*ACTA BIOLOGICA CRACOVIENSIA Series Botanica* 57/1: 31–43, 2015
DOI: 10.1515/abcsb-2015-0002

# INTRASPECIFIC MOLECULAR VARIATION OF *ALLIUM URSINUM* (AMARYLLIDACEAE) ACROSS THE BORDER OF TWO SUBSPECIES DISTRIBUTION RANGES

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Received March 25, 2014; revision accepted December 28, 2014

The study investigates the genetic differentiation among two subspecies of *Allium ursinum* L., namely *A. ursinum* subsp. *ursinum* and subsp. *ucrainicum* as well as their putative hybrid that is represented by individuals with intermediate morphology. Inter-Simple Sequence Repeats (ISSR) were applied to determine the status of intermediate morphotypes in terms of their genetic pattern and to assess the level of genetic variability within and between various populations of *A. ursinum*. The study comprises 144 specimens from nine populations along the east-west transect in Poland, which includes localities of both subspecies and their putative hybrid. Among the examined populations, 48 bands were amplified, of which 45 were found to be polymorphic. The principal coordinate analysis (PCoA), the neighbour-net analysis and Mantel test showed a strong correlation between genetic variability and geographic distance. Analysis of molecular variance (AMOVA) revealed that a greater proportion of total genetic variation resided within populations rather than among them. The Structure Bayesian clustering analysis revealed the presence of three distinct genetic groups within studied populations, where 'eastern' genotypes correspond to *A. ursinum* subsp. *ucrainicum*, and 'western' to subsp. *ursinum*; whereas the third genetic group has the largest share in the individuals occurring at the border of the distribution ranges of both subspecies. The emergence of the third genetic group is probably an effect of hybridization events occurring within the secondary contact zone. Typical morphologically intermediate populations occur only in a relatively narrow geographical zone, but the hybrid zone revealed by molecular markers is actually much wider than it is suggested by the morphological pattern of individuals. The current distribution pattern of both subspecies of *A. ursinum* and their hybrid zone is related to the two main directions of postglacial migration of *Fagus sylvatica* to the area of Poland. The hybrid zone arose as an effect of the secondary contact of two divergent lineages of *A. ursinum*.

**Key words:** Distribution pattern, genetic variability, hybrid zone, intermediate morphotype, ISSR.

## INTRODUCTION

The postglacial migration of plant species is an important factor responsible for the patterns of intraspecific variation observed in natural populations (Hewitt, 2001). The northward postglacial migration of many species from the southern allopatric refugia resulted in the secondary contact of divergent lineages and the formation of hybrid zones in Europe. The area at the border between the Carpathians and Sudetes is an example where different migratory elements meet (Hewitt, 1999). The existence of phenotypically intermediate forms of many species, in which mixed genotypes are additionally determined by molecular studies, demonstrate the occurrence of evident hybrid zones in this geographical region. Such phytogeographical relationships have been observed in the case of several

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PL ISSN 0001-5296

TABLE 1. Geographical origin of the studied populations of *Allium ursinum*.

| Population name | Number of individuals | Locality | Latitude | Longitude | Altitude (m a.s.l) |
|---|---|---|---|---|---|
| *Allium ursinum* subsp. *ucrainicum* | | | | | |
| B1 | 17 | Beskid Śląski Mts, Bielsko-Biała, Wapienica, along Potok Żydowski stream | 49° 46' 24.32"N | 18° 58' 55.78"E | 506 |
| B2 | 17 | Beskid Śląski Mts, along Wapienica river | 49° 47' 9.51"N | 18° 58' 48.52"E | 428 |
| BG | 16 | Beskid Żywiecki Mts, Masyw Babiej Góry Mts | 49° 35' 39.58"N | 19° 29' 18.27"E | 933 |
| CG | 15 | Beskid Niski Mts. Cergowa Góra Mt., N slope | 49° 31' 58.63"N | 21° 42' 29.85"E | 559 |
| KAL | 17 | Bieszczady Zachodnie Mts, Kalnica village | 49° 11' 1.00"N | 22° 25' 48.74"E | 582 |
| *Allium ursinum* subsp. *ursinum* | | | | | |
| GZ | 15 | Eastern Sudetes, Góry Złote Mts, between Złoty Stok village and Orłowiec village | 50° 24' 44.05"N | 16° 50' 20.51"E | 642 |
| JN | 16 | Eastern Sudetes, Masyw Śnieżnika Mts, Kletno village near Jaskinia Niedźwiedzia cave | 50° 14' 14.96"N | 16° 50' 54.34"E | 761 |
| Intermediate morphotype | | | | | |
| ZP | 16 | Kraków-Częstochowa Upland, Złoty Potok village | 50° 41' 40.20"N | 19° 25' 20.79"E | 338 |
| SG | 15 | Kraków-Częstochowa Upland, Sokole Góry Mts,Wzgórze Knieja hill | 50° 44' 10.95"N | 19° 17' 38.25"E | 355 |

pairs of plant species/taxa, e.g. from *Dentaria*, *Senecio* and *Aconitum* genera (Hodálová, 2002; Lihova et al., 2007; Sutkowska et al., 2013).

*Allium ursinum* is a perennial geophyte widely distributed in Europe (e.g. Stearn, 1978). The species represents the connective Sub-Atlantic-Central-European-Mediterranean geographical element (Zając and Zając, 2009). It occurs from western Europe (Portugal, Spain, France, Switzerland, Belgium, Netherlands, Great Britain, Ireland, Germany, Austria) running through Italy, central Europe (Czech Republic, Poland, Slovakia, Hungary), the countries of the former Yugoslavia, Bulgaria, Romania, Moldavia, Ukraine, Belarus, Scandinavian countries and Baltic states up to the European part of Russia, Caucasus and Anatolia (Bordzilovskij, 1950; Tutin, 1957; Soják, 1968; Zahariadi, 1966; Conti et al., 2005; Karpavičiene, 2006; Aguiar and Aedo, 2006; Chifu et al., 2006; Krahulec and Duchoslav, 2010; Aedo, 2014). It is a typical understory species, having a rather narrow range of ecological tolerance (Kevey, 1977; Grime et al., 1988). The species is strongly confined to nutrient-rich deciduous woodlands and mixed beech-fir or beech-spruce forests (Tutin, 1957; Karpavičiene, 2006; Kovács, 2007). Two subspecies within *Allium ursinum* were described, i.e. *A. ursinum* L. subsp. *ursinum* and *A. ursinum* subsp. *ucrainicum* Kleop. et Oxner. However, such taxonomic recognition has not been definitively resolved; some authors do not distinguish intraspecific taxa (e.g. Czerepanov, 1995), whereas others treat the aforementioned subspecies at a subspecific or species level (see Bordzilovskij, 1950; Stearn, 1980; Krahulec and Duchoslav, 2010). Regardless of the taxonomic rank, the division seems to be reasonable due to the distinct differences in the morphology of pedicels and generally non-overlapping geographical ranges of taxa (Soják, 1968; Rola, 2012). *Allium ursinum* subsp. *ursinum* has scabrous pedicels with numerous papillae and occurs in the western part of the species distribution area (Stearn, 1978, 1980; see also Soják, 1968), whereas subsp. *ucrainicum*, the eastern-distributed form, is characterized by smooth pedicels (Bordzilovskij, 1950; Stearn, 1978; Kovács, 2007). Additionally, intermediate morphotypes occur along the shared borderline of the distribution of both taxa. The first reports on morphologically intermediate forms came from Romania and former Czechoslovakia (Zahariadi, 1966; Soják, 1968). Their occurrence was later also recognized in other parts of Europe (e.g. Karpavičiene, 2006; Rola, 2012). The recognition of the genetic relationships of three considered forms of *A. ursinum* is

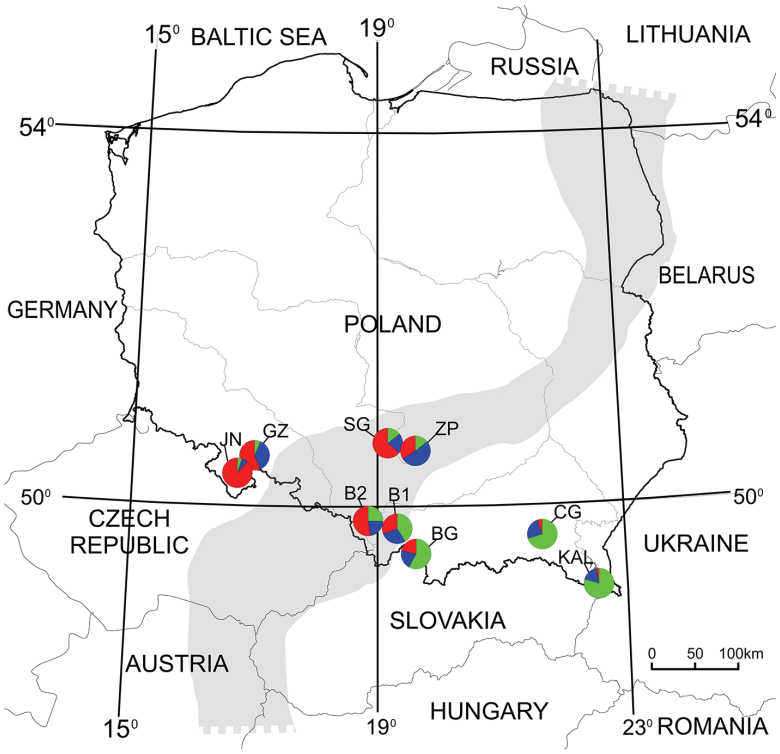


**Fig. 1**. Location of nine populations of *Allium ursinum* sampled along the east-west transect that includes localities of both subspecies and their putative hybrid. Details of populations are given in Table 1. Pie charts refer to the three ISSR genetic groups (K = 3) resolved using Bayesian analysis (see also, Fig. 7b). Particular colours denote the proportions of the genetic groups present in the populations. The grey zone indicates the approximate area where morphologically intermediate specimens occur; based on Soják (1968) and Rola (2012).

interesting both in the context of their taxonomic position and biogeographical relationships. However, the status of intermediate morphotypes as genetic hybrids remains unresolved so far.

In the present work Inter-Simple Sequence Repeat (ISSR) markers were chosen to study the genetic diversity and relatedness of *A. ursinum*. ISSR markers are highly polymorphic and proved to be useful in studies on genetic diversity, phylogeny, and evolutionary biology (Reddy et al., 2002). In particular, this technique is very efficient in plant population genetic studies (Bonin et al., 2007). Furthermore, the ISSR resolving power reveals the intraspecific variation and therefore allows us to easily differentiate closely related individuals (Ziętkiewicz et al., 1994). The ISSR was also successfully employed to assess hybridization and detect hybrid taxa (e.g. Wolfe et al., 1998; Conte et al., 2007; Goldman, 2008; Kramina et al., 2012; Sutkowska et al., 2013). Many studies have demonstrated the efficacy of those markers, both in the phylogenetic analyses and taxonomic relationships of *Allium* species (Hao et al., 2002; Son et al., 2012) as well as in population and cultivar genetics studies (Hur et al., 2006; Jabbes et al., 2011).

This study was aimed specifically to (1) assess the genetic differentiation and variability of morphologically defined groups of individuals representing two subspecies of *A. ursinum* and their putative hybrid; (2) find out whether geographic distribution of examined populations is correlated with their genetic distances; (3) determine whether the morphologically intermediate individuals can be regarded as a hybrid in terms of genetic pattern.

## MATERIALS AND METHODS

### SAMPLING AND PLANT MATERIAL

The study area covered the populations along the east-west transect crossing the morphologically-determined borderline separating the distribution ranges of both subspecies. This strategy enabled the collection of all three considered morphotypes, i.e. *A. ursinum* subsp. *ursinum*, *A. ursinum* subsp. *ucrainicum* and their putative hybrid. The locations of the examined populations are provided in Table 1 and shown in Fig. 1. 144 randomly chosen specimens from nine populations were collected during

one vegetative season in May and June 2013. Well-developed young leaves from individuals, distributed a minimum distance of 4 m from each other, were silica-dried in zipper bags and stored until DNA extraction. One leaf from one specimen constituted a single sample designated for further molecular analysis. The morphological determination of each individual from particular populations was based on the detailed examination of pedicel morphology using a stereoscopic microscope (Nikon SMZ 1500). Voucher specimens were deposited in the herbarium of the Institute of Botany at the Jagiellonian University in Kraków (KRA), Poland.

### DNA EXTRACTION AND ISSR ANALYSIS

Total genomic DNA was extracted from silica-gel-dried leaf fragments for each sample separately using a Genomic Mini AX SPIN kit (A&A Biotechnology) according to the manufacturer's instruction. DNA extracts were directly used as templates ISSR-PCR. Initially, six primers: ISSR1, ISSR2, ISSR3, ISSR4, ISSR6 and ISSR7 (Stepansky et al., 1999) were tested on the subset of eight samples from different populations, for quality and polymorphism of generated banding patterns. For further analysis three of them (ISSR1 – 5'- TCTCTCTCTCTCTCTCC-3', ISSR2 – 5'- AGAGAGAGAGAGAGAGT-3', ISSR3 – 5'- GGGTGGGGTGGGGTG-3') were selected, as they produced the richest and most polymorphic profiles. In order to verify the reproducibility of ISSR-PCR reactions the analysis was performed in two independent ISSR amplifications for each sample. Reactions were performed in 12.5 µl volume containing: 1× green DreamTaq reaction buffer (Thermo Fischer Scientific) 2.0 mM $MgCl_2$ (premixed with buffer) 0.2 mM dNTP mix, 1u of DreamTaq DNA polymerase (Thermo Fischer Scientific) and 1.6 µM of ISSR1, ISSR2 or ISSR3 primer (Stepansky et al., 1999). Amplification was run in T100 thermal cycler (BioRAD) with the following conditions: five minutes of initial denaturation at 94°C, 35 cycles composed of: 35 seconds of denaturation at 94°C, 40 seconds of primer annealing at 43°C (primer ISSR2) or 47°C (primers ISSR1 and ISSR3) and 35 seconds of primer elongation at 72°C followed by seven minutes of final elongation at 72°C. ISSR amplification products along with DNA size marker were separated in 1% agarose gel with the addition of SimplySafe™ (EURx) fluorescent stain (1µl/100ml of gel), visualized in ultraviolet light and photographed.

### DATA ANALYSIS

For each sample, results from two separate amplifications were compared in order to identify the replicable bands. Then the markers were scored by eye and encoded as 1 (present) or 0 (absent) in the binary matrix and used for further calculations. Only the bands present in two independent runs were scored.

For each population the following allelic variation parameters were calculated using GenAlEx 6.5 (Peakall and Smouse, 2006): number of bands, number of bands with frequency ≥5%, number of private bands, number of locally common bands (frequency ≥5%) found in ≤25% and ≤50% of populations and mean expected heterozygosity (He).

The genetic structure of populations was determined based on the following parameters: band frequency, frequency of p and q alleles in locus, number of bands (Na), the effective number of alleles (Ne), the Shannon genetic diversity index (I), expected heterozygosity (He) for each locus per population, the mean over loci per population and the percentage of polymorphic loci (P) for each population. Moreover, the genetic structure of the populations was assessed by calculating the Nei's genetic distance values (Nei, 1978) as well as PhiPT values (Peakall and Smouse, 2006). Genetic distances were expressed as a pairwise population matrix. Analysis of molecular variance (AMOVA) was used to determine the main source of variation observed in ISSR data set. This analysis was performed for the whole data set, each subspecies and putative hybrid separately. We also assessed the level and tested the significance of genetic differentiation among *A. ursinum* subsp. *ursinum*, subsp. *ucrainicum* and their putative hybrids. Next, the pairwise PhiPT values among these three groups were calculated. The test of significance for the AMOVA was carried out on 999 permutations of the data. The Mantel test (Mantel, 1967) was performed to determine the relationship and statistical correlation between the two matrices of distance, i.e. the genetic distance matrix (PhiPT values) obtained from AMOVA and the geographical distance matrix obtained after the conversion of geographical coordinates to geodetic coordinates X,Y.

The overall genetic structure and relationships among the studied populations/taxa were explored using the principal coordinate analysis (PCoA, performed using XLSTAT 2014.1.09), the neighbour-net analysis and the Bayesian nonhierarchical clustering. PCoA based on Nei's genetic distances (Nei, 1978) allowed us to illustrate populations grouped according to the ISSR similarity pattern. A neighbour-net diagram was based on the individual matrix of Nei & Li genetic distances (Nei and Li, 1979) bootstrapped using 1000 replicates with SPLITSTREE 4.13 software (Huson and Bryant, 2006).

The Bayesian model-based clustering algorithm was applied using STRUCTURE, 2.3.3 (Pritchard et al., 2000). The analysis was performed in 60000

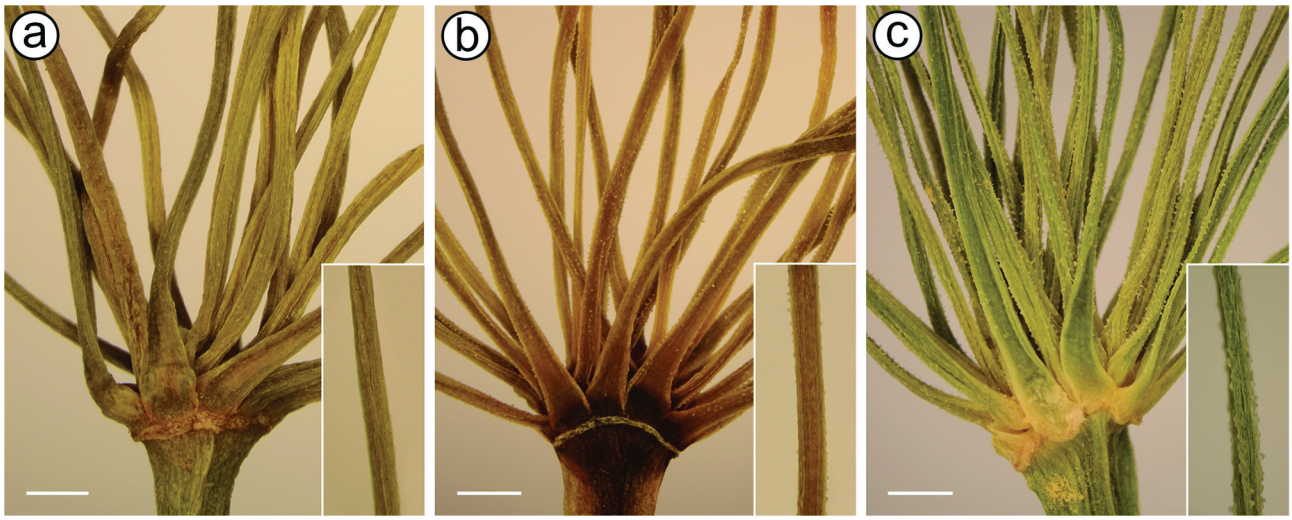


**Fig. 2.** The pedicel morphology of *Allium ursinum*. (**a**) *A. ursinum* subsp. *ucrainicum* – population KAL, (**b**) Intermediate morphotype – population ZP, (**c**) *A. ursinum* subsp. *ursinum* – population JN. Bar = 2 mm. Abbreviations of population names are explained in Table 1.

(first 10000 burn-in) iterations testing K = 1 to 9 clusters in 25 runs each. The Structure Harvester (Dent and von Holdt, 2012) online application was used to analyse the structure results according to Evano et al. (2005) algorithm. The number of clusters K with the highest Delta K value was chosen for further calculations. As the study is focused on the comparison of two subspecies in the hybrid zone, results for the simplest scenario of K = 2 were also taken into account. The coefficients of similarity among 25 runs for particular K values were calculated according to Nordborg et al. (2005) with Structure-sum script (Ehrich et al,. 2006). Then, the results for 25 runs of selected K were summarized with CLUMPP 1.1.2. (Jakobsson and Rosenberg, 2007), while the graphic visualization of summarized structure results was acquired with DISTRUCT software (Rosenberg, 2004).

## RESULTS

The detailed examination of pedicel morphology revealed that individuals within one population represent only one morphotype. Seven populations clearly referred to morphologically defined subspecies of *Allium ursinum*. Populations JN, GZ consisted of individuals possessing all pedicels with distinct papillae, thus they refer to subsp. *ursinum*. Whereas, individuals from populations B1, B2, BG, CG, KAL had all of the pedicels smooth within a single inflorescence and refer to subsp. *ucrainicum*. Individuals from SG and ZP populations represented various types of intermediate morphotypes (see Table 1 and Fig. 2) and were characterized by: (1) some pedicels smooth and some with papillae within a single inflorescence; (2) all pedicels within a single inflorescence papillate, but only in some parts of the pedicel; (3) the combination of the two abovementioned cases in relation to a single inflorescence or the whole individual.

The analysis of electropherograms allowed the distinction of 48 ISSR loci of which 45 were found to be polymorphic. This situation allowed us to perform a variety of reliable statistical analyses despite the relatively low overall number of detected loci. The maximum number of 45 bands was observed in four of the examined populations (B1, B2, CG, KAL; for abbreviations see Table 1). All of them appeared with a frequency of at least 5%. Each of the analysed populations had specific private bands. Two locally common bands in ≤50% of populations were found in CG and ZP populations, whereas one such band was found in B1, B2, GZ and KAL populations. The percentage of polymorphic loci ranged from 54.17% (JN) to 83.33% (GZ), while the average for the species equalled 70.60%. The mean of alleles over loci for all populations equalled 1.620, whereas the mean effective number of alleles was 1.423. The expected heterozygosity (He) ranged from 0.179 to 0.279; whereas Shannon genetic diversity index ranged from 0.270 to 0.420. Concerning Nei's genetic distance values, the populations that proved to be the most similar to each other were: SG and ZP, GZ and ZP, as well as BG and B1, B2. On the other hand, KAL and CG populations were found to be the most genetically distant from GZ, SG, JN and ZP populations (Tab. 2).

AMOVA showed highly significant ($p < 0.001$) genetic differentiation among all examined populations (Tab. 3). A large proportion of genetic variation

TABLE 2. Parwise Nei's genetic similarities (Nei, 1978) for populations of *Allium ursinum* from Poland based on ISSR markers. Abbreviations of population names as in Table 1.

| | CG | GZ | SG | JN | B1 | B2 | BG | ZP | KAL |
|---|---|---|---|---|---|---|---|---|---|
| CG | 0.000 | | | | | | | | |
| GZ | 0.150 | 0.000 | | | | | | | |
| SG | 0.178 | 0.079 | 0.000 | | | | | | |
| JN | 0.227 | 0.083 | 0.086 | 0.000 | | | | | |
| B1 | 0.085 | 0.105 | 0.100 | 0.113 | 0.000 | | | | |
| B2 | 0.131 | 0.078 | 0.108 | 0.094 | 0.085 | 0.000 | | | |
| BG | 0.118 | 0.098 | 0.107 | 0.112 | 0.063 | 0.063 | 0.000 | | |
| ZP | 0.163 | 0.034 | 0.060 | 0.121 | 0.099 | 0.091 | 0.104 | 0.000 | |
| KAL | 0.078 | 0.180 | 0.172 | 0.252 | 0.084 | 0.153 | 0.098 | 0.152 | 0.000 |

TABLE 3. Analyses of molecular variance (AMOVA) based on markers acquired with three ISSR primers for nine populations of *Allium ursinum*.

| Variation | df | SS | MS | Est. var. | Per. var. | PhiPT | P |
|---|---|---|---|---|---|---|---|
| *Allium ursinum* | | | | | | | |
| Among populations | 8 | 272.751 | 34.094 | 1.759 | 23% | | |
| Within populations | 135 | 805.226 | 5.965 | 5.965 | 77% | | |
| Total | 143 | 1077.977 | | 7.723 | 100% | 0.228 | <0.001 |
| *Allium ursinum* subsp. *ursinum* vs. *A. ursinum* subsp. *ucrainicum* | | | | | | | |
| Among subspecies | 1 | 80.382 | 80.382 | 1.206 | 14% | | |
| Among populations | 5 | 133.338 | 26.668 | 1.268 | 15% | | |
| Within populations | 106 | 648.732 | 6.120 | 6.120 | 71% | 0.288 | <0.001 |
| *Allium ursinum* subsp. *ucrainicum* | | | | | | | |
| Among populations | 4 | 112.620 | 28.155 | 1.343 | 18% | | |
| Within populations | 77 | 472.801 | 6.140 | 6.140 | 82% | | |
| Total | 81 | 585.421 | | 7.483 | 100% | 0.179 | <0.001 |
| *Allium ursinum* subsp. *ursinum* | | | | | | | |
| Among populations | 1 | 20.526 | 20.526 | 0.958 | 14% | | |
| Within populations | 29 | 164.958 | 5.688 | 5.688 | 86% | | |
| Total | 30 | 185.484 | | 6.646 | 100% | 0.144 | <0.001 |
| Intermediate morphotype | | | | | | | |
| Among populations | 1 | 16.131 | 16.131 | 0.669 | 10% | | |
| Within populations | 29 | 167.467 | 5.775 | 5.775 | 90% | | |
| Total | 30 | 183.598 | | 6.444 | 100% | 0.104 | 0.010 |

df – degrees of freedom, SS – sum of squares, MS – mean square, Est. var. – estimated variance, Per. ver. – percentage of variation.

(77%) resided among individuals within populations, whereas only 23% resided among populations. The major part of genetic variation was also within populations of both subspecies and the intermediate form (Tab. 3). The PhiPT values among all pairs of subspecies and putative hybrid were very similar, ranging from 0.114 to 0.191 (Tab. 4). However, it was observed that the most distant are two subspecies, whereas the putative hybrid is more closely related to *A. ursinum* subsp. *ursinum*. Moreover, AMOVA allowed us to obtain PhiPT values for each pair of populations and they ranged from 0.059 (GZ vs. ZP) to 0.435 (KAL vs. JN).

The PCoA based on pairwise Nei's genetic distances between populations revealed a clear geographical distribution pattern. Populations from particular geographical regions are horizontally scattered along Axis 1 (Fig. 3). Moving from the left to the right side of the diagram, the geographical position changes from the west to the east. The most

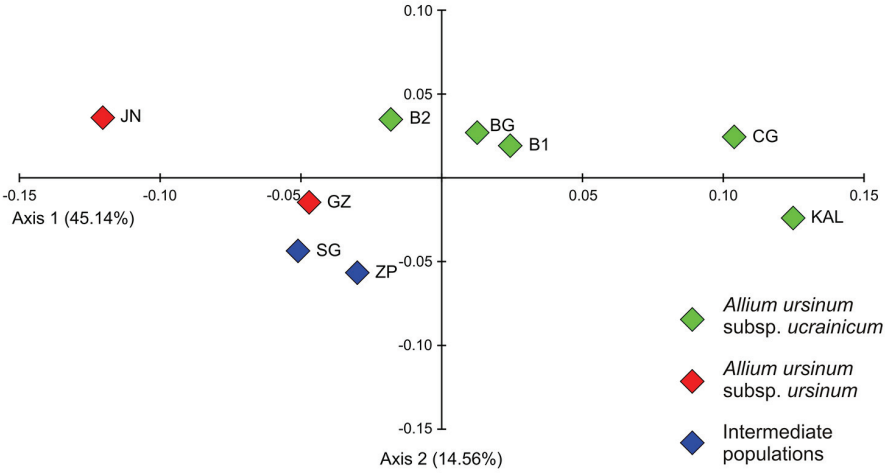


**Fig. 3.** Principal coordinate analysis (PCoA) of ISSR data from nine studied populations of *Allium ursinum* based on Nei's genetic distances (Nei, 1978). Abbreviations of population names are explained in Table 1.

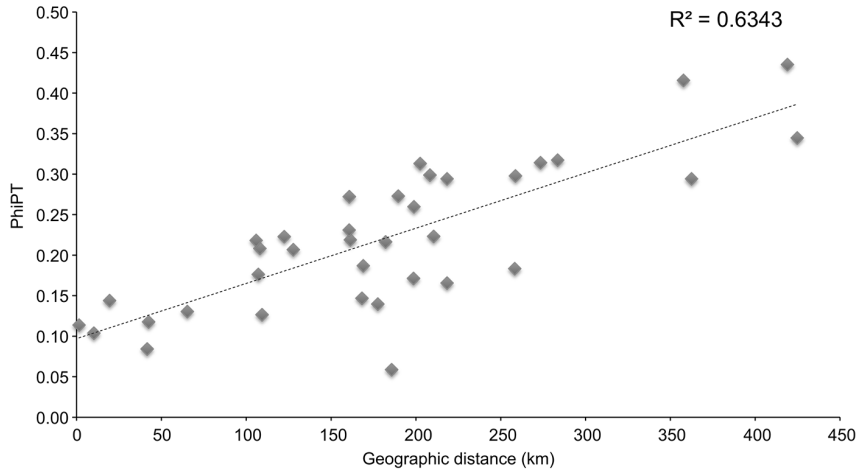


**Fig. 4**. The results of the Mantel test. The correlation between genetic distance (the values of PhiPT coefficient) and geographical distance for 144 individuals of *Allium ursinum*.

eastern populations (KAL and CG) are grouped on the right side of the scatterplot, whereas the populations from the Beskid Żywiecki and Beskid Śląski Mts (BG, B1, B2) are located in the middle part. All of the aforementioned populations represent *A. ursinum* subsp. *ucrainicum*. The populations from the Kraków-Częstochowa Upland area (ZP, SG) represented by individuals with intermediate morphology are grouped on the opposite side of Axes 1 and 2, close to each other, but are also very close to the population from the Góry Złote Mts (GZ). The last population represents *A. ursinum* subsp. *ursinum*. The population from the Masyw Śnieżnika Mts (JN) is the most distantly located on the left side of the scatterplot. This pattern is also strongly supported by the results of the Mantel test, which showed a significant correlation (R = 0.80, P = 0.010) between genetic and geographic distance. The graph (Fig. 4)

TABLE 4. The matrix of pairwise PhiPT values among two subspecies of *Allium ursinum* and their putative hybrid. PhiPT values are shown below diagonal; probabilities (P) based on 999 permutations are shown above the diagonal.

| | *Allium ursinum* subsp. *ucrainicum* | *Allium ursinum* subsp. *ursinum* | Intermediate morphotype |
|---|---|---|---|
| *Allium ursinum* subsp. *ucrainicum* | 0.000 | 0.001 | 0.001 |
| *Allium ursinum* subsp. *ursinum* | 0.191 | 0.000 | 0.001 |
| Intermediate morphotype | 0.146 | 0.114 | 0.000 |

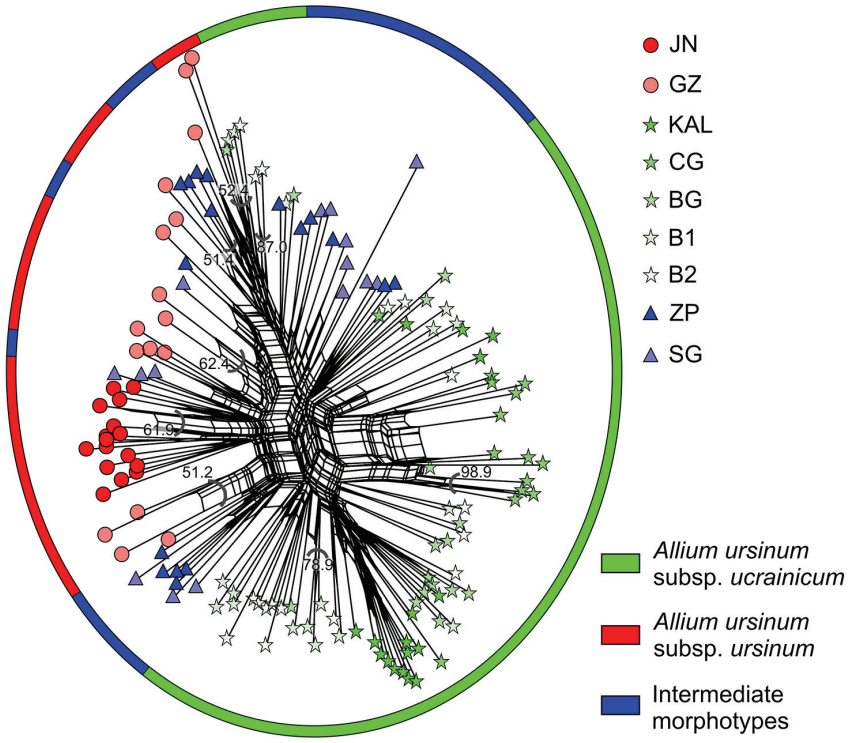


**Fig. 5.** Neighbour-net diagram of the *Allium ursinum* individuals based on the Nei & Li coefficient (Nei and Li, 1979). Abbreviations of population names are explained in Table 1. Bootstrap values ≥ 50 are given.

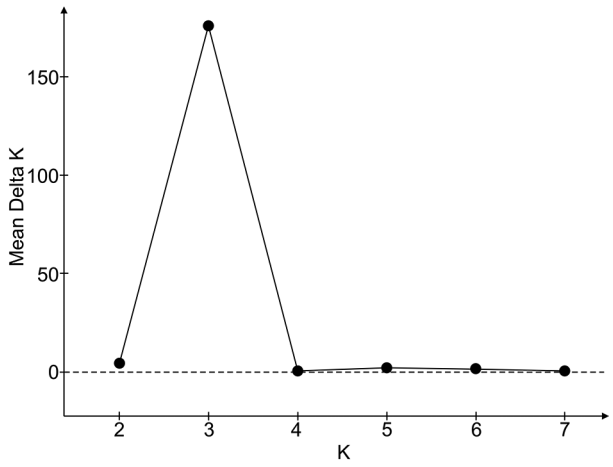


**Fig. 6.** Values of Delta K criterion for the detection of the optimal number of genetic clusters K in populations of *Allium ursinum*.

represents 63.43% of the explained variance. This indicates that the genetic differentiation among *A. ursinum* populations increases with increased geographical distance. The neighbour-net analysis showed low genetic variability within *A. ursinum*. On the other hand, the genetic split between individuals representing both subspecies could be observed (Fig. 5). This analysis also gave an additional insight. Some individuals of intermediate morphotype are located within *A. ursinum* subsp. *ursinum* and some of them within *A. ursinum* subsp. *ucrainicum*.

The STUCTURE runs for K = 2 and K = 3 were the most congruent as they reached the similarity coefficients 0.99 and 0.98, respectively. The analysis indicated that the highest Delta K value (175.73) was found for three distinct genetic groups (K = 3) that are distributed in nine populations (Fig. 6). The results for two and three genetic groups are summarized in Figure 7. Further interpretation assumes that the number of genetic group K = 3 is relevant. The relative share of each cluster composed of an individual genetic group of *A. ursinum* specimens is clearly correlated with the geographical distribution of the populations (Figs. 1, 7). Genetic group 1 appears to be more characteristic for western populations of *A. ursinum* morphologically identified as subspecies *ursinum*, and in the genotypes of individuals from the westernmost population (JN) the genetic group 1 clearly dominates as a rule. On the other hand, the genetic group 2 is found mostly in the eastern populations that morphologically refer to *A. ursinum* subsp. *ucrainicum*. The third genetic group is distributed

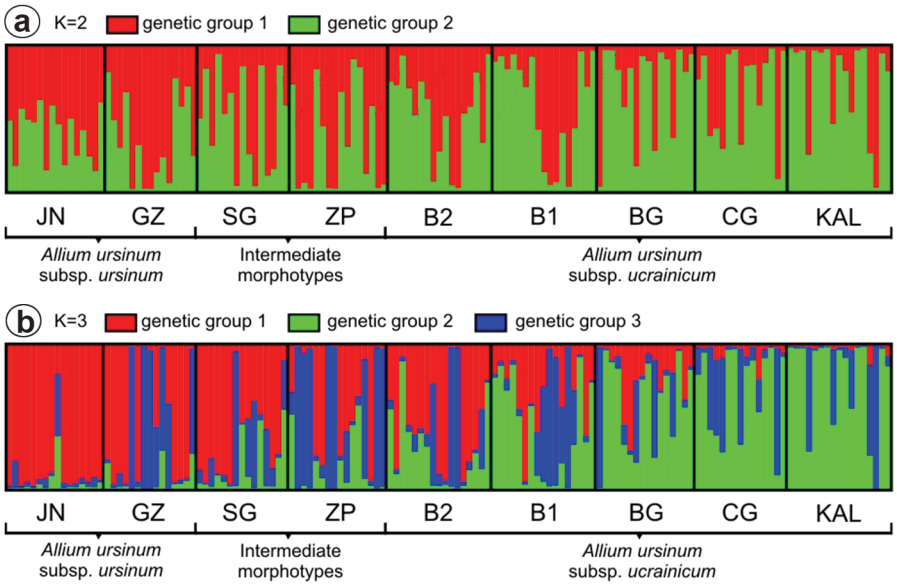


**Fig. 7.** Genetic relationships among populations revealed by Bayesian analysis of *Allium ursinum* ISSR dataset using STRUCTURE software (Pritchard et al., 2000). (**a**) K = 2 (**b**) K = 3. The colours of genetic groups are the same as in Fig. 1. Abbreviations of population names are explained in Table 1

throughout the whole west-east geographical transect, but it is the most common in the intermediate locations (Fig. 1). The most symptomatic is the fact that the populations from the Kraków-Częstochowa Upland area are the most differentiated both in the context of genetic structure and morphology of particular individuals. Furthermore, the proportion of 'western' and 'eastern' genotypes is relatively higher for respectively more western or more eastern locations of the population (Fig. 1). These results suggest that genetically defined groups 1 and 2 correspond to both subspecies while the third group is characteristic for the line of shared ranges of both subspecies as its proportion decreases with increasing geographic distance from this hybrid zone (Fig. 1). Such results confirm the existence of populations at the distribution margins of both subspecies represented by individuals in which the genetic material of parent taxa have mixed. This is also reflected in the pedicel morphological characters of individuals (Fig. 2). However, other studied populations also comprise individuals with mixed genotype (Fig. 7), but their ultrasculpture of pedicels corresponds either to *A. ursinum* subsp. *ucrainicum* or subsp. *ursinum*. This proved that the hybrid zone represented by mixed genotypes are actually much wider than it is suggested by morphological characters. Similarly, the results of STRUCTURE analysis for K = 2 suggest that the vast majority of the studied individuals have mixed genotypes of *A. ursinum* subsp. *ursinum* and subsp. *ucrainicum* (Figs 1, 7).

## DISCUSSION

Although in the genus *Allium* strong crossing barriers exist in some groups, even between morphologically similar species, spontaneous hybridization is not as rare as formerly believed (Fritsch and Friesen, 2002). Hybrid zones are frequently formed when populations of two related taxa meet together in a secondary contact zone, which causes the hybrid offspring to arise (Hewitt, 1999, 2001). The presumed hybrids are often firstly noticed when individuals with morphological characters intermediate between the parent taxa are found. However, the hybrid status of such specimens often remains hypothetical. This was the case of *Allium ursinum*, for which morphologically intermediate forms between both recognized subspecies have been observed in Europe for a long time (e.g., Zahariadi, 1966; Soják, 1968; Karpavièiene, 2006; Rola, 2012). The geographic distribution of the hybrid zone mirrors the contact between the distribution ranges of both subspecies (Fig. 1, see also Soják, 1968; Rola, 2012).

Natural hybrid zones of many plant species have been recorded (Hewitt 2001) and play an important role in intermediating gene introgression between parental populations (Zhang et al., 2014). Morphological and molecular evidence suggest the presence of natural hybrids in the contact zone between the distribution ranges of both subspecies of *Allium ursinum*. The delta K plot revealed the clear peak at K = 3 (Fig. 6), which is interpreted as the optimal number of genetic groups. The distribu-

tion of these groups throughout the studied populations is closely correlated with geographic origin (Figs. 1, 7). The proportion of the 'western' cluster (genetic group 1) is the highest in the westernmost populations and its share continuously decreases moving to the east. The same phenomenon, but in the opposite direction, may be observed for the 'eastern' cluster (genetic group 2). The third genetic group appears in all populations, but its proportion is the highest in the geographically intermediate populations. Such a pattern indicates that the 'western' genetic group is associated with *A. ursinum* subsp. *ursinum* while the 'eastern' one corresponds to *A. ursinum* subsp. *ucrainicum*. The emergence of the third genetic group seems to be an effect of hybridization events occurring within the secondary contact zone. Only two populations from the Kraków-Częstochowa Upland area (SG, ZP) represent clear intermediate phenotypes (Fig. 2). Both these populations are dominated by genetic groups 1 and 3 and the proportion of the 'eastern' genotype is rather limited (Figs. 1, 7b). The more western populations (GZ, JN) are characterized by an even lower fraction of the 'eastern' genotype and even eastern-genotype-free individuals were observed (Fig. 7b). The proportion of particular genetic groups in the populations from the Beskid Śląski Mts (B1, B2) is quite similar, however they morphologically represent 'pure' *A. ursinum* subsp. *ucrainicum*. Nevertheless, intermediate morphotypes were previously observed within this region (Rola, 2012). The three remaining populations (BG, CG, KAL) are characterized by the highest proportion of the 'eastern' genotype (Figs. 1, 7b). This may suggest that the development of papillae on the pedicels go along with the lack or at least limited share of the 'eastern' genotype. Consequently, a high proportion of the 'eastern' genetic group, even balanced by the other groups, results in the 'pure' *A. ursinum* subsp. *ucrainicum* phenotype. Although the changes in the proportion of particular genetic groups are clearly associated with the geographical distribution of the examined populations (Fig. 1), there are some individuals within particular populations whose genetic pattern do not fit strictly to this general trend (Fig. 7). This may be the effect of human activity. *Allium ursinum* subsp. *ucrainicum* is commonly cultivated as a garden or medicinal plant, being often planted in parks and cemeteries (Hanelt, 2001; Seidemann, 2005). Consequently, the phenomenon of the presence of the 'eastern' genotype in the representatives of natural western populations might take place.

The results of STRUCTURE analysis for both K = 2 and K = 3 suggest that the vast majority of the studied individuals have mixed genotypes of *A. ursinum* subsp. *ursinum* and subsp. *ucrainicum* (Figs 1, 7). The pattern of the revealed genetic groups can, however, be described as an example of introgression resulting from gene flow from intersubspecies hybrids towards both parental forms. This idea is supported by the results of the Mantel test and PCoA, as both these analyses indicate a strong correlation between genetic diversity and geographic distance (Figs. 3, 4). The individuals represented by genetic group 3 (Fig. 7b) are widespread in populations located both to the west and east of the morphologically determined hybrid zone. The STRUCTURE analysis indicated that gene flow between the two studied subspecies is fairly possible and backcrossing could occur intensively in both directions (Fig. 7). Consequently, a distinguishable genetic boundary between *A. ursinum* subsp. *ursinum* and subsp. *ucrainicum* does not exist and direct gene introgression between the two subspecies is rather evident. We found that typical morphologically intermediate populations occur only in a relatively narrow geographical zone (Fig. 1, see also Rola, 2012), but intensive introgression with both parental subspecies in both directions could be observed in a wider geographical zone (Fig. 7). Consequently, the hybrid zone revealed by ISSR markers is actually much wider than it was suggested by the morphological pattern of individuals. Thus, we can suppose that "pure" populations of both subspecies have not been included in the present study. This fact implies the need for research that covers a wider range of the distribution of the species. Nevertheless, our results based on ISSR markers provide an important insight into the extent of the intersubspecific hybrid zone of *A. ursinum*, although they don't deal with the whole genetic variation and geographic ranges of both subspecies. This approach is a sort of prelude to a fully-scaled investigation of the *A. ursinum* population structure, planned to be undertaken in the future, which will be based on molecular markers more suitable to confirm the hybridisation and will include data acquired from populations covering a representative part of the species distribution range.

Genetic studies of *A. ursinum* showed that all populations are characterized by balanced and very high levels of genetic variation within a population. The results of AMOVA showed that more genetic variation of the studied *A. ursinum* is distributed within populations than between them (Tab. 3). This indicates a relatively restricted population differentiation in Poland. Genetic variations in populations are dependent on several factors, such as the demographic pattern, historical distribution, geographical isolation and particularly the mating system (see e.g. Antonovics, 1984). *Allium ursinum* reproduces both generatively and vegetatively by producing daughter bulbs. Although the species tends to reproduce clonally, sexual reproduction is assumed to outrank vegetative reproduction when compared to other herb

layer plants (Tutin, 1957; Ernst, 1979; Bierzychudek, 1982; Grime et al., 1988; Oborny et al., 2011). This explains its great intrapopulation genetic differentiation revealed during this study. This high genetic variability protects *A. ursinum* against potential genetic erosion and decrease in genetic diversity. This is particularly important given that the species is mainly connected with large and mostly ancient forest complexes and is unable, or almost unable, to establish itself in secondary forests (Hermy, 1992; Dzwonko and Loster, 2001; see also Karpavièiene, 2006; Rola, 2012).

*Allium ursinum* is a habitat specialist, having a rather narrow range of ecological tolerance (Grime et al., 1988; Kevey, 1977; Oborny et al., 2011). It is strongly confined to deciduous woodlands, mixed beech-fir and beech-spruce forests in Europe (Tutin, 1957; Karpavièiene, 2006; Kovács, 2007), and especially to beech forests (with *Fagus sylvatica*) in Poland (Rola, 2012). Such species are supposed not to survive for a long time outside mesic forest complexes (Ellenberg, 1996). Consequently, the current distribution patterns of such species should bear the imprint of postglacial migratory history (Willner et al., 2009). The migration routes of plant species, confined to forest undergrowth, after the last glacial period is closely connected with the migration history of forest tree species, which is especially apparent in the case of mountain species in Poland (Szafer, 1929). Because the distribution of *A. ursinum* and *Fagus sylvatica* in most parts of Europe are consistent and the first species is frequently observed in ancient forest complexes in Poland, it seems that this two taxa could migrate northwards following similar routes as the glacial withdrawal. The colonization of *F. sylvatica* from European glacial refugia northwards did not proceed with a closed front, but with a diffuse spread from scattered nuclei (Magri, 2008). Based on palynological data, the northwards migration of *F. sylvatica* progressed from southern Bohemia and Moravia (Czech Republic), areas considered the main source regions for the colonization of Central Europe by *F. sylvatica* (Magri et al., 2006; Magri, 2008). The migration to southern Poland progressed from two main directions (Szafer, 1929; Latałowa et al., 2004). The first one was south-west and west from the Sudetes and the second one south-east from the Eastern Carpathians. These two waves of migration are clearly reflected by the current borders of distribution ranges of some species associated with the undergrowth of beech forests, e.g. *Dentaria gladulosa* and *D. enneaphyllos* (Jalas and Suominen, 1994; Lihová et al., 2007) as well as *Allium ursinum* subsp. *ursinum* and *A. ursinum* subsp. *ucrainicum*. It should also be mentioned that the genetic variability of *Allium ursinum* found in this study is higher than recorded in the populations from Germany detected by RAPD fingerprinting (Herden et al., 2012). Low genetic variability could be explained by a rapid spread of the species after glaciation in central Germany (Hewitt, 1999; Herden et al., 2012), in contrast to the more complex postglacial migration history of *A. ursinum* in Poland.

## AUTHORS' CONTRIBUTIONS

KR original idea and study design; KR, PO data collection; KR morphological analysis; ALB, PB molecular analysis; KR statistical analysis; KR, ALB, PB interpretation of the results; KR drafting of manuscript. The authors declare that they have no conflicts of interest.

## ACKNOWLEDGEMENTS

This study was supported by the Faculty of Biology and Earth Sciences at the Jagiellonian University (subvention No K/DSC/001682) in Kraków, Poland.

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