis (PCA) · Principle coordinate analysis (PCO)

Introduction

Haberlea Friv. is a genus in the family Gesneriaceae (Weber 2004; Weber and Skog 2007). It was established on material from the Rhodope Mountains in Bulgaria, and the Hungarian botanist Frivaldszky (1835) described and published *Haberlea rhodopensis* Friv. (Szeląg and Somlyay 2009). The species is distributed in Central (Balkan Mountains) and southern Bulgaria (Rhodope Mountains) and in Greece (Menikion, Pangeon, Falakron, Rhodope, Papikion Mountains and the Nestos river gorge) (Strid 1991; Bazos and Petrova 2010). The species is listed in the *Red Data Book of the Republic of Bulgaria* (Biserkov 2011) and the IUCN Red List of Threatened Plants (Walter and Gillet 1998) as rare, and is protected in Bulgaria since 2002 (Biodiversity Law 2002).

At the beginning of the 20th century, a second species, *Haberlea ferdinandi-coburgii* Urum., from the northern mountains in Bulgaria, near 'Lovec' (=Lovech), was described (Urumoff 1902). It was distinguished from *H. rhodopensis* on the basis of several quantitative characters. In the Botanisches Centralblatt, Adamović (1903) commented that it is difficult to determine whether the new species is distinct from the existing *H. rhodopensis* since Urumoff neither included a drawing nor an extensive diagnosis of the new species. He stated that from

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H. rhodopensis, the new species is apparently distinguishable by a smaller habit, adaxially glabrous leaves, smaller flowers, narrower and more pointed sepals, dark-blue corolla and by an elongated capsule which gradually tapers out.

H. ferdinandi-coburgii was classified as a neo-endemic species in Bulgaria by Turrill (1951). In the Mountain Flora of Greece, Strid (1991) synonymised *H. ferdinandi-coburgii* with *H. rhodopensis*, but without further comments or supporting data. In the most recent Flora of Bulgaria (Markova 1995), both species are included, but *H. ferdinandi-coburgii* is considered as a variety of *H. rhodopensis*. *H. ferdinandi-coburgii* has not been recollected since its first description. Plants with this name are in cultivation in many botanical gardens, such as in Edinburgh and at the Royal Horticultural Society (RHS) site in Wisley. However, these have usually no locality records, do not conform to the description and are often indistinguishable from *H. rhodopensis* plants. *Haberlea* are commonly propagated asexually from cuttings and many forms and cultivars are maintained as clones in cultivation, masking any natural intra-population variation that exists. For this reason, we did not rely on cultivated material to investigate whether *H. ferdinandi-coburgii* represents an independent taxonomic entity, and collected *Haberlea* material from their natural environment.

Haberlea plants grow in shady rock crevices on limestone at altitudes of 100–1,700 m (Petrova and Vladimirov 2010), forming dense tufts of leaves, every rosette bearing in spring one to five inflorescences, each with two to four flowers (Szelag and Somlyay 2009). Individual plants are rather long-lived and can grow for several decades. Detailed knowledge of the reproductive biology of the species is, however, scant. It is known that *Haberlea* reproduces vegetatively, sometimes through short stolons, and by seeds (Ganchev 1950; Vassilev 1984). The seeds are relatively small (ca. 450 μm long), have no obvious morphological trait that would support long distance dispersal (Beaufort-Murphy 1983). *H. rhodopensis* shows a prominent entomophilous floral syndrome, with flowers possessing an open tube and bright corolla markings with anthers that are present deep in the corolla tube behind the receptive stigma. This spatial arrangement increases outcrossing rates, since pollen on visiting pollinator is first placed on the stigma before new pollen is deposited on the back of the pollinator. From the flower morphology, supported by observations on plants growing at the Royal Botanic Garden Edinburgh, it is highly likely that this species is entomophilous in the wild, similar to other European members of the family, like *Ramonda myconi* and *Jancaea heldreichii* (Vokou et al. 1990; Dubreuil et al. 2008). It is expected that the homogenising effects of gene flow decrease with increasing geographical distances between the populations. Thus, it is expected that there is little or no gene flow between populations separated by physical barriers, such as the Balkan and Rhodope Mountains. Since *Haberlea* is an endangered species, knowledge of the taxonomic status and genetic make-up of its populations are essential for future conservation initiatives (Xiao and Gong 2006).

Molecular techniques have provided many tools for studying the genetic diversity at the population level (Avise 2000). Molecular markers such as random amplified polymorphic DNA (RAPD; Munthali et al. 1992), amplified fragment length polymorphism (AFLP; Vos et al. 1995), simple sequence repeats (SSR; Zietkiewicz et al. 1994) and inter-simple sequence repeat (ISSR; Wolfe and Randle 2001; Kiani et al. 2012), although having limitations (e.g. Archibald et al. 2006), have been widely used to detect genetic diversity in plants, where no detailed genomic information is available (e.g. Wolfe and Liston 1998). RAPDs have been applied successfully in populations of *R. myconi*, another European Gesneriaceae genus (Dubreuil et al. 2008). Here we genotyped *Haberlea* populations with ISSR markers which can be highly variable within a species and have the advantage over RAPDs in utilising longer primers that allow more stringent annealing temperatures (Camacho and Liston 2001) and revealing more polymorphic fragments (Fang and Roose 1997).

In Gesneriaceae, single or few morphological characters in taxonomy have proven problematic in the past (e.g. Burtt 1977, 1997). However, the application of multivariate approaches, such as principle component analysis (PCA), on a suite of characters has proven to result in taxonomic patterns that are closely resembling geographic distributions of taxa and enabling the delineation of species (e.g. Möller et al. 2007). In the present study, we use PCA to investigate the distribution of morphological patterns among *Haberlea* populations.

H. ferdinandi-coburgii was described as being smaller in most morphological aspects from *H. rhodopensis* that are reminiscent of differences in ploidy levels (Leitch and Bennett 1997). To elucidate whether these morphological differences are perhaps due to variation in ploidy levels or due to genetic or epigenetic factors, we complemented the DNA analyses with flow cytometry data. Cytological data are only available for *H. rhodopensis*. With counts of $2n = 48$ (Contandriopoulos 1966), $2n = 44$ (Lepper 1970; Milne 1975) and $2n = 38$ (Borhidi 1968), a variation in polyploidy level is not indicated. However, *H. ferdinandi-coburgii* has not been investigated cytologically. Variation in genome size and ploidy levels were recently reported in the Iberian peninsula species of *Ramonda* (Gesneriaceae) and their associated effects on morphology are observed (Siljak-Yakovlev et al. 2008).

Here, we analysed samples from 12 natural *Haberlea* populations covering the entire distribution range of this

genus in Bulgaria, including plants from the area of Lovech, the type locality of *H. ferdinandi-coburgii*. We gathered molecular, morphological and genome size data to determine the levels of genetic and morphological diversity among populations of *Haberlea*, and to investigate the taxonomic status of the genus in Bulgaria. The provided data can be used further for the establishment of conservation practices for the preservation of these endangered plants.

Materials and methods

Plant material

Plants from 12 populations were collected during the period April–August 2008 and 2009 (Fig. 1; Table 1), covering the entire distribution range of *Haberlea* in Bulgaria as defined in the latest Flora of Bulgaria (Markova 1995). These represent all known major locations based on information from the available literature (Petrova 2006; Petrova et al. 2010) and from specimens deposited in the Herbaria of Sofia University, Institute of Botany at Bulgarian Academy of Sciences, and the Agricultural University, Plovdiv.

DNA extraction

Leaves were taken from plants collected in the field, and rapidly dried and stored in silica gel until DNA extraction. Genomic DNA was extracted from 50 mg of dried leaf tissue according to the procedure described by Dellaporta et al. (1983).

Inter-simple sequence repeat (ISSR) analysis

PCR conditions and amplification product analysis

ISSRs for a total of 120 plants (10 per population) were obtained. Ten primers (Microsynth, Balgach, Switzerland) were selected (Table 2), after the screening of 15 primers on a subset of all samples from the 12 populations. Polymerase chain reactions (PCRs) 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 thermal cycler GeneAmp® PCR System 2700 (Applied Biosystems, Foster City, CA, USA). To determine the optimal annealing temperature (T_a) 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 best T_a was selected for each primer for routine ISSR fingerprinting. The reproducibility of the technique was tested by replicating each amplification reaction twice. To further ensure the quality, PCRs were performed with one positive as well as one negative control.

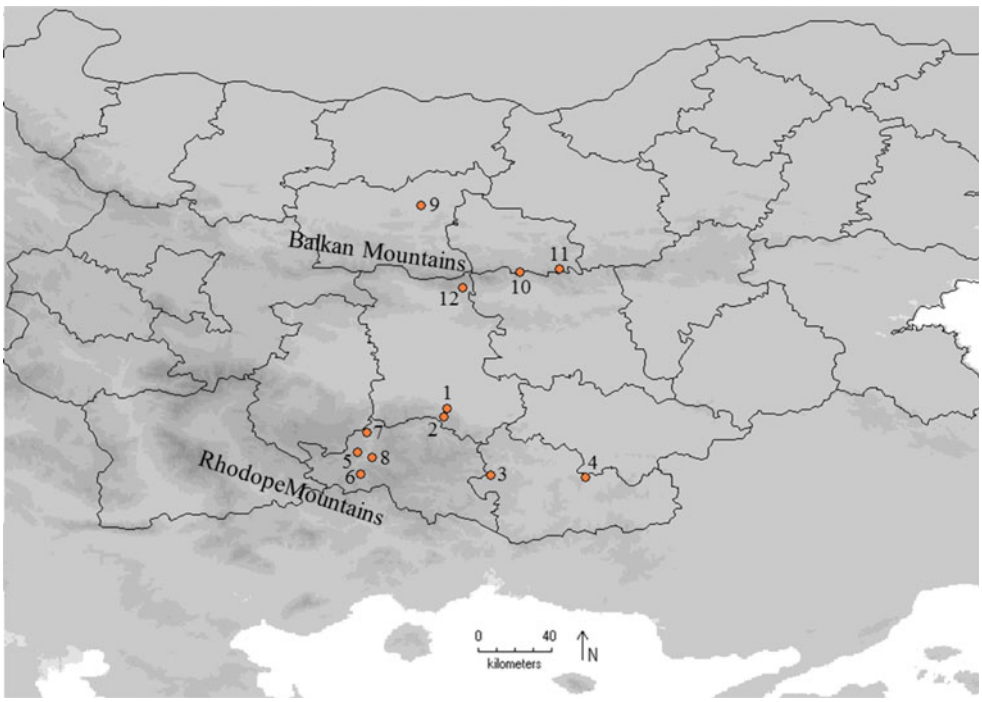


Fig. 1 Locations of the investigated 12 populations of *Haberlea* in Bulgaria. *9* Lovech. Population code as in Table 1

Table 1 Collection details for the 12 *Haberlea* populations from Bulgaria analysed here for ISSR polymorphisms, morphology, and nuclear DNA content determination

Region	Locality	Population code	Latitude (N)	Longitude (E)	Altitude (m a.s.l.)	ISSRs	No of plants morphology	DNA content
Rhodope mountains	Asenova krepost	Pop01	41°59′17″	24°52′15″	410	10	19	11
	Bachkovo	Pop02	41°56′39″	24°51′25″	372	10	24	5
	Dyavolski most, Ardino	Pop03	41°37′13″	25°06′53″	416	10	33	5
	Studen kladenec	Pop04	41°36′27″	25°38′48″	153	10	23	10
	Devin	Pop05	41°44′44″	24°22′27″	812	10	32	5
	Trigrad	Pop06	41°37′27″	24°23′34″	786	10	29	9
	Mihalkovo	Pop07	41°51′29″	24°25′23″	550	10	30	5
	Shirokolashka reka	Pop08	41°42′56″	24°27′12″	786	10	30	5
Balkan mountains	Lovech	Pop09	43°07′25″	24°43′34″	202	10	10	10
	Malusha	Pop10	42°44′59″	25°16′54″	1,312	10	26	5
	Plachkovci	Pop11	42°45′57″	25°30′07″	1,061	10	20	5
	Byala reka, Kalofer	Pop12	42°39′47″	24°57′36″	1,278	10	26	5
Total						120	301	80

Table 2 Primer sequences, total number and number of polymorphic bands and annealing temperature for ten ISSR markers used in this study on 12 *Haberlea* populations from Bulgaria

Primer sequence (5′-3′)	Total number of bands	Number of polymorphic bands	% of polymorphic bands	Annealing temperature (°C)
AGA GAG AGA GAG AGA GAG AGC	23	16	69.6	60
AGA GAG AGA GAG AGA GAG AGT	28	17	60.7	50
ACA CAC ACA CAC ACA CG	26	16	61.5	57
GAG AGA GAG AGA GAG AC	32	20	62.5	50
GAG CAA CAA CAA CAA CAA	27	12	44.4	50
AGA GAG AGA GAG AGA GAG AGG	16	16	100	60
AGA GAG AGA GAG AGA GT	13	13	100	48
AGA GAG AGA GAG AGA GG	13	12	92.3	57
DBD ACA CAC ACA CAC AC	15	10	66.7	57
AGA GAG AGA GAG AGA GC	16	15	93.8	50
Total	209	147	70.3	

The PCR products were analysed on 2 % agarose gels (Fermentas, Vilnius, Lithuania) in 0.5× TBE buffer. A 100-bp DNA ladder size standard (Fermentas, Vilnius, Lithuania) was used to estimate the length of PCR products (100–3,000 bp). 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 135 V and the DNA visualised with a UV transilluminator (e.g. Online Resource F1) and analysed with a video image analyser (BioImaging Systems, Cambridge, UK).

Genetic diversity analysis

The ISSRs were treated as dominant genetic markers. Only strong reliably scorable bands were included (e.g. Online Resource F1). Amplified fragments were scored for the presence (1) or absence (0) of homologous bands. The assignment of ISSR bands to genetic loci was done semi-automatically using GelAnalyser 2010a image analysis software (http://www.gelanalyzer.com/). The binary data were used for characterising the genetic diversity among the *Haberlea* populations. The percentage of polymorphic loci (*P*) and Shannon's information index (*I*) were calculated using GenAlEx ver.6.5 (Peakall and Smouse 2006, 2012). This software was also used to calculate analysis of molecular variance (AMOVA) (Excoffier et al. 1992), among and within populations and between the Balkan vs Rhodope Mountain populations and population Lovech vs the remaining populations (Excoffier et al. 1992; Huff et al. 1993).

To illustrate the spatial genetic relationships among the samples, a principle coordinate analysis (PCO) was

conducted using the Jaccard index (Jaccard 1908) in the R-package Le Progiciel v.4 (Casgrain et al. 2005). To further assess the genetic relationships in the Bulgarian *Haberlea* populations, a neighbor-joining (NJ) clustering analysis based on Nei and Li's genetic distance index was constructed with PAUP 4.0b10 (Swofford 2002). Both indices exclude the shared absence of bands, which can introduce errors (e.g. Archibald et al. 2006). A Mantel test was conducted to test isolation-by-distance using Nei's genetic distance (Nei 1972) uGD and geographic distance in GenAlEx.

Morphometric multivariate analysis

Floral morphological characters

A total of 24 morphological characters were obtained in the field for a total of 301 plants (10–33 plants per population, average 25 plants, for Lovech only 10 plants were available reflecting the small size of the population). All but one character came from flowers as defined in Online Resource F2; character 23 concerned the indumentum on the adaxial leaf surface. All but two were continuous characters; character 16 and 24 were discrete characters (Online Resource T1). Only a few data were missing amounting to 3.1 % of the total data. These were dealt with listwise deletion.

Principal component analysis (PCA)

Procedures for the PCA (Sneath and Sokal 1973) followed Möller et al. (2007) using the R-package and JMP 3 (SAS Institute Inc., Cary, NC), respectively. Because the data consisted of continuous and discrete data, the PCA was carried out using the correlation matrix (see Möller et al. 2007). Prior to PCA, the characters were tested for normal distribution (no character showed a significant skewness and no data transformation were necessary). The results were displayed as two-dimensional scatter plots with JMP, after testing for the number of axes to be displayed in a scree test (Cattell 1966).

The 22 continuous characters were subjected to an ANOVA and pairwise mean differences tested with a two-tailed *t* test in Excel (Microsoft Office 2010).

Nuclear DNA content determination

The 2C DNA levels were determined for 80 *Haberlea* plants (for 5–11 plants per population, average 6.7). Fresh leaves were sampled from plants collected from natural habitats and cultivated in greenhouses at the Agrobioinstitute, Sofia. The nuclear DNA amount was assessed by flow cytometry on triplicate samples following the procedure of Costich et al. (1993) and Siljak-Yakovlev et al. (2008). Tomato (*Solanum lycopersicum*) cv. 'Gardener's Delight' (2C = 2.00 pg) (Obermayer et al. 2002) was used as internal standard.

Briefly, somatic nuclei were isolated by chopping separately 50 mg fresh leaves of *H. rhodopensis* and the internal standard in 1 ml nuclei isolation buffer [$MgSO_4$ buffer, β-mercaptoethanol (5 µl/ml), and 10 % w/v Triton-X-100]. The suspension was filtered through 30 µm nylon mesh and centrifuged at 13,000 rpm for 1 min. After removing the supernatant, the pellet was re-suspended in 500 µl PI/RNase staining buffer (10 µg/ml PI and 300 µg/ml RNase; Cat. No. 550825; BD Biosciences, New York, USA) and incubated for 15 min at 37 °C. The standard and experimental samples were mixed at this point and analysed.

We used a BD FACSCalibur flow cytometer with two lasers, a red diode laser (635 nm) and an argon laser (15 mW, 488 nm). A minimum of 10,000 events were measured for each sample. The data were processed on a Power Macintosh G4 using the software Cell Quest Pro (BD Biosciences). Absolute 2C DNA values were calculated according to the formula:

$$(\text{mean of the test sample peaks/mean of the calibration standard peaks}) \times \text{known 2C value of the calibration standard used.}$$

The estimated DNA contents were compared with a Kruskal–Wallis test and Dunn's multiple comparison test in GraphPad Prism (La Jolla, CA, USA).

Results

ISSR diversity

For the 120 plants from the 12 *Haberlea* populations, the 10 selected ISSR primers yielded a total of 209 reproducible amplification bands in the size ranging between 200 and 2,500 base pairs (bp) of which 147 (70.3 %) showed variation across all samples. At the individual primer level, of these 44.4–100 % were polymorphic across all populations with an average of 70.3 % (Table 2).

Population genetic diversity

The polymorphic loci at the population level ranged from 38.1 to 61.9 %, with an average of 51.0 %. The Shannon's information index (*I*) was on average 0.268, ranging from 0.2 to 0.319. In all genetic diversity indices, populations from the Rhodope Mountains had lower values, with population Asenova krepost having the lowest values.

Table 3 Genetic diversity indices for 12 populations of *Haberlea* from Bulgaria, based on ten ISSR loci

Population	Pop code	*P* (%)	*I*	Private bands
Rhodope Mountains				
Asenova krepost	Pop01	38.1	0.200 (0.022)	1
Bachkovo	Pop02	49.7	0.271 (0.024)	0
Dyavolski most, Ardino	Pop03	52.4	0.262 (0.023)	0
Studen kladenec	Pop04	51.0	0.269 (0.024)	0
Devin	Pop05	46.9	0.256 (0.024)	0
Trigrad	Pop06	46.9	0.234 (0.022)	0
Mihalkovo	Pop07	51.0	0.272 (0.024)	2
Shirokolashka reka	Pop08	51.7	0.279 (0.024)	0
Balkan Mountains				
Lovech	Pop09	60.5	0.319 (0.023)	2
Malusha	Pop10	61.9	0.318 (0.023)	0
Plachkovci	Pop11	45.6	0.243 (0.023)	0
Byala reka, Kalofer	Pop12	56.5	0.297 (0.024)	2
Mean (SE)		51.0 (1.89)	0.268 (0.007)	0.58

P Percentage of polymorphic loci, *I* Shannon's information index

Those from the Balkan Mountains, except Plachkovci had higher values, with population Lovech having the highest, followed by population Malusha (Table 3).

Among the eight southern populations, only two had a total of three private alleles (Table 3), one in population Asenova krepost, and two in population Mihalkovo, while among the four northern populations, Lovech and Kalofer had two private alleles each.

Population genetic differentiation

The AMOVA without geographic structuring showed about two-thirds (63 %) of the variation to reside within populations, and the rest among populations (Table 4). When geographically structured into the northern Balkan and southern Rhodope Mountains, 11 % of the genetic variation was distributed among the northern and southern populations, with significant differentiation between populations ($F_{PR} = 0.336$; $P = 0.001$) and regions ($F_{RT} = 0.111$; $P = 0.001$) (Table 4). When analysing population Lovech against the remaining Balkan populations, a significant differentiation was observed ($F_{RT} = 0.056$; $P = 0.017$) (Table 4).

In the PCO, the individuals clustered relatively strongly according to their population assignation (Fig. 2). The population clusters were separated strongly along a line representing the northern and southern localities. Population Lovech fell in the interphase between the two geographic clusters, and here overlapping with samples of the Shirokolashka reka population from the Rhodope Mountains. The NJ tree (Fig. 3) divided the populations into two main clusters that corresponded to the geographic areas, the Rhodope and Balkan Mountains, with the only exception of the Shirokolashka reka population. This southern population from the Rhodope Mountains clustered with the northern populations from the Balkan Mountains. Half the populations fell in single population clusters. The remaining ones formed mixed clusters: populations Bachkovo and Ardino, Studen kladenec and Devin, and Plachkovci and Kalofer.

The Mantel test showed a significant relationship between genetic distance and geographic distance ($P = 0.037$) (Online Resources F3, T2).

Morphological data

Principle component analysis (PCA)

In the PCA, the first 3 axes contained 32.7, 16.4 and 7.9 % variance (total = 57.1 %), and the scree test suggested that the first three axes should be displayed (Online Resource F4). There was little structure in the resulting PCA plot, and samples from the Balkan and Rhodope Mountains were mixed together, irrespective of the axis observed (Fig. 4). The samples from Lovech fell as a loosely spread group scattered among those from both regions. One sample from Lovech (sample 09–10) fell quite separate (Fig. 4a, b). Inspection of the values for the individual characters revealed that this plant had the smallest flowers observed in the field, with the smallest dimensions for all but three characters (and for those the values were near the smallest). This was also the only plant with glabrous adaxial leaf surfaces.

Individual characters

At the regional level, flowers from plants from the Balkan Mountains had, on average, smaller dimensions in all but five continuous characters. Those, where larger dimensions

Table 4 Analysis of molecular variance (AMOVA) based on 10 ISSR markers for the 12 populations of *Haberlea* from Bulgaria analysed

Source of variance	*df*	SS	MS	Variance component	Percentage total (%)	*F* statistic	*P*
All populations							
Among populations	11	1,119.625	101.784	8.720	37		
Within populations	108	1,574.700	14.581	14.581	63	$F_{PT} = 0.374$	0.001
Total	119	2,694.325	23.301		100		
Balkan vs Rhodope mountains populations							
Among regions	1	234.688	234.688	2.741	11	$F_{RT} = 0.111$	0.001
Among populations within regions	10	884.938	88.494	7.391	30	$F_{PR} = 0.336$	0.001
Within populations	108	1,574.700	14.581	14.581	59	$F_{PT} = 0.410$	0.001
Total	119	2,694.325		24.713	100		
Lovech vs Balkan mountains populations							
Among regions	1	114.017	114.017	1.403	6	$F_{RT} = 0.056$	0.017
Among populations within regions	2	185.933	92.967	7.699	31	$F_{PR} = 0.325$	0.001
Within populations	36	575.000	15.972	15.972	63	$F_{PT} = 0.363$	0.001
Total	39	874.950		25.075	100		

df Degree of freedom, *SS* sum of squares, *MS* expected mean squares

* *P* value denotes the probability of null hypothesis

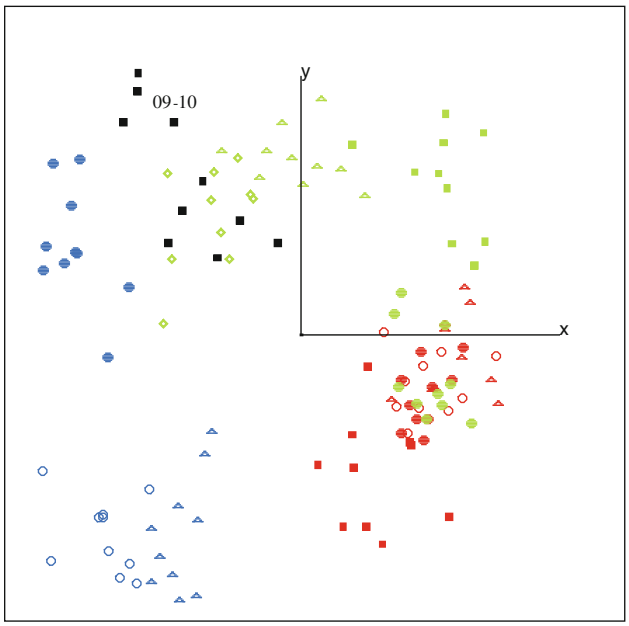


Fig. 2 Scatterplot of the principal coordinate analysis (PCO) based on ISSR polymorphisms of 120 samples from 12 populations of *Haberlea* from Bulgaria based on the Jaccard index. Populations from the Balkan Mountains in *blue* and *black* (population Lovech), the remaining from the Rhodope Mountains *red open triangles* Pop01, *red open circles* Pop02, *red circles* Pop03, *red squares* Pop04, *green circles* Pop05, *green squares* Pop06, *green open triangles* Pop07, *green open diamonds* Pop08, *black squares* Pop09, *blue circles* Pop10, *blue open circles* Pop11, *blue open triangles* Pop12. Population code as in Table 1. *09–10* indicates morphologically distinct sample of the Lovech population

were observed all concerned sepal characters (Online Resource T3).

Comparisons of the population means for individual characters, using population Lovech as bench mark, revealed a mixed picture of the distribution of characteristics. For most characters, plants from Lovech had significantly smaller measurements compared to all other population means (Online Resource T3). For most characters of the calyx (character 17–23), the measurements were similar across all populations, including Lovech. For some Rhodope populations, significantly smaller dimensions were recorded compared to Lovech plants. Observing those characters that were deemed critical for the distinction of the two *Haberlea* species, concerning the flower size (e.g. length of the corolla tube, characters 4 and 5, corolla width and height, characters 10 and 11, corolla colour, character 16, sepal width characters 21–23) and leaf indumentum (character 24), some populations had larger values, but others identical or smaller values (e.g. corolla tube length in populations 10 and 11, or corolla face width in populations 4, 10,11 and 12) (Online Resource T3, highlighted grey).

Nuclear DNA content

The majority of CV % of G0/G1 for single peaks of *Haberlea* samples was between 3 and 4 (e.g. Online Resource F5). The 2C nuclear DNA values ranged from 2.62 pg (Studen kladenec) to 3.00 pg (Trigrad) ($P = 0.01$), a difference of 14.5 % (Table 5). The content of population

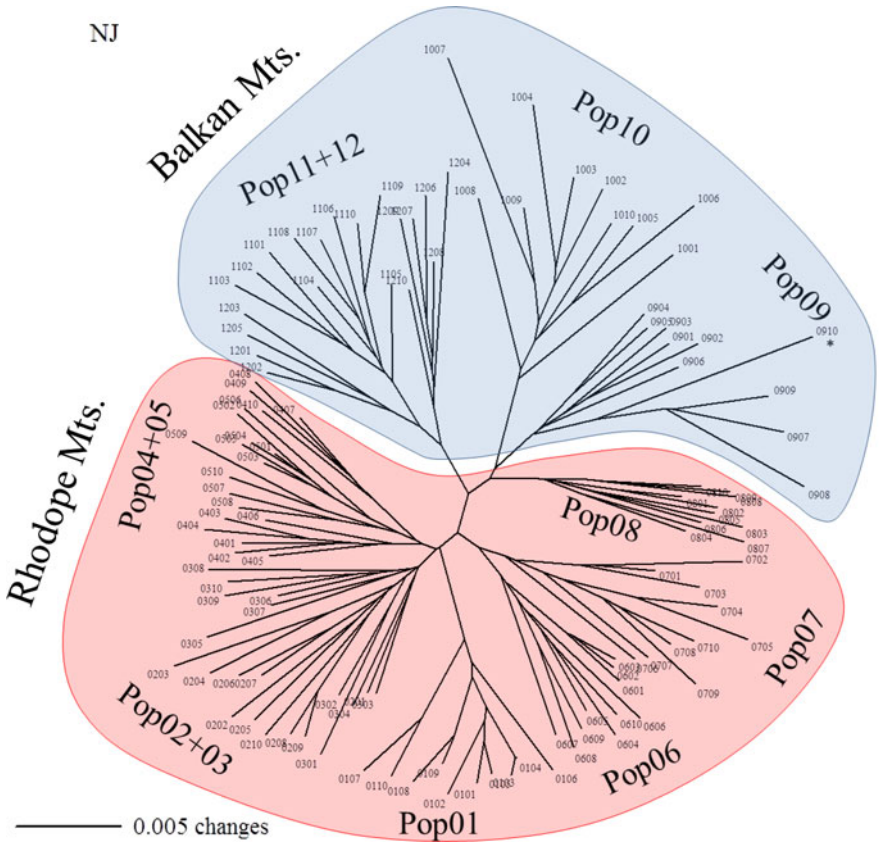


Fig. 3 Unrooted neighbor-joining tree of 120 individuals of 12 populations of *Haberlea* from Bulgaria based on ISSR markers and Nei and Li's genetic distance. Individual sample numbers are a combination of the population code and individual within population numbers. Population codes as in Table 1. *Asterisk* indicates morphologically distinct sample of the Lovech population (09–10)

Shirokolashka reka (2.62 pg) was also significantly different from population Trigrad ($P = 0.05$). There was no statistical difference between the Balkan Mountains (mean 2C = 2.83 pg, SD = 0.13) and Rhodope Mountains (mean 2C = 2.81 pg, SD = 0.15) population averages (*t* test; $P = 0.308$) (Online Resource T4). The 2C DNA value for the Lovech population was 2.75 pg (SD = 0.11), close to the overall average of 2.82 pg (SD = 0.14).

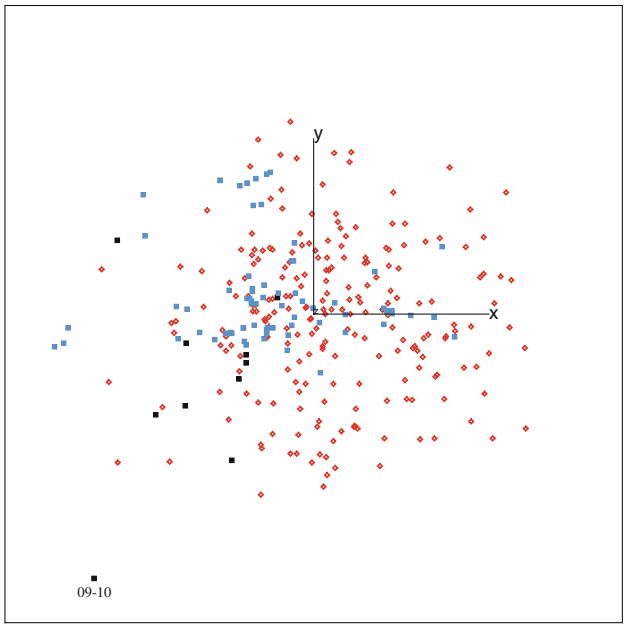
Discussion

The genus *Haberlea* is distributed in Bulgaria and Greece. We present a study that is focussed on the populations from Bulgaria to address the genetic diversity status of the genus in Bulgaria from a conservation perspective and to determine whether the genus contains separate entities in Bulgaria, specifically *H. rhodopensis* and *H. ferdinandi-coburgii*. A population genetics study across the entire range of the genus and an unravelling of the biogeographic history will be dealt with in a forthcoming paper. The discussion has to be interpreted in the light of this aspect.

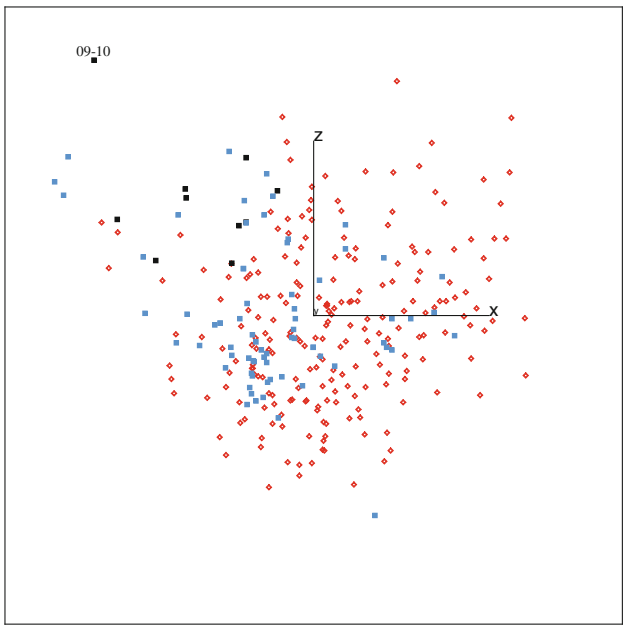
Genetic diversity and differentiation

The population genetic diversity and structure of a species reflect the interaction of various factors, including the long-term evolutionary history of the species, genetic drift, gene flow, breeding system, seed dispersal, geographic range (Hogbin and Peakall 1999). Of these factors, the breeding system is the main source affecting the genetic diversity both among and within populations (Hamrick and Godt 1989). We found relatively low levels of genetic diversity among the populations of *Haberlea* in Bulgaria (Table 3). This is somewhat surprising since the genus has been regarded as a tertiary relict in Europe (e.g. Thompson 2005). However, this may indicate that the populations are relatively young and have been affected by the last glacial maximum in Europe, and have recently become established after the glaciers retreated ~20,000 years ago (e.g. Clark et al. 2009). A comparable scenario was found for another European Gesneriaceae, *R. myconi*, from the Pyrenees using RAPDs (Dubreuil et al. 2008).

The levels of genetic differentiation among regions (11 %; $P = 0.001$) and particularly among populations within regions (30 %; $P = 0.001$) in *Haberlea* suggest that gene flow between populations is not high. The breeding system of plants greatly affects the genetic differentiation, with outcrossing and long-lived seed plants maintain most of the genetic variations within populations, while predominantly selfing, short-lived species harbour comparatively higher variation among populations (Hamrick and Godt 1989). *Haberlea* as a putative outcrossing long-lived plant follows the trend here.



(A) Axis 1(x) and 2 (y)



(B) Axis 1(x) and 3 (z)

Fig. 4 Scatterplot of the principal component analysis (PCA) on 301 samples from 12 populations of *Haberlea* from Bulgaria, based on 24 morphological characters. Populations from the Balkan Mountains in *blue* with population Lovech in *black*, those from the Rhodope Mountains in *red*. *09–10* indicates morphologically distinct sample of the Lovech population

Species with restricted distribution ranges usually have lower genetic diversity and, usually, tend to be genetically homogeneous at the population level. The habitats of *Haberlea*, separated by inter-mountains valleys and mountains, have seemingly reduced the gene flow among populations. Pollinator behaviour is another factor that can lead to this limited gene flow. The potential pollinators of *Haberlea*, diverse species of honeybees do not fly over longer distances of a few kilometres (Eckert 1933; Ratnieks 2000). *Haberlea* seeds are primarily gravity dispersed over short distances, a factor contributing to higher levels of genetic differentiation (Hamrick and Godt 1989). These facts combined with the effects of the last glaciation may explain the combination of low genetic diversity and higher differentiation among populations of the relict *Haberlea*.

Considerations for conservation

From a conservation genetics point of view, there were some discernible structures separating populations from the Balkan Mountains in the north and the Rhodope Mountains in the south, with the former harbouring a slightly higher genetic diversity (Table 3). This separation was also well illustrated in the PCO (Fig. 2) and NJ tree with the only exception of population Shirokolashka reka (Pop08, Fig. 3). The populations from the Balkan Mountains occur at much higher altitudes (1,061–1,312 m, except population Lovech at 202 m). This may suggest the presence of a high altitude glacial refugial area in Bulgaria. It is interesting that the low altitude Lovech population from the Balkan Mountains clustered in the PCO closer to the lower altitude populations from the Rhodope Mountains (Fig. 2). Here, altitude seems to indicate a closer relationship than geography. The isolated status of population Lovech within the Balkan populations was also indicated in the AMOVA (Table 4). This suggests that it may be a separate genetic entity in this region, though not in Bulgaria and overlaps with populations from the Rhodope Mts. (Fig. 2), and is linked to the high altitude population Malusha (Pop10) from the Balkan Mountains in the NJ tree (Fig. 3). With this discontinuity in geography and genetic affiliations, population Lovech is an interesting link between the northern and southern populations of *Haberlea* in Bulgaria, and an important entity for conservation efforts. The population is rather small (<20 plants) and is extremely endangered due to its relative close vicinity to the city's suburbs. Other entities for conservation foci could include populations with high levels of genetic diversity and presence of private ISSR bands, such as Kalofer in the Balkan Mountains, and Mihalkovo and the two close-by localities of Asenova krepost and Backkovo. Though other considerations, such as population sizes and threats need to be taken into considerations as well.

Morphological data

Unlike the ISSR PCO, the morphological PCA did not result in a separation between the northern and the southern

Table 5 Average nuclear DNA content per plant for 12 populations of *Haberlea* in Bulgaria arranged by increasing values

Region	Population code	Locality	*N*	2*C* nuclear DNA content (pg)	SD	%
Rhod.	Pop04	Studen kladenec	10	2.62**[a]	0.19	100.0
Rhod.	Pop08	Shirokolashka reka	5	2.62*	0.11	100.0
Rhod.	Pop07	Mihalkovo	5	2.68	0.13	102.3
Balkan	Pop10	Malusha	5	2.69	0.13	102.7
Balkan	Pop09	Lovech	10	2.75	0.11	104.9
Rhod.	Pop01	Asenova krepost	11	2.79	0.30	106.5
Rhod.	Pop02	Bachkovo	5	2.91	0.24	111.1
Balkan	Pop11	Plachkovci	5	2.91	0.15	111.1
Rhod.	Pop03	Ardino	5	2.91	0.06	111.1
Rhod.	Pop05	Devin	5	2.94	0.14	112.2
Balkan	Pop12	Byala reka	5	2.96	0.06	113.0
Rhod.	Pop06	Trigrad	9	3.00	0.10	114.5
Mean				2.82	0.14	
Balkan mountains			25	2.83	0.13	ns[b]
Rhodope mountains			52	2.81	0.15	

Population codes as in Table 1

[a] Significantly different from population Trigrad at $*P = 0.05$ and $**P = 0.01$, based on a Dunn's multiple comparison test (Online Resource T3)

[b] No significant difference between regions

Bulgarian populations (Fig. 4), indicating that they do not harbour consistent morphological discontinuities. This is in contrast to other studies, such as on *Taxus* in China, where a strong correlation (>90 %) between genetic and morphological structures and geographic distributions was found (Gao et al. 2007; Möller et al. 2007).

The reason for this lack of congruence in *Haberlea* lies most likely in the inconsistency of distribution of extreme characteristics between the northern and southern populations in Bulgaria. For instance, the southern Rhodope populations have significantly higher values for most of the corolla characters, but have lower values for some sepal characters (e.g. characters 18, 19 and 20, Online Resource T3). This likely causes conflicting signals in the PCA and results in a mixing of the samples from the Balkan and Rhodope Mountains.

On the basis of average values for the morphological characters, the Balkan populations can be distinguished from the Rhodope populations by a shorter and narrower corolla tube, smaller corolla face, but larger calyx and narrower sepal lobes (except the middle lobe) (Online Resource T3). The Lovech population follows this trend and represents a population with extremely low corolla and some sepal measurements (Online Resource T3). From this point of view, this population could be morphologically distinguished from most other populations, even within the Balkan populations.

Nuclear DNA content

The relatively low CV % values indicated the stability and comparatively good quality of the data (e.g. Voglmayr 2000; Doležel and Bartoš 2005). While we found variation among the 12 populations of Bulgarian *Haberlea* in the region of around 15 %, which is high at the intraspecific level (c.f. Greilhuber 2005), we seemingly have no cases of polyploidy among the studied populations, which would result in significantly higher values. Variation in ploidy levels has been found among populations of *Ramonda serbica* from the Balkan peninsula (6×, 8×, 10×, Siljak-Yakovlev et al. 2008), accompanied by an almost proportional increase in cellular DNA levels. Our observed variations in *Haberlea* here are thus unlikely involving variation at the ploidy level (Table 5).

Previous chromosome counts of *H. rhodopensis* show some variation in somatic number and include $2n = 48$ (Contandriopoulos 1966), $2n = 44$ (Lepper 1970; Milne 1975) and $2n = 38$ (Borhidi 1968). Only the last study provided locality information, and the plants originated near the Monastery of Batshkovo (=Bachkovo), Rhodope Mountains. This locality appears to have the lowest chromosome count, but in our study, this population had an average 2C DNA level (Table 5). Our values are based on several plants averaged across a population, and whether intra-population variation exists in chromosome numbers

requires much more detailed investigations, which are beyond the scope of the present study. Though, such variation could account for the high intraspecific variation of 14.5 % found across the Bulgarian populations.

We did not find a consistent difference in the nuclear DNA content between the Balkan and Rhodope Mountains, with the maximum values occurring in populations from the Rhodope Mountains, with the Balkan populations possessing mid-range values (Table 5). This does not support the findings from our morphological studies, and it can be concluded that there is no link between DNA levels and morphological variation in *Haberlea*. This is further confirmed by the average genome sizes observed for Lovech plants that have the smallest floral dimensions (but a large calyx). The absence of a ploidy difference for the Lovech population was further indicated by a chromosome count of $2n = 48$, albeit only for one plant from this population (M. Möller, personal observation).

One or two species of *Haberlea* in Bulgaria?

Urumoff (1902) listed several morphological characteristics in comparison to *H. rhodopensis* to establish *H. ferdinandi-coburgii*:

Smaller habit Habit is a rather variable character in *Haberlea*, and any population observed shows a range of plant sizes, likely linked to its microhabitat with view to perhaps water and nutrient availability. Size differences of this kind are not likely to be persistent. We thus used nuclear DNA levels to investigate whether ploidy level differences are involved in size variation in *Haberlea*, but could not find an indication of ploidy variation in *Haberlea*.

Glabrous adaxial leaf surface We only found one plant with a few trichomes on the upper leaf surface, in population Devin from the Rhodope Mountains, and only one with glabrous leaves, from Lovech, Balkan Mountains. All other plants had a strong pubescence, indicating that the absence of hairs is a very rare, but not population defining characteristic.

Smaller flower size Based on average values, plants of population Lovech possessed the smallest flowers and could be distinguished statistically from other populations, though not from all, since some populations had similar or smaller dimensions.

Narrower sepals Narrow sepals were found in populations both from the Balkan and Rhodope Mountains, and are not useful to distinguish the two putative species.

Dark-blue corolla Colour is a difficult character to score and is environmentally very labile. We found plants with some darker coloured corollas and scored them as medium, since they were not very darkly coloured. The darker coloured corollas we found across all populations were observed with an almost even distribution with lighter coloured ones.

Elongated capsule which gradually tapers out Capsules were not available to us for study.

Thus, of the characters listed by Urumoff (1902), none of the discrete ones (e.g. glabrous adaxial leaf surface, dark-blue corolla) were found exclusively in any population (e.g. Online Resource F6). Characterising floral dimensions indicated some quantitative differences in mean values between populations and the Rhodope and Balkan Mountains regions specifically. However, when observing the floral characters across populations, it becomes impossible to distinguish any population since there is almost complete overlap of ranges (for virtually all characters) between the populations (Online Resource T3). Thus, flower dimensions alone are not able to distinguish the two putative species in Bulgaria. Because of the conflicting signals form corolla and sepal dimensions, analysing all characters together in a PCA also failed to characterise *H. ferdinandi-coburgii*. Urumoff (1902) described this species from a collection near Lovech. Our plants from near Lovech showed some differences in morphology compared to other populations but not consistently. One plant that fell quite isolated in the PCA (plant 09–10) showed extremely small values for corolla and sepal characters. This is also the only plant with a glabrous leaf and had a darker corolla. This is perhaps one plant that most closely represents Urumoff's *H. ferdinandi-coburgii*. However, genetically it forms an integral part of the Lovech population and is genetically not disjunct from other plants in this population (Fig. 3). This plant represented a single individual in an otherwise morphologically different population. Such a scenario of a combination of morphological discontinuity and genetic continuity within a single population does not allow the assignment of this population to *H. ferdinandi-coburgii*, or the recognition of *H. ferdinandi-coburgii* at the species level. Since most known populations of *Haberlea* in Bulgaria were sampled here (only missing a few localities in the Rhodope Mountains), it is unlikely that a pure stand of *H. ferdinandi-coburgii* exists. It is more likely that a rare, atypical phenotype was the basis for Urumoff's species.

Conclusions

We found relatively low levels of genetic diversity among the populations of *Haberlea* in Bulgaria, but with some spatial differentiation and a higher level of diversity among the high altitude populations in the Balkan Mountains. These might represent refugial populations in Bulgaria. A forthcoming study on populations from the entire distribution range of *Haberlea* will shed light on the origin and

biogeographic history of the genus. We found no morphological groupings among the 12 populations analysed by PCA and a relatively high level of intra-population variation in size of floral components. For conservation purposes not only the levels and distribution of genetic diversity is important, but knowledge of the number of species is equally significant, if not more so. The obtained genetic, morphological and nuclear DNA level data suggest that the population from Lovech, the putative type locality for *H. ferdinandii-coburghii*, is overlapping with other populations from the Balkan and Rhodope Mountains. Thus, it is correct to synonymise *H. ferdinandi-coburgii* with *H. rhodopensis*. It has been indicated before, but without concrete data. Here, we provide the data.

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