

## ISSR analysis points to relict character of *Aconitum bucovinense* Zapal. (Ranunculaceae) at the range margin

Piotr Boroń<sup>1</sup>, Joanna Zalewska-Gałosz<sup>1</sup>, Agnieszka Sutkowska<sup>2</sup>, Bogdan Zemanek<sup>1</sup>, Józef Mitka<sup>1\*</sup>

<sup>1</sup> Institute of Botany, Jagiellonian University, Kopernika 27, 31-501 Kraków, Poland

<sup>2</sup> Department of Plant Breeding and Seed Science, Agricultural University, Łobzowska 24, 31-140 Kraków, Poland

### Abstract

*Aconitum bucovinense*, a high-mountain species endemic to the Eastern and Southern Carpathians, including the Apuseni Mountains, is legally protected and classified in the Polish Red Data Book of Plants. It attains its NW geographical range in two peripheral populations in the Western Bieszczady Mountains (Polish Eastern Carpathians), isolated by a distance of 13.1 km. PCR-ISSR analysis has been used to elucidate the within- and among-population levels of species genetic diversity. A UPGMA and block clustering showed discreteness of the populations and subpopulations based on ISSR banding pattern. Analysis of Molecular Variance (AMOVA) revealed significant divergence ( $P = 0.024$ ) of the two marginal populations and highly significant ( $P < 0.001$ ) differentiation of subpopulations within populations. The theta index calculated for the two marginal populations and the core population in the Carpathians was  $0.131 \pm 0.030$  S.D. Most of the population-genetic diversity indices of the marginal populations were not different from those in the core area but the Shannon's and rarity indices were lower in the marginal populations. It seems that founder effect and subsequent genetic bottleneck resulted in a fine-scale population genetic structure. The marginal populations under study need a relevant recovery program to maintain their genetic diversity.

**Keywords:** Carpathian Mountains, conservation genetics, endemic species, marginal populations, schizoendemism

### Introduction

Previous phenetic studies on the variability of *Aconitum bucovinense* Zapal. (Bucovina's monkshood, Ranunculaceae) in the Eastern Carpathians showed morphological differences of the Bieszczady's populations against those located deeper in the Eastern Carpathians [1]. The presumed phenetic and genetic distinctness of the Bieszczady's populations could be interpreted in historical-biogeographical terms as an example of a schizoendemism (endemocarism), i.e. continuous diversification of an ancestral taxon into derived taxa of identical chromosome numbers in various parts of the range [2]. It accepts the possibility of a parallel, independent origin of the close, low systematic units (e.g. geographic race or subspecies and biotype) in long-term isolated regions from a common, previously widely distributed, ancestral form. In the isolated regions, new forms arise, and the region of their occurrence are centers of neo-endemism, as for example the eastern Iberian peninsula and the Balearic islands [3]. The Eastern Carpathians

represent one of the centers of the neo-endemism [4], and the Bieszczady Mountains are of special importance because of they are situated at the geographical border between the Eastern and Western Carpathians [5]. Twenty nine the Eastern/Southern Carpathian species reach their geographical limits here, in these *Aconitum bucovinense* Zapal. [6]. This species is confined to the alpine and subalpine zones in the main ranges of the Eastern and Southern Carpathians with only two extant localities in Poland (Fig. 1, Fig. 2). As with most peripheral populations, it can be found in local patches of favorable environment [7], which are isolated from each other and limited in the area studied.

In the present paper we report the results of studies on two peripheral populations of *Aconitum bucovinense* by means of PCR-ISSR fingerprinting. Archibald et al. [8] stressed that ISSR markers are most useful for detecting if the clusters have at least one of a combination of attributes, including small population size, isolated distribution, self-compatibility, and heterozygote deficiency at allozyme loci. The ISSR technique is relatively fast, cheap and reproducible. They do not require genome sequence information and leads to multilocus and highly polymorphous patterns suitable for reliable estimation of the genetic variability at the population level, e.g. [9,10]. Recently it has been also used for phylogenetic analyses involving species that are closely related and represent recent radiations at the population level [8,11-13], clonal diversity [14] and conservation concern (e.g. [15]). In addition to the information about population genetic diversity, ISSR markers seem provide relevant information for identifying the spatial

\* Corresponding author. Email: [j.mitka@uj.edu.pl](mailto:j.mitka@uj.edu.pl)

This is an Open Access digital version of the article distributed under the terms of the Creative Commons Attribution 3.0 License ([creativecommons.org/licenses/by/3.0/](http://creativecommons.org/licenses/by/3.0/)), which permits redistribution, commercial and non-commercial, provided that the article is properly cited.



**Fig. 1** Locality of *Aconitum bucovinense* on a local ridge (on the right in front) situated at the S slope of Halicz Mt, W Bieszczady Mts, Eastern Carpathians. In foreground Kopa Bukowska Mt (on the right) and Krzemień ridge (on the left). Open areas above artificially lowered tree-line in the E Carpathians are formed with tall-grass communities (Połoninas) consist of *Deschampsia caespitosa* L. (P. Beauv.) and *Calamagrostis arundinacea* (L.) Roth (in front). Photo by J. Mitka (2006.09.22).

scales and the genetic processes that take place in the populations [16] and are a suitable alternative to isozymes, especially in cases involving rare or clonal species that typically express low levels of variation, a case found in the present study.

Our specific emphasis was given to within- and among-population genetic variation of *Aconitum bucovinense* at the edge of its range. The general prediction is that geographically peripheral populations should exhibit lower diversity and higher differentiation in comparison to the core area [17]. Such information will aide in understanding the microevolutionary processes typical for peripheral populations and elaborate recovery guidelines in face of the threats. The only two existant, peripheral populations in the Western Bieszczady Mts (WBM), Poland, have altogether <150 individuals, and according to population-genetic theory are prone to processes typical for small populations: genetic drift and inbreeding leading to genetic erosion, and likely extinction from random environmental fluctuations [18-21]. The two populations differ threefold in population size, and both have the clear sub-population spatial structure. From theoretical predictions and a number of empirical studies, the small populations might be expected to show reduced levels of polymorphism and allelic richness [22]. The small population, even genetically depauperized, may possess private molecular markers. Alternatively, it can be only a subset of the larger population. Thus, we addressed

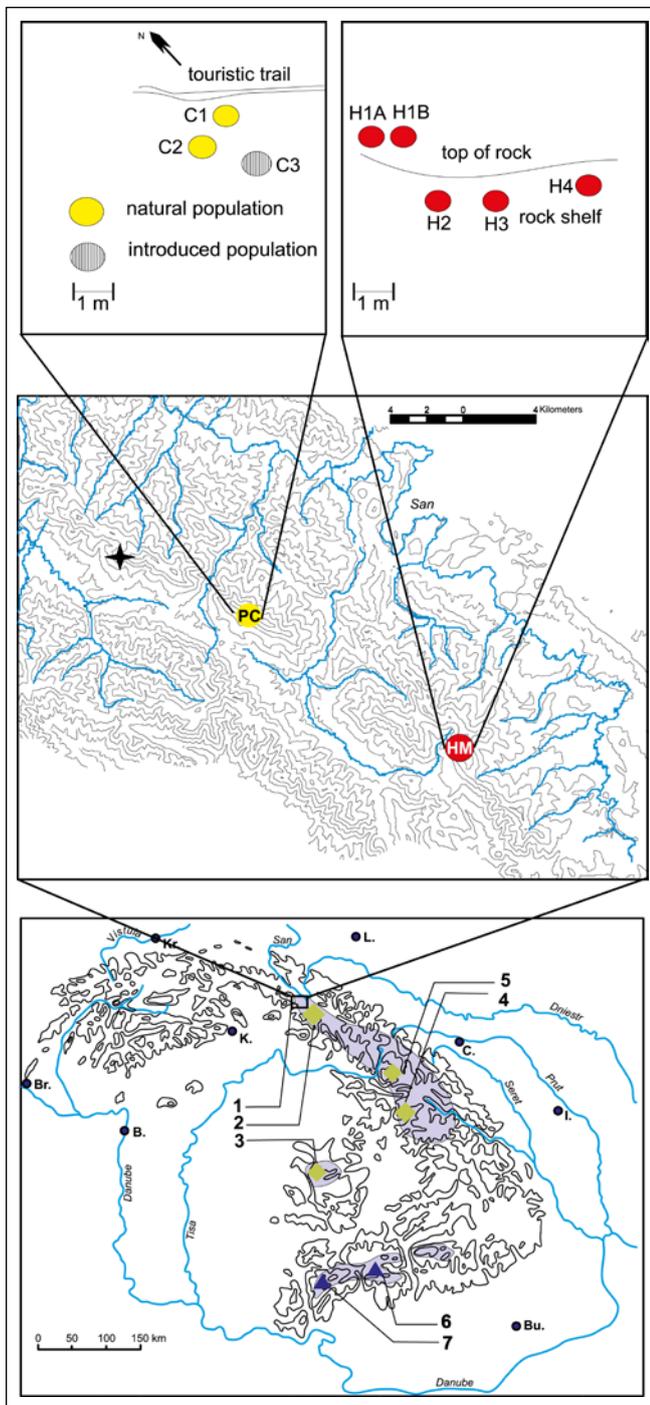
the following questions: (i) do the two peripheral populations differ from each other, (ii) does the smaller population form a subset of the larger population, (iii) is it a sub-population genetic structure within the populations? The general (iv) question is what conservation measures should be recommended to preserve the genetic integrity and diversity of the marginal populations?

## Material and methods

### Study area

The NW limit of the geographical distribution of *Aconitum bucovinense* is in the Western Bieszczady Mts (WBM) [23]. It is a mountain range of moderate heights (Mt Tarnica 1346 m), situated in south-eastern Poland close to Slovakia and Ukraine (Fig. 1, Fig. 2). This is the only part of the Eastern Carpathians within the borders of Poland [24]. The WBM is protected within trans-boundary, trilateral Biosphere Reserve the Eastern Carpathians. They form one of the Carpathian “hot spots” with valuable fauna (large mammals), flora and vegetation [24].

The WBM are a part of the Eastern Beskids Mountains, known as “Waldenkarpaten” [25], forming the most westerly wing. This mountain range is a strong barrier to at least



**Fig. 2** Generalized distribution of *Aconitum bucovinense* (shaded area) and localities of sampled populations or OTUs. 1 – Western Bieszczady; 2 – Eastern Bieszczady; 3 – Bihor; 4 – Rodna; 5 – Giumalau-Rarau; 6 – Fogarași; 7 – Retezat (see Tab. 1). Br – Bratislava; Bu – București; B – Budapest; C – Černivce; I – Iasy; K – Košice; Kr – Kraków; L – Lviv. Locality of populations and subpopulations in the Western Bieszczady Mountains: HM – Halicz Mount; PC – Polonina Caryńska; asterisk – vanished population on Hnatowe Berdo Mt.

postglacial migrations of many mountain plant species from the Eastern Carpathians towards Western Carpathians, as evidenced by the distribution of cytotypes, molecular variants and morphological-anatomical studies, e.g. [26-29]. The barriers and resulting phytogeographical divisions were first recognized and described by plant biogeographers (reviewed by Zemanek [5]). It can be argued that in the case of some alpine/subalpine species, including *A. bucovinense*, the Quaternary interglacial

periods of warming could divide their more or less continuous distribution into several isolated ranges, now of the relict character. The alpine species in the WBM are linked with relict outcrops on the ridges and questas determined by lithological properties (Fig. 1).

### Species and populations

*Aconitum bucovinense* is a rare, herbaceous perennial that is under strict legal protection and classified in the Polish Red Data Book of Plants and in the Vascular Plants of Red Data Book of the Polish Carpathians as a threatened species with VU category [30]. It is an endemic species to the Eastern and Southern Carpathians, including the Apuseni Mountains (see Fig. 2 and [31]) and an alpine-subalpine species, presumably with its center of occurrence in the inner Eastern Carpathians: the Rodna, Maramureș and Bistricei Mountains.

Its taxonomical status was recently clarified [31-34] and the cytogenetic studies showed that it is a stabilized tetraploid with presumably two genomes [35]. Field observations of visitors and the crossing data suggest that geitonogamy is the predominant breeding system of the species of *Aconitum*; sometimes floral damage is observed as an effect of pollen and nectar robbery by insects, mainly bumblebees (personal observation; see also [36,37]). Spatial isolation of the populations in the WBM (Fig. 2) suggests limited gene flow [38,39].

Preliminary observations made during the recovery program held within the plan for species protection in the Bieszczady National Park suggest that the factor limiting population development is seedling recruitment. No seedlings (excluding those sown within a recovery program on Polonina Caryńska) were found at either location. In garden conditions seedlings of *A. bucovinense* germinated two years after sowing, and their mortality was more than 80%.

The population at Polonina Caryńska (PC) was noted for the first time in [40]. It grows near to the touristic trail. In 1970 its size was ca. 40 individuals. In the next 11 years (to 1981) it diminished to 22 individuals (herbarium collections were one of the threat factors). In the following 13 years, by the year 1994, its size again shrunk to 17 individuals, reaching a stable size with 5-6 flowering individuals in 2008. It covers ca. 7 m<sup>2</sup> and grows in rock fissures covered by the *Vaccinietum myrtilli* association. The population consists of three subpopulations: C1, C2 and C3 (Fig. 2).

Subpopulation C3 was established within a recovery program under the auspice of the Bieszczady National Park in the years 2006-2008. The seeds were collected in 2005, germinated in garden conditions, and then 30 individuals reintroduced in situ in 2008. By 2009 twenty five of them (83%) survived. Nowadays, the whole population at PC consists of 39 individuals, in this 8 in flowers (Tab. 1).

The second population, discovered in the 1990's, is situated on the south slope of Halicz Mt (HM; Fig. 1) on a local ridge in the *Aconitum bucovinense*-*Festuca airoides*-*Saxifraga paniculata* plant community in a distance from the tourist trail. It consists of 111 individuals, of which 14 were flowering (2008), and is distributed in four subpopulations H1-H4 on ca. 20 m<sup>2</sup> (Tab. 1, Fig. 2). Its size has been stable for the last 15 years. The subpopulation on the top rock, H1, is much more isolated than the remaining ones.

In the WBM a third population on Mt Hnatowe Berdo existed (Fig. 2). It was found in 1994 and at that time 5 flowering individuals were counted on a square meter [23]. It was not found again, so it is probably became extinct.

**Tab. 1** Geographical location, subpopulation/population size, including non-flowering specimens and sampling size of *Aconitum bucovinense* in Western Bieszczady Mountains and in other Carpathian mountain ranges.

Region	Locality	No. of region/ subpopulation	Elevation (m a.s.l.)	N	Sampling size
WBM	PC	1/C1	1220	8	8
WBM	PC	1/C2	1220	6	2
WBM	PC	1/C3	1220	25	8
WBM	HM	1/H1	1260	42	17
WBM	HM	1/H2	1260	54	17
WBM	HM	1/H3	1260	7	5
WBM	HM	1/H4	1260	8	5
E Bieszczady	Pikuj	2	1380	<250*	4
Bihor	Cărligatele	3	1550	<250*	5
Giulalau-Rarău	Chiril	4	920	<250*	3
Chornogora	Seşul	5	1440	<500*	1
Fogaraş	Paltinului	6	1980	<500*	3
Retezat	Gura Zlata	7	1820	<500*	3
Retezat	Stanişoara	7	1700	<500*	3

HM – Mount Halicz; N – non-flowering specimens; PC – Polonina Caryńska; WBM – Western Bieszczady Mountains. \* Population size estimated in proportion to habitat area.

### Sampling

Individuals of *Aconitum bucovinense* were sampled from two populations in the WBM Mountains and from the localities scattered across the whole Carpathian range (Fig. 2), in 2003–2006 during field-trips to the region by one of the authors (JM). Fragments of leaves were stored in silica-gel. A leaf from the Chornogora Mts was taken from the herbarium material deposited in Krakow, Poland (KRA). WBM specimens with less than five leaves were not sampled.

### DNA extraction and ISSR survey

Total genomic DNA was extracted from silica-gel-dried leaf fragments for each sample separately using modified CTAB method [41]. Thirty-fold diluted (20–100 ng/µl) DNA extracts were used as templates in DNA amplification. ISSR amplification was performed in 12.5 µl reaction volume containing 1.25 µl of 10-fold concentrated reaction buffer (500 mM Tris-HCl pH 8.9, 500 mM KCl, 15 mM MgCl<sub>2</sub> – Kucharczyk TE), 0.25 µl MgCl<sub>2</sub> (25 Mm – Fermentas), 0.25 µl dNTP mix (10 mM each – Invitrogen), 0.5 µl Taq DNA polymerase (2 u/l – Kucharczyk TE) and 2 µl of ISSR primer (10 mM – Integrated DNA Technologies). Reaction was run in Mastercycler Gradient (Eppendorf) with following conditions: 94°C for

5 minutes – initial denaturation, 35 cycles with: 94°C for 35 seconds – denaturation, 43°C (primers ISSR2, ISSR4, ISSR7) or 47°C (primers ISSR1, ISSR6) for 40 seconds – primer annealing and 72°C for 35 seconds – primer extension followed by 72°C for 7 minutes of final extension. Full primer sequences are listed in Tab. 2. ISSR amplification products and molecular weight marker (100 bp ruler) were separated in 1% agarose gel containing ethidium bromide (0.5 µg/ml), visualized in ultraviolet light and photographed.

For analysis of band patterns GelScan ver. 1.45 (Kucharczyk Techniki Elektroforetyczne) software was used. Thanks to the opportunity to create a calibration curve based on the band pattern of markers length (GeneRuler TM 100 bp – Fermentas), it was possible to determine the molecular weight of the resulting amplification products. With the application of a manual checking function, PCR products sizes were precisely determined (within the customary error borders).

### ISSR data analyses

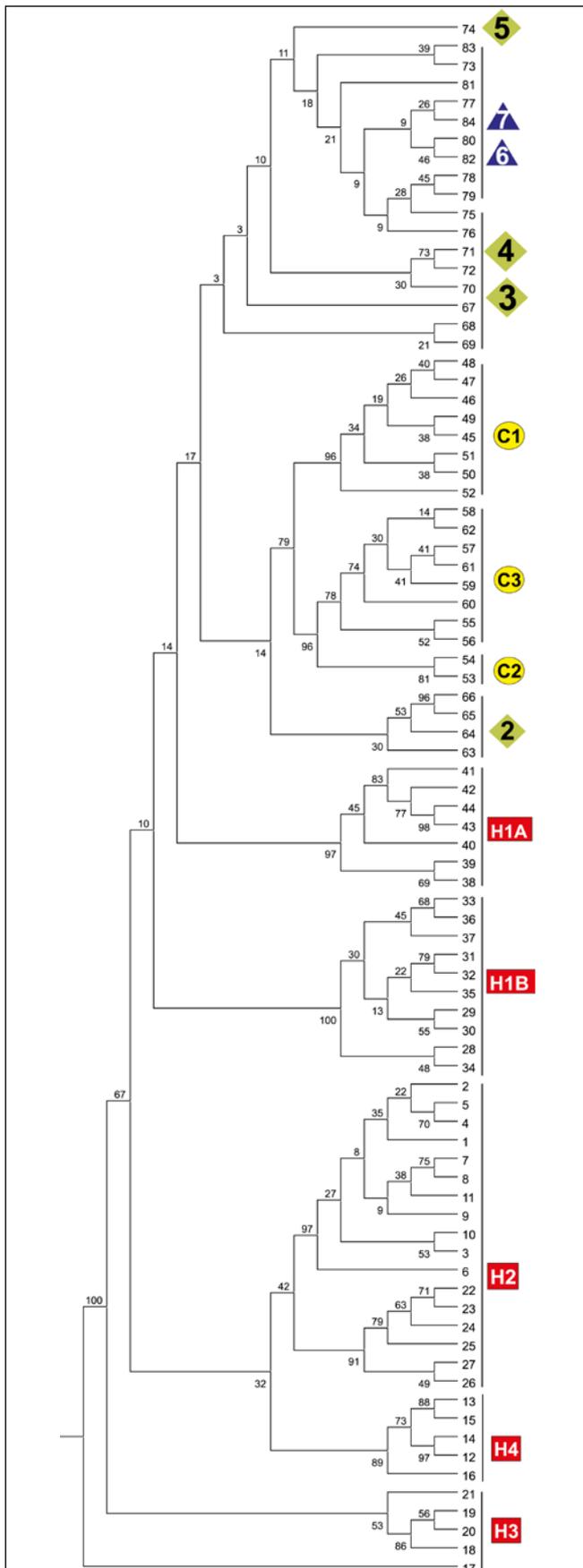
The amplification products were scored as a presence/absence matrix of binary data. The matrix, after removing single and non-interpretable products encompassed 258 ISSR bands found in 84 *Aconitum bucovinense* samples (Tab. 1). The resulting binary data were analysed using POPGENE ver. 1.32 [42]. Genetic diversity within and among populations were measured by the percentage of polymorphic bands (PLP), the effective number of alleles ( $n_e$ ), observed number of alleles ( $n_o$ ), Nei's [43] gene diversity or expected heterozygosity ( $H_e$ ) and Shannon's index ( $I$ ). Additionally, rarity index corresponding to "frequency-down-weighted marker values" per population was calculated [44] and its statistical significance based on 1000 permutation was assessed. R scripts to calculate DW permutation test are available from AFLPdat software [45].

Then, the block structure of the ISSR data was revealed including the following steps: data normalization to unit length, detection of outliers, selection of clustering method, checking within-group diversity, analyzing of the resulting block structure of data, rearrangement of samples and bands within the groups and finally the selection of the informative bands [46]. In the effect the ISSR data set was reduced to 67 the most informative bands using the weighting algorithm based on the sum of the  $F$  statistics [47]. To produce a sample resemblance matrix, the van der Maarel's distances, and to produce a band resemblance matrix, the Euclidean distances were calculated. Both matrices were used in a minimum variance clustering (Ward's clustering, [48]) to classify samples and bands. A correspondence analysis was carried out to rearrange samples and bands within groups [49]. All the calculations were carried out with MULVA-5 software [50].

Genetic dissimilarities (distances) were calculated using the transformed formula ([51],  $D = 1 - \text{Nei coefficient}$ ) based on

**Tab. 2** Primers used in ISSR analysis and banding characteristics in samples of *Aconitum bucovinense*.

Primer	Size (pb)	Sequence 5' – 3'	No. of bands	Max.	Mean	Median
ISSR 1	270–1190	TCT CTC TCT CTC TCT CC	332	22	6.3	4.0
ISSR 2	290–750	AGA GAG AGA GAG AGA GT	498	44	11.1	6.0
ISSR 4	250–960	ATG ATG ATG ATG ATG ATG	407	81	11.0	5.0
ISSR 6	230–1240	ACA CAC ACA CAC ACA CG	311	18	5.0	4.0
ISSR 7	230–1350	ACA CAC ACA CAC ACA CT	435	46	7.1	4.0



**Fig. 3** UPGMA clustering based on the reduced set of ISSR bands of *Aconitum bucovinense* from the WBM and other mountain ranges in the Carpathians. C1-C3 – subpopulations on PC; H1-H4 – subpopulations on HM; 2-7 – samples from other Carpathian mountain ranges (see Fig. 2 and Tab. 1). HM – Halicz Mount; PC – Połonina Caryńska; WBM – Western Bieszczady Mountains.

67 bands. The DISTANCE procedure based on 1000 bootstrap iterations was applied using PhylTools software [52]. The trees were obtained with the use of the ultrametric UPGMA clustering method implemented in the PHYLIP package [53]. Relative bootstrap support values were computed with the CONSENSE option in the PHYLIP package. The OUTREE file was drawn with MEGA4 software [54].

Analysis of molecular variance (AMOVA) was carried out using ARLEQUIN software version 3.1 [55,56]. Variance components were defined between two peripheral WBM populations and among subpopulations within the two populations. Significance of the group portioning was tested against alternative random distribution (null hypothesis) through 1000 permutations of individuals among groups, what is analogous to Fisher's exact test [57]. Additionally, theta index, equivalent to  $F_{st}$  [58], and its confidence interval based on the jackknifing over loci (to obtain variance estimate), and bootstrapping over loci was calculated using a TFPGA software [59].

## Results

A UPGMA clustering based on the ISSR band pattern points to substantial differences between PC and HM populations (Fig. 3). Each of the H and C subpopulations could be distinguished on the basis of a specific combination of band pattern (Tab. 3), and subpopulation H1 was additionally internally diversified into clusters H1A and H1B with a strong 97 and 100% support. Internally diversified was also the subpopulation H2, however with a weak 42% support. The four samples from Mt Pikuj (2), the neighbour population in the Eastern Bieszczady Mts, joined the C1-C3 subpopulations, however with a weak 14% support. The remaining samples from the Eastern and Southern Carpathians formed weakly supported, internally diversified and sister cluster to the Bieszczady's populations (Fig. 3).

The numerical analysis with the use of the multivariate algorithms enabled the block structure of samples and bands to be uncovered (Tab. 3). Some of the bands were characteristic only for the particular subpopulation, as for example ISSR1-8, 46 for the subpopulation C3 or ISSR7-254 for H1B or had optimum in the one, two or more subpopulations, as for example ISSR1-31 in the Eastern and Southern Carpathians, ISSR2-72 in the H3 and H4 or ISSR7-244 in C and H1B. The most conspicuous was ISSR4-105, common for most of the Southern-Eastern Carpathian populations and present in a part of the H2. The most common was ISSR4-98, however absent in the H1A. Internal diversification of the H2 subpopulation was supported by some bands, for example by absence of ISSR2-58 and ISS4-105 or presence of ISSR7-224. ISSR2-89 connected Mt Pikuj (the E Bieszczady Mts) with C3 and H4, ISSR4-103 with C, and ISSR6-181 with H3.

In the AMOVA, the difference in genetic structuring of the two peripheral populations was statistically significant ( $P = 0.024$ ; Tab. 4). In PC relatively less molecular variation was attributable to individuals within subpopulations (52.73%), in comparison to HM, where the value reached 69.28% of the total molecular variability. The differences between the subpopulations in the two peripheral populations in the WBM were statistically significant ( $P < 0.001$ ) and account for 29% of the total variance. On PC the among-subpopulational component of the variance reached 47.27% and on H – 30.72%. The theta statistics among the two marginal populations and





the remaining, core population in the E and S Carpathians equaled to 0.131 and its 95% confidence interval was within 0.080-0.193. Among the genetic-population indices (Tab. 5) the Shannon's index  $I$  and the rarity index  $DW$  were higher in the core area than in the marginal populations. The remaining indices, especially  $H_e$ , did not differentiate among the populations compared.

## Discussion

The molecular ISSR analysis pointed to the genetic distinctness of neighbouring populations of *Aconitum bucovinense* in the WBM. The known closest population in Mt Pikuj (E Bieszczady Mts), located ca. 35 kilometers away [23], displayed some genetic ISSR similarity with the Bieszczady's populations. Both of WBM's populations differed not only from each other, but there was also a conspicuous difference in within-population variability of the species. Its internal diversification is clearly seen in block structure of ISSR bands, where each of the subpopulations is clearly defined by the characteristic bands (Tab. 3), seen in the UPGMA clustering (Fig. 3), and AMOVA analysis (Tab. 4). Thus, we can suppose that the specific mating system linked with the presumably long-term spatial isolation of the two the marginal populations were sufficient to accumulate some differentiation not only between the populations but also among subpopulations in the WBM. It could be linked with within-patch ecological Allee effects as a result of pollen limitation in low-density or isolated patches. Both germination rate and offspring survival were lower for seeds produced in low-density patches of *A. napellus* in northern France [60]. Also, significant genetic structure at small distances is to be found at some plant species due to restricted seed dispersal leading to limited seedling recruitment around the maternal plant (for discussion see [61]). For example, in *Delphinium nuttallianum* Pritzl, a species with similar reproductive biology, spatial autocorrelations at the scale of a few cm to m calculated for several allozyme loci showed genetic differentiation among distant populations not different from that among subpopulations 5-20 m apart. The overall genetic variance among individuals was mostly determined by fixation within the smallest spatial scale of cm to m [38]. According to authors, it could be the result of the postpollination selection that may further modify genetic structure during the life cycle leading to transient local kin

structure that is regenerated during every episode of seedling recruitment. An opposite opinion has also been expressed, that perhaps the directional, e.g. selection against homozygous rare alleles, is less possible because stochastic or demographic factors, often combined with habitat destruction, have most certainly played a significant part in the reduction of genetic variation or are of more immediate importance than population genetics [62,63] (critically reviewed in [64]).

Another factor could be inbreeding, as for example in *A. lycoctonum* studied across Europe. The investigations show inbreeding in more than half the populations under study [65]. It resulted in the deficiency of heterozygotes. It can be a result of geitonogamy and pollen robbery [36,37]. In patches, where floral density is low, the frequency of self-fertilization or pollination between closely related genotypes may increase, thereby reducing seed production as a consequence of partial self-incompatibility or inbreeding depression, or seed quality as a consequence of inbreeding depression [60]. Generally inbreeding, either as a result of mating among close relatives in small populations or as a result of selfing, would tend to fix morphological differences among populations [66,67], lowers genetic variation and facilitates genetic drift in species that are normally highly outbred [68,69]. The increased level of inbreeding in small populations in combination with inbreeding depression may have contributed to their fitness reduction, i.e. "genetic erosion" [70].

In spite of the general genetic impoverishment of small populations, on PC the population possesses private markers, as for example ISSR1-8, and 46 or ISSR7-228. The data obtained do not allow to give a conclusive answer to the question. In the dispersalistic "stepping stone" model, the repeated founding of a small population would likely have resulted in the stochastic loss of genetic variation, mainly rare alleles [22,71]. ISSR analysis does not solve the problem: most bands are common for HM and PC or some of them are absent in the larger HM population, as for example ISSR4-99 (Tab. 3).

None of the individuals from the marginal populations intermingled in UPGMA clusters, nevertheless many ISSR bands were common for the both populations. It seems that the number of private bands was sufficient to make the within-population structuring of both populations statistically significant ( $P < 0.001$ ). In the effect 15.29% ( $P = 0.024$ ) of the molecular variance could be attributable to the differences between the populations in the WBM. The molecular variance among subpopulations within populations in the WBM was

**Tab. 4** Analysis of molecular variance (AMOVA) of *Aconitum bucovinense* in the W Bieszczady (E Carpathians, Poland).

Source of variation	d.f.	SS	Variance components	Percentage of variation	P-value
A. HM - among subpopulations	3	196.867	5.241	30.720	<0.001
Among individuals within subpopulations	41	484.555	11.818	69.280	<0.001
B. PC - among subpopulations	2	88.373	6.569	47.270	<0.001
Among individuals within subpopulations	15	109.905	7.327	52.730	<0.001
C. WBM - among populations	1	134.681	2.913	15.290	0.024
Among subpopulations within populations	5	285.240	5.525	29.000	<0.001
Among individuals within subpopulations	56	594.460	10.615	55.710	<0.001

HM – Halicz Mt; PC – Połonina Caryńska; WBM – W Bieszczady Mts.

**Tab. 5** Genetic diversity within populations and subpopulations of *Aconitum bucovinense* based on 258 ISSR bands (loci).

Population	<i>n</i>	PLP (%)	$H_e$	$n_a$	$n_e$	<i>I</i>	DW	Rsign
1/PC1	8	23.6	0.0502	1.2054	1.0734	0.0822	2.45886	ns
1/PC2	3	12.8	0.0247	1.0415	1.0415	0.0369	2.49221	ns
1/PC3	8	19.8	0.0711	1.3256	1.1096	0.1170	2.63456	ns
PC total	19	50.0	0.0746	1.5000	1.0450	0.1321	2.55487	small
1/HM1	17	29.1	0.0831	1.4225	1.1224	0.1395	2.97267	ns
1/HM2	17	21.3	0.0575	1.3411	1.0813	0.0999	2.12293	small
1/HM3	5	18.6	0.0423	1.1628	1.0626	0.0686	2.26828	ns
1/HM4	5	14.7	0.0325	1.1124	1.0501	0.0513	2.10847	small
HM total	44	69.77	0.0800	1.6977	1.0790	0.1476	2.46171	small
EC (2)	4	30.6	0.0819	1.2984	1.1249	0.1307	4.42738	large
EC (3+4+5)	8	48.8	0.0876	1.4806	1.1147	0.1543	4.37728	large
SC (6+7)	7	30.2	0.0647	1.2868	1.0898	0.1087	3.21438	ns
EC+SC	19	68.60	0.0864	1.6860	1.1069	0.1635	4.02093	large

DW – rarity index corresponding to “frequency-down-weighted marker values” per population [44]; EC – Eastern Carpathians;  $H_e$  – Nei’s [43] gene diversity, with  $F_{is}$  assumed to be 0.2 (see [65]); HM – Mount Halicz; *I* – Shannon’s information index; *n* – sampling size;  $n_a$  – observed No. of alleles;  $n_e$  – effective No. of alleles; ns – no significant; PC – Połonina Caryńska; PLP – percentage of polymorphic loci; Rsign – significance of rarity index ( $P \leq 0.05$ ) based on 1000 permutations [45]; SC – Southern Carpathians. See also Tab. 1.

also high and amounted 29.00% ( $P < 0.001$ ), proving the distinctness of even the particular subpopulations within the peripheral populations of *A. bucovinense* (Tab. 3). The theta index  $0.131 \pm 0.030$  S.D. points to moderate genetic differentiation among the two margin and core populations of *Aconitum bucovinense* in the Carpathians. According to Reynolds et al. [72] the derivation of theta index assumes that genetic divergence among the groups is due to only drift and it measures divergence on a short evolutionary time scale. Previous phenetic studies [1] showed the morphological coherence of the Bieszczady’s population and its differentiation against the remaining Eastern-Carpathian populations. The morphological distinctness (seen on the indumentum and in shape of the spur) was discussed in terms of the schizoendemism. The present results of PCR-ISSR analysis persuade that the phenetic differences found at the edge of the geographical distribution of the species may have some genetic background in the effect of genetic drift, neutral mutagenesis or response to local selective forces. The loss of genetic diversity in the marginal populations (Shannon’s and rarity indices) and, in the same time, the existence of private markers advocates for genetic drift accompanied by the presumably reduced of the number of alleles per locus [73].

An experimental cross-pollination between the HM and PC populations may shed some light on the problem. The study should be carried out to determine if seed production and seedling survival is any higher after the outcrossing. Also, reciprocal seed transplatantation experiments should be carried out to demonstrate local adaptations. Additionally, health condition and reproductive parameters of the newly established C3 subpopulation, linked with their adaptation to local conditions, should be assessed in the future. As Fahselt [74] noted, long-term monitoring of the newly established population is necessary because although initially transplant survival rates may be high they are often followed by remarkable reversals over time. Probably, it may concern also specimens obtained from the seedlings.

What protection measures should be recommended? In

the long-term conservation concern genetic variation has been seen as a crucial to success in the management of rare and endangered species. In the short-term, given the limited number of individuals, it is necessary to protect all the existing populations [75]. It seems also feasible to establish a transplanted population of *Aconitum bucovinense* from seeds obtained from the population HM, which is the most genetically diversified. Conservation goal can be achieved by careful choice of the transplant site, taking into consideration the phytosociological criteria, in order to increase the number of conserved populations [76,77]. Additionally, the main ex situ conservation policy is maintenance of a germplasm bank [78]. The seeds of *Aconitum bucovinense* are stored in the Center for Biological Conservation of the Polish Academy of Sciences, Warsaw. Additionally, specimens obtained from seeds should be maintained in the botanical gardens.

The preservation population of the species was established in 2006 year in the experimental garden of the educational-scientific station of the Bieszczady National Park in Suche Rzeki. The cultivation of the species should be continued to ensure that there is a stock of individuals on hand for reintroduction.

## Acknowledgements

The authors would like to thank Jacqueline Trojan (New Mexico State University) for linguistic corrections and to an anonymous reviewer for constructive comments. To Elżbieta and Leopold Bekier for their hospitality in Wołosate, the Western Bieszczady Mts. The work was supported by a MNiSzW grant No. 8144/B/P01/2011/40.

## References

1. Mitka J. Phenetic and geographic pattern of *Aconitum* sect. *Napellus* (Ranunculaceae) in the Eastern Carpathians – a

- numerical approach. *Acta Soc Bot Pol.* 2002;71:35-48.
2. Richardson IBK. Endemic taxa and the taxonomist. In: Street H, editor. *Essays in plant taxonomy*. London: Academic Press; 1978. p. 245-262.
  3. Rossello JA. Testing taxonomic and biogeographical relationships in a narrow Mediterranean endemic complex (*Hippocrepis balearica*) using RAPD markers. *Ann Bot.* 2002;89:321-327. <http://dx.doi.org/10.1093/aob/mcf044>
  4. Pawłowski B. Remarques sur l'endémisme dans la flore des Alpes et des Carpates. *Vegetatio.* 1970;21:181-243. <http://dx.doi.org/10.1007/BF02269663>
  5. Zemanek B. The phytogeographical boundary between the East and West Carpathians – past and present. *Thaiszia.* 1991;1:59-67.
  6. Zemanek B, Winnicki T. Rośliny naczyniowe Bieszczadzkiego Parku Narodowego. Ośrodek Naukowo-Dydaktyczny BdPN; 1999. (Monografie Bieszczadzkie; vol 3).
  7. Brown JH, Stevens GC, Kaufman DM. The geographic range: size, shape, boundaries, and internal structure. *Annu Rev Ecol Syst.* 1996;27(1):597-623. <http://dx.doi.org/10.1146/annurev.ecolsys.27.1.597>
  8. Archibald JK, Crawford DJ, Santos-Guerra A, Mort ME. The utility of automated analysis of inter-simple sequence repeat (ISSR) loci for resolving relationships in the Canary Island species of *Tolpis* (Asteraceae). *Am J Bot.* 2006;93(8):1154-1162. <http://dx.doi.org/10.3732/ajb.93.8.1154>
  9. Alexander JA, Liston A, Popovich SJ. Genetic diversity of the narrow endemic *Astragalus oniciformis* (Fabaceae). *Am J Bot.* 2004;91(12):2004-2012. <http://dx.doi.org/10.3732/ajb.91.12.2004>
  10. Tsumura Y, Ohba K, Strauss SH. Diversity and inheritance of inter-simple sequence repeat polymorphisms in Douglas-fir (*Pseudotsuga menziesii*) and sugi (*Cryptomeria japonica*). *Theor Appl Genet.* 1996;92:40-45. <http://dx.doi.org/10.1007/BF00222949>
  11. Bussell JD, Waycott M, Chappill JA. Arbitrarily amplified DNA markers as characters for phylogenetic inference. *Perspectives in Plant Ecology, Evolution and Systematics.* 2005;7(1):3-26. <http://dx.doi.org/10.1016/j.ppees.2004.07.001>
  12. Sutkowska A, Mitka J. Molecular PCR-ISSR analysis in *Bromus* subgenus *Festucaria* – preliminary results. In: Frey L, editor. *Biology of grasses*. Kraków: W. Szafer Institute of Botany, Polish Academy of Sciences; 2005. p. 1-10.
  13. Mitka J, Sutkowska A, Ilnicki T, Joachimiak A. Reticulate evolution of high-alpine *Aconitum* (Ranunculaceae) in the Eastern Sudetes and Western Carpathians (Central Europe). *Acta Biol Cracov Ser Bot.* 2007;49(2):15-26.
  14. Esselman EJ, Jianqiang L, Crawford DJ, Windus JL, Wolfe AD. Clonal diversity in the rare *Calamagrostis porteri* subsp. *insperata* (Poaceae): comparative results for allozyme and random amplified polymorphic DNA (RAPD) and intersimple sequence repeat (ISSR) markers. *Mol Ecol.* 1999;8:443-451. <http://dx.doi.org/10.1046/j.1365-294X.1999.00585.x>
  15. Camacho FJ, Liston A. Population structure and genetic diversity of *Botrychium pumicola* (Ophioglossaceae) based on inter-simple sequence repeats (ISSR). *Am J Bot.* 2001;88(6):1065-1070. <http://dx.doi.org/10.2307/2657089>
  16. Heywood V. Plant conservation: old problems, new perspectives. *Biol Conserv.* 2003;113:321-335. [http://dx.doi.org/10.1016/S0006-3207\(03\)00121-6](http://dx.doi.org/10.1016/S0006-3207(03)00121-6)
  17. Sagarin RD, Gaines SD. The “abundant centre” distribution: to what extent is it a biogeographical rule? *Ecol Lett.* 2002;5:137-147. <http://dx.doi.org/10.1046/j.1461-0248.2002.00297.x>
  18. Lande R, Schemske DW. The evolution of self-fertilization and inbreeding depression in plants. I. genetic models. *Evolution.* 1985;39(1):24-40. <http://dx.doi.org/10.2307/2408514>
  19. Goodman D. The demography of chance extinction. In: Soulé ME, editor. *Viable populations for conservation*. Cambridge: Cambridge University Press; 1987. p. 11-34. <http://dx.doi.org/10.1017/CBO9780511623400.003>
  20. Lesica P, Allendorf FW. Are small populations of plants worth preserving? *Conserv Biol.* 1992;6:135-139. <http://dx.doi.org/10.1046/j.1523-1739.1992.610135.x>
  21. Dolan RW. Patterns of isozyme variation in relation to population size, isolation, and phylogeographic history of royal catchfly (*Silene regia*; Caryophyllaceae). *Am J Bot.* 1994;81:965-972. <http://dx.doi.org/10.2307/2445289>
  22. Chang C-S, Choi DY, Kim H, Park TY, Kim Y-S. Patterns of allozyme variation in relation to population size of the threatened plant *Megaleranthis saniculifolia* (Ranunculaceae) in Korea. *J Plant Biol.* 2005;48:339-350. <http://dx.doi.org/10.1007/BF03030575>
  23. Mitka J, Zemanek B. Rare and threatened plant species of the Bieszczady National Park (Western Bieszczady, Eastern Carpathians). *Roczniki Bieszczadzkie.* 1997;5:19-41.
  24. Winnicki T, Zemanek B. *Nature in the Bieszczady National Park*. Ustrzyki Dolne: Bieszczady National Park; 2003.
  25. Pax F. *Grundzüge der Pflanzenverbreitung in der Karpathen*. Leipzig: Verlag von Wilhelm Enegelman; 1898. (vol 1).
  26. Konnert M, Bergmann F. The geographical distribution of genetic variation of silver fir (*Abies alba*, Pinaceae) in relation to its migration history. *Plant Syst Evol.* 1995;196:19-30. <http://dx.doi.org/10.1007/BF00985333>
  27. Boratyńska K, Muchewicz E. *Pinus mugo* Turra geographic differentiation based on needle characters. *Dendrobiology.* 2004;51:9-17.
  28. Ronikier M, Cieślak E, Korbecka G. High genetic differentiation in the alpine plant *Campanula alpina* Jacq. (Campanulaceae): evidence for glacial survival in several Carpathian regions and long-term isolation between the Carpathians and the Alps. *Mol Ecol.* 2008;17:1763-1775. <http://dx.doi.org/10.1111/j.1365-294X.2008.03664.x>
  29. Tešitel J, Malinová T, Štech M, Herbstová M. Variation in the *Melampyrum sylvaticum* group in the Carpathian and Hercynian region: two lineages with different evolutionary histories. *Preslia.* 2009;81:1-22.
  30. Mitka J. Tojad bukowiński (T. niski) *Aconitum bucovinense* Zapał. In: Mirek Z, Piękoś-Mirkowa H, editors. *Red data book of the Polish Carpathians, vascular plants*. Kraków: W. Szafer Institute of Botany, Polish Academy of Sciences; 2008. p. 62-63.
  31. Mitka J. Systematics of *Aconitum* subgen. *Aconitum* in the Eastern Carpathians – preliminary results. *Roczniki Bieszczadzkie.* 2000;9:79-116.
  32. Seitz W. Die Taxonomie der *Aconitum napellus*-Gruppe in Europa. *Feddes Repert.* 1969;80(1):1-76. <http://dx.doi.org/10.1002/fedr.19690800102>
  33. Starmühler W, Mitka J. Systematics and chorology of *Aconitum* sect. *Napellus* (Ranunculaceae) and its hybrids in the Northern Carpathians and Forest Carpathians. *Thaiszia.* 2001;10:115-136.

34. Mitka J. The genus *Aconitum* L (Ranunculaceae) in Poland and adjacent countries. A phenetic-geographic study. Kraków: Institute of Botany of the Jagiellonian University; 2003.
35. Ilnicki T, Mitka J. Chromosome numbers on *Aconitum* sect. *Aconitum* (Ranunculaceae) from the Carpathians. *Caryologia*. 2009;62(3):198-203.
36. Utelli A-B, Roy BA. Pollinator abundance and behavior on *Aconitum lycoctonum* (Ranunculaceae): an analysis of the quantity and quality components of pollination. *Oikos*. 2000;89:461-470. <http://dx.doi.org/10.1034/j.1600-0706.2000.890305.x>
37. Utelli A-B, Roy BA. Causes and consequences of floral damage in *Aconitum lycoctonum* at high and low elevations in Switzerland. *Oecologia*. 2001;127:266-273. <http://dx.doi.org/10.1007/s004420000580>
38. Williams CF, Waser NM. Spatial genetic structure of *Delphinium nuttallianum* populations: inferences about gene flow. *Heredity*. 1999;83:541-550. <http://dx.doi.org/10.1038/sj.hdy.6885920>
39. Zhang D, Chen S, Chen S, Zhang D, Gao Q. Patterns of genetic variation in *Swertia przewalskii*, an endangered endemic species of the Qinghai-Tibet Plateau. *Biochem Genet*. 2007;45:33-50. <http://dx.doi.org/10.1007/s10528-006-9057-7>
40. Jasiewicz A, Zarzycki K. *Carex rupestris* Bell ex All. and *Aconitum tauricum* Wulf. subsp *nanum* (Baumg.) Gáy., two new plants for the Polish flora from the West Bieszczady. *Fragm Flor Geobot*. 1959;2:24-27.
41. Gawel N, Jarret RL. Cytoplasmic genetic diversity in banana and plantain. *Euphytica*. 1991;52(1):19-23. <http://dx.doi.org/10.1007/BF00037852>
42. Yeh F, Yang R, Boyle T. POPGENE version 1.32. Microsoft-based freeware for population genetic analysis. Canada: Molecular Biology and Biotechnology Center, University of Alberta; 1999.
43. Nei M. Analysis of gene diversity in subdivided populations. *PNAS*. 1973;70(12):3321-3323. <http://dx.doi.org/10.1073/pnas.70.12.3321>
44. Schönswetter P, Tribsch A. Vicariance and dispersal in the Alpine perennial *Bupleurum stellatum* L. (Apiaceae). *Taxon*. 2005;54:725. <http://dx.doi.org/10.2307/25065429>
45. Ehrich D. AFLPdat: a collection of r functions for convenient handling of AFLP data. *Mol Ecol Notes*. 2006;6:603-604. <http://dx.doi.org/10.1111/j.1471-8286.2006.01380.x>
46. Feoli E, Orlóci L. Analysis of concentration and detection of underlying factors in structured tables. *Vegetatio*. 1979;40:49-54. <http://dx.doi.org/10.1007/BF00052015>
47. Jancey RC. Species ordering on a variance criterion. *Vegetatio*. 1979;39(1):59-63. <http://dx.doi.org/10.1007/BF00055329>
48. Ward JH. Hierarchical grouping to optimize an objective function. *J Am Statist Assoc*. 1963;58(301):236-244. <http://dx.doi.org/10.2307/2282967>
49. Hill MO. Reciprocal averaging: an eigenvector method of ordination. *J Ecol*. 1973;61(1):237-249. <http://dx.doi.org/10.2307/2258931>
50. Wildi O, Orlóci L. Numerical exploration of community patterns: a guide to the use of MULVA-5. 2nd ed. Hague: SPB Academic; 1996.
51. Nei M, Li WH. Mathematical model for studying genetic variation in terms of restriction endonucleases. *PNAS*. 1979;76(10):5269-5273. <http://dx.doi.org/10.1073/pnas.76.10.5269>
52. Buntjer JB. Phylogenetic Computer Tools ver. 1.3. Netherlands: Wageningen University; 1997.
53. Felsenstein J. PHYLIP (Phylogeny Inference Package) ver. 3.67 [Internet]. 2010 [cited 2010 Jun 18]; Available from: <http://evolution.genetics.washington.edu/phylip.html>
54. Tamura K, Dudley J, Nei M, Kumar S. MEGA4: Molecular Evolutionary Genetics Analysis (MEGA) software version 4.0. *Mol Biol Evol*. 2007;24(8):1596-1599. <http://dx.doi.org/10.1093/molbev/msm092>
55. Excoffier L, Smouse PE, Quattro JM. Analysis of molecular variance inferred from metric distances among dna haplotypes: application to human mitochondrial DNA restriction data. *Genetics*. 1992;131(2):479-491.
56. Arlequin ver. 3.1. An integrated software package for population genetic analysis [Internet]. 2010 [cited 2010 Jun 18]; Available from: <http://cmpg.unibe.ch/software/arlequin3>
57. Raymond M, Rousset F. An exact test for population differentiation. *Evolution*. 1995;49(6):1280-1283. <http://dx.doi.org/10.2307/2410454>
58. Weir BS, Cockerham CC. Estimating *F*-statistics for the analysis of population structure. *Evolution*. 1984;38(6):1358-1370. <http://dx.doi.org/10.2307/2408641>
59. Tools for population genetic analyses (TFPGA) 1.3: a Windows program for the analysis of allozyme and molecular population genetic data [Internet]. 2010 [cited 2010 Jun 18]; Available from: <http://www.marksgeneticssoftware.net/tfpga.htm>
60. Le Cadre S, Tully T, Mazer SJ, Ferdy J-B, Moret J, Machon N. Allee effects within small populations of *Aconitum napellus* ssp. *lusitanicum*, a protected subspecies in northern France. *New Phytol*. 2008;179(4):1171-1182. <http://dx.doi.org/10.1111/j.1469-8137.2008.02529.x>
61. Wallace LE. Spatial genetic structure and frequency of interspecific hybridization in *Platanthera aquilonis* and *P. dilatata* (Orchidaceae) occurring in sympatry. *Am J Bot*. 2006;93(7):1001-1009. <http://dx.doi.org/10.3732/ajb.93.7.1001>
62. Lande R. Genetic variation and phenotypic evolution during allopatric speciation. *Am Nat*. 1980;116(4):463-479. <http://dx.doi.org/10.1086/283642>
63. Widén B, Andersson S. Quantitative genetics of life-history and morphology in a rare plant, *Senecio integrifolius*. *Heredity*. 1993;70:503-514. <http://dx.doi.org/10.1038/hdy.1993.73>
64. Frankham R. Genetics and extinction. *Biol Conserv*. 2005;126:131-140. <http://dx.doi.org/10.1016/j.biocon.2005.05.002>
65. Utelli AB, Roy BA, Baltisberger M. History can be more important than "pollination syndrome" in determining the genetic structure of plant populations: the case of *Aconitum lycoctonum* (Ranunculaceae). *Heredity*. 1999;82:574-584. <http://dx.doi.org/10.1038/sj.hdy.6885070>
66. Baker HG. Reproductive methods as factors in speciation in flowering plants. *Cold Spring Harb Symp Quant Biol*. 1959;24:177-191.
67. Hamrick JL, Linhart YB, Mitton JB. Relationships between life history characteristics and electrophoretically detectable genetic variation in plants. *Annu Rev Ecol Syst*. 1979;10:173-200. <http://dx.doi.org/10.1146/annurev.es.10.110179.001133>
68. Templeton AR. Off-site breeding of animals and implications for plant conservation. In: Falk DA, Holsinger KE,

- editors. Genetics and conservation of rare plants. New York: Oxford University Press; 1991. p. 189-194.
69. Philipp M, Nielsen LR. Reproductive ecology of *Scalesia cordata* (Asteraceae), an endangered species from the Galápagos Islands. Bot J Linn Soc. 2010;162:496-503. <http://dx.doi.org/10.1111/j.1095-8339.2010.01034.x>
70. Fischer M, Matthies D. Effects of population size on performance in the rare plant *Gentianella germanica*. J Ecol. 1998;86(2):195-204. <http://dx.doi.org/10.1046/j.1365-2745.1998.00246.x>
71. Lande R. Genetics and demography in biological conservation. Science. 1988;241:1455-1460. <http://dx.doi.org/10.1126/science.3420403>
72. Reynolds J, Weir BS, Cockerham CC. Estimation of the coancestry coefficient: basis for a short-term genetic distance. Genetics. 1983;105(3):767-779.
73. Nei M, Maruyama T, Chakraborty R. The bottleneck effect and genetic variability in populations. Evolution. 1975;29(1):1-10. <http://dx.doi.org/10.2307/2407137>
74. Fahselt D. Is transplanting an effective means of preserving vegetation? Can J Bot. 2007;85:1007-1017. <http://dx.doi.org/10.1139/B07-087>
75. Huang Y, Zhang C-Q, Li D-Z. Low genetic diversity and high genetic differentiation in the critically endangered *Omphalogramma souliei* (Primulaceae): implications for its conservation. J Syst Evol. 2009;47:103-109. <http://dx.doi.org/10.1111/j.1759-6831.2009.00008.x>
76. Holl KD, Hayes GE. Challenges to introducing and managing disturbance regimes for *Holocarpha macradenia*, an endangered annual grassland forb. Conserv Biol. 2006;20(4):1121-1131. <http://dx.doi.org/10.1111/j.1523-1739.2006.00416.x>
77. Viana e Souza HA, Lovato MB. Genetic diversity and structure of the critically endangered tree *Dimorphandra wilsonii* and of the widespread in the Brazilian Cerrado *Dimorphandra mollis*: Implications for conservation. Biochem Syst Ecol. 2010;38:49-56. <http://dx.doi.org/10.1016/j.bse.2009.12.038>
78. López-Pujol J, Bosch M, Simon J, Blanché C. Population genetics and conservation priorities for the critically endangered island endemic *Delphinium pentagynum* subsp. *formenterianum* (Ranunculaceae). Biodiversity Conserv. 2003;12:1937-1951. <http://dx.doi.org/10.1023/A:1024103714274>