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Genetic variation of the relict and endangered population of *Chamaedaphne calyculata* (Ericaceae) in Poland

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Abstract: *Chamaedaphne calyculata* is rare and endangerded species of Polish flora. The genetic variation within and among ten polish population of leatherleaf was analysed by ISSR and ISJ markers. The analysis revealed a total of 160 loci with an average of 13.3 bands per primer. We expected a low level of genetic diversity of this narrowly distributed species in Poland, but our results indicate that *Ch. calyculata* revealed a high level of genetic diversity at species level (P=88.7% of polymorphic loci, A_E=1.468, H_E=0.290). At the population level, the variation of *Ch. calyculata* was significantly lower (P=27.6%, A_E=1.140, H_E=0.098). There was significant correlation between ecological properties (population size; number of flowering ramets) and genetic diversity parameters. Analysis of molecular variance showed that most of variation (62%) in *Ch. calyculata* occurred among population. Gene flow (Nm) between the ten studied populations, determined based on the G_{ST} index, was very low at 0.239. It indicated that the fragmentation and isolation of populations might result from specific evolutionary history of this plant and postglacial recolonization.

Additional key words: *Chamaedaphne calyculata*, genetic diversity, ISSR and ISJ, conservation, rare and endangered plant species

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Introduction

The past decades witnessed a drastic drop in the number of plant species as an inseparable consequence of a deteriorating natural environment, climatic change and excessive urbanisation. The danger of extinction particularly applies to rare and endangered plant species and peripheral population of widespread species which occur at a limited number of geographically isolated localities. The conservation of those species is the main objective of research teams who pursue this goal by combining the experience of ecologists and population geneticists. Various protection programs have been initiated to preserve habitats which support the development of the endangered species and the existing degree of their genetic variation.

One of the most important factors in the preservation of rare and endangered species is genetic diversity. Genetic diversity influences adaptive flexibility of species to physical and biological environment changes (Vrijenhoek 1994). The majority of rare and endangered species grow in habitats marked by extreme conditions and fluctuating changes. Those factors have a critical influence on the survival of those populations, often reducing their abundance and leading to the extinction of individuals in populations

(Hewitt 1996). The above results in the loss of allelic richness and a higher degree of homozygosis. The uniformity of genotypes is also supported by inbreeding which reveals unfavourable alleles in populations (Hamrick and Godt 1996).

Most important role in the process of shaping the genetic structure of a population plays gene flow (Hamrick and Godt 1989). In populations of rare and threatened species, which are usually widely dispersed, gene flow is relatively obstructed and in some cases, it may not take place at all. The above leads to a loss of allelic richness and it increases the degree of divergence between isolated populations (Evans et al. 2001). The absence or presence of gene flow also influences inbreeding efficiency and determines the rate of inbreeding depression in a population (Vellend and Geber 2005).

Maintenance of genetic diversity is therefore an important factor for the survival of endangered species, and assessment of genetic diversity is vital for their conservation management (Hamrick and Godt 1996). Information on genetic diversity patterns provides insight into evolutionary and demographic history of taxon (Miligan et al. 1994). Understanding the importance processes that structure diversity within and among population can provide both means to asses future risk of erosion of diversity and means to designing effective conservation strategies for the taxa.

Rare and endangered plants account for around 15% of all flora species in Poland (Kazimierczakowa and Zarzycki 2001). The largest group comprises taxa which occur in boreal and arctic areas. One of such taxa is *Chamaedaphne calyculata*. This plant's main geographic range covers the tundra zone. In Poland, it is considered to be a postglacial relict, which most probably spread from the south-eastern refuge in Siberia (Kruszelnicki 2001). The species has its most southbound localities in Poland. Until recently, 13 *Chamaedaphne calyculata* populations were identified, of which only 9 are presently confirmed and 1 introduced population. The plant inhabits mostly raised unforested bogs with acidic soils and a high moisture content (*Sphagnetum magellanici* communities), but it is also found in *Vaccinio uliginosi-Pinetum* marshy coniferous forests (Kloss 1996, 1999, Kruszelnicki 2001). In the distant past, this species was most probably marked by wide geographic coverage and high population abundance throughout Central Europe. Changing climatic conditions during the Pleistocene, which involved sudden temperature drops, and subsequent interglacial periods resulted in the mass migration of entire floras (Huntley and Webb 1988, Hewitt 1996, Taberlet et al. 1998, Walter and Epperson 2001), and this process shaped and limited the geographic range of this taxon as it is known today. The majority of *Ch. calyculata* localities in Poland are found in the northern parts of the country, in lakeland regions and in the Mazowsze-Podlasie Lowland, which were among the few European regions to be covered by glacial ice in the last glaciation period (Huntley and Webb 1988). The region's specific, well preserved habitat and climatic conditions as well as the local abundance of peatlands contributed to the plant's survival at those localities. All Polish localities feature small populations of the analysed species and are geographically isolated. In most localities, the number of individuals per population has been declining steadily in the recent years due to an unstable level of ground waters which hinders the preservation of *Ch. calyculata* localities (Szkudlarz 1995, Kloss 1996, 1999, Klimko et al. 2000, Kruszelnicki 2001). *Chamaedaphne calyculata* populations are also adversely affected by succession changes which lead to the encroaching of trees to open marshy areas and the expansion of deciduous species which block light access. The deterioration of habitat conditions at those localities is also a consequence of human activity, such as intensive land reclamation and peat mining.

Chamaedaphne calyculata populations have been monitored continuously for several years. The species genetic structure has to be researched to foster a better understanding of the mechanisms that shape its populations. This knowledge will support an evaluation of the risk of genetic erosion and it will pave the way to the initiation of effective protection programs covering this endangered species to preserve environmental biodiversity (Neel and Ellstrand 2003). Biodiversity should be preserved primarily at the genetic level. The knowledge of genetic structure supports the adequate selection of samples for reinstating and reintroducing the species to the environment (Brown 1989).

Previous studies on *Chamaedaphne calyculata* mainly concerned on characteristic of localities, of this plant in Poland (Zabawski and Matuła 1975, Szkudlarz 1995, Kloss 1996, Falkowski and Krechowski 1999, Kruszelnicki 2001), variability of leaves (Klimko et al. 2000) and propagation from seeds and shoot cuttings (Malinowska et al. 2004). However genetic structure of this plant remains unknown. The purpose of the present study was to investigate the genetic diversity of Polish population of *Ch. calyculata* using ISSR and ISJ markers

The main objective of this study were to a) asses levels of genetic diversity of natural and not natural population of *Ch. calyculata* in Poland, b) determine whether small populations of this species maintain a low level of genetic variation in comparison with large populations, c) evaluate the degree of differentiation among population and determine whether genetic differences between the populations are correlated with geographical distance which could be indicative of their genetic isolation.

Among various molecular tools, inter-simple sequence repeats (ISSR) have been widely used for population genetic studies (Gupta et al. 1994), especially in plants (Wolfe et. al. 1998, Jin et. al 2006) because of their high level of polymorphism and reproducibility (Kojima et al. 1998, Bornet and Branchard 2001, Wolfe et al. 1998).

Semi-specific ISJ (Intron-Exon Splice Junction) markers are based on the sequences which are commonly found in plants and which are indispensable for post-transcription DNA processing (Weining & Langridge 1991). ISJ primers are partly complementary to the sequences on the exon-intron boundary.

These markers had been successfully applied in studies of genera *Polygonatum* (Szczecińska et. al 2006), *Sphagnum* (Sawicki and Zieliński 2008) and *Aneura* (Bączkiewicz et al. 2008). Application of these two categories of DNA markers enables estimating a variability within investigated populations both in non-coding (SSR markers) and encoding (ISJ markers) regions of genome, what might have great weight during surveying a real adaptative variability. Nowadays it is considered more and more frequently that quantifying a population "fitness" basing on an analysis of highly polymorphic but at the same time non-coding microsatelite loci might not reflect the real adaptative capability of population which is determined mainly by functoinal genes. Therefore the use of both SSR and ISJ markers seems to be justified as ISJ markers associate to a sequence on the exon-intron boundary and they are able to amplify functional regions of a genome. An estimation of gene variation with these categories markers will allow us to draw objective conclusions.

Methods

Population studying

All nine known Polish populations of *Chamaedaphne calyculata* and one reintroduced population located in the Kampinos National Park (Długie Bagno) were sampled in this study (Fig. 1). The geographical distance between the studied populations ranged from 3 km to 600 km.

Experimental material was collected from July to August 2007–2008. Fresh leaves were collected randomly from each individual and they were dried in silica gel (Chase and Hills 1991). The distances between sampled plant were from 5 to 250 m, which depended on the population size. Leaf samples were collected carefully in order to minimize potential damage to populations.

Molecular analysis

DNA was extracted from 40 mg dry leaf tissue extraction using the DNeasy Plant extraction kit (Qiagen). The isolated DNA was dissolved in water and stored at –20°C. Two marker categories were used in the analysis of genetic variation: microsatellite ISSR markers, (Inter-simple Sequence Repeat) developed by Zietkiewicz et al. (1994), and ISJ markers (Weining and Langridge 1991). Primer sequences are presented in Table 2. Similarly to RAPD and AFLP markers, the target sequence of ISSR markers does not require prior identification which makes those markers suitable for studying species for which species-specific primers amplifying microsatellite loci (SSR-simple sequence repeats) have not yet been developed. Yet contrary to SSR markers, ISSR primers are complementary to repeated sequences rather than to fragments flanking those sequences. Semi-specific ISJ markers are based on sequences which are commonly found in plants and which are indispensable for post-transcription DNA processing (Weining and Langridge 1991). ISJ primers are partly complementary to the sequences on the exon-intron boundary. More details concerning PCR-ISJ methodology are given by Sawicki and Szczecinska 2007. The sequences of ISSR and ISJ primers used for DNA amplification in this study are given in Table 2.

ISSR-PCR and ISJ-PCR reactions were performed in 20 μl of a reaction mixture containing 40 ng genomic DNA, 1 μM primer, 1.5 mM $MgCl_2$, 200 μM dNTP (dATP, dGTP, dCTP, dTTP), 1× PCR buffer (Sigma, supplied with polymerase), 1μl BSA and 1 U Genomic Red Taq polymerase (Sigma). ISSR marker reactions were performed under the following thermal conditions: (1) initial denaturation – 5 minutes at a temperature of 94°C, (2) denaturation–1 minute at 94°C, (3) annealing – 1 minute at 50°C, (4) elongation – 1'30'' at 72°C, final elongation–7 minutes at 72°C. Stages 2–4 were repeated 34 times. The follow-

Fig. 1. Geographical distribution of analysed population of *Chamaedaphne calyculata*

Table 1. Studied populations of *Chamaedaphne calyculata*

N°	Population code	Locality	Community type	N
1	KR	Country Krasnopol	Marshy coniferous forest	10
2	CZ	Country Czarna Białostocka Knyszyńska Forest	Swampy pine forest Vaccinio uliginosi-Pinetum	10
3	GO	Country Michałowo, Gorbacz Nature Reserve	Marshy coniferous forest	30
4	LJ	Country Siemień, Lisie Jamy peatbog	Disappearing peatbog	5
5	MA	Mazurski Lanscape Park, Forestry Kołowin	Raised bog Sphagnetum magellanicii	45
6	SO	Country Grom, Sołtysek Nature Reserve	Swampy pine forest Vaccinio-uliginosi Pinetum and Caricion lasiocarpe	30
7	DB	Kampinos National Park, Długie Bagno peat bog (reintroduced population)	Raised bog Sphagnetum magellanicii	5
8	KA	Kampinos National Park, Sieraków Nature Reserve	Swampy pine forest Vaccinio uliginosi-Pinetum	17
9	TU	Tucholski Lanscape Park, Forestry Sowiniec	Raised bog Sphagnetum magellanicii	20
10	DR	Drawieński National Park, Ścienko Nature Reserve, Forestry Martew	Raised bog Sphagnetum magellanicii	20

N-is number of collected samples per population

Table 2. Sequence of 12 primers successfully used in the ISSR and ISJ analysis and number of amplified bands per primer

Primer	Seguence (5'–3')	Number of the amplified bands
IS807	$(AG)_8T$	13
IS810	$(GA)_8T$	12
IS822	$(TC)_8A$	14
IS825	$(AT)_8G$	18
IS828	$(TG)_8A$	16
IS834	$(ATG)_6$	13
IS831	$(ACC)_6$	15
IS843	CATGGTGTTGGTCATTGTTCCA	13
ISJ2	ACTTACCTGAGGCGCCAC	14
ISJ4	GTCGGCGGACAGGTAAGT	13
ISJ5	CAGGGTCCCACCTGCA	10
ISJ6	ACTTACCTGAGCCAGGGA	9
Total		160

ing reaction conditions were applied to ISJ primers: (1) initial denaturation–5 minutes at 94°C, (2) denaturation–1 minute at 94°C, (3) annealing – 1 minute at 48°C, (4) elongation – 2'50'' at 72°C, final elongation – 5 minutes at 72°C. The products of the ISSR-PCR and ISJ-PCR reaction were separated on 2% agarose gel, followed by DNA staining with ethidium bromide. After rinsing in deionised water, agarose gel was analysed in a transilluminator under UV light at a wavelength of 302 nm with the application of the Felix 1010 system.

Statistical analysis

Statistical analysis was based on 160 loci. The analyses of genetic data were performed using PopGene v. 1.32 (Yeh & Boyle 1999) and Arlequin v. 3.01 (Excoffier et al. 2006). The following parameters were used to estimate genetic diversity at the population level and at the species level: the percentage of polymorphic loci (P%), number of alleles per locus (A_E), genetic diversity (H_E) and Shannon information index of diversity (I_S) (Lewontin 1972). Allele frequency in locus was identified in view of band presence or absence. It was assumed that every observed band resulted from the amplification of a single locus, therefore, the number of observed bands corresponded to the number of investigated loci. Only allele "1" (band present) or allele "0" (band absent) were observed in every locus. Differences in allele frequencies between the investigated populations were determined with the use of Statistica software (Statsoft 2003). Nei's unbiased genetic identity (I) and genetic distance (D) were estimated for each population (Nei 1972). Genetic differentiation among population was estimated by Nei's gene diversity statistics (Nei 1973). The amount of gene flow among those populations was estimated at $N_m = (1/Gst-1)/5$ (Slatkin 1987). The analysis of molecular variance

Table 3. Genetic diversity within population of *Chamaedaphne calyculata*

Population	N	n	P%	A_E	I_S	H_E
KR	82	14	8.75	1.041 (0.018)	0.040 (0.015)	0.026 (0.010)
CZ	114	32	20.00	1.140 (0.078)	0.116 (0.034)	0.079 (0.019)
GO	124	48	30.00	1.196 (0.038)	0.165 (0.030)	0.112 (0.021)
LJ	78	20	17.50	1.097 (0.029)	0.085 (0.022)	0.056 (0.015)
MA	130	78	48.75	1.299 (0.044)	0.250 (0.033)	0.169 (0.023)
SO	120	46	28.75	1.140 (0.034)	0.116 (0.027)	0.079 (0.019)
DB	104	30	18.75	1.135 (0.035)	0.109 (0.026)	0.075 (0.018)
KA	114	48	30,00	1.162 (0.035)	0.143 (0.027)	0.094 (0.019)
TU	132	62	38.75	1.265 (0.043)	0.219 (0.033)	0.149 (0.023)
DR	126	56	35.00	1.239 (0.043)	0.193 (0.032)	0.132 (0.022)
Species*	160	142	88.75	1.468 (0.341)	0.427 (0.229)	0.290 (0.169)

*Total genetic diversity of *Chamaedaphne calyculata* at species level

N is the number of amplified bands per population, n is the number of polymorphic loci per population, P is the percentage of polymorphic loci, A_E is the effective number of allels, I_S is Shannon,s information index (Lewontin 1972), H_E is Nei's (1973) gene diversity. Data in the bracket is the standard deviation

(AMOVA) was used to partition the genetic variance into within and among populations. AMOVA analyses were carried out using ARLEQUIN v. 3.01 (Excoffier et al. 2006).

The correlation between genetic (D) and geographical distance (in km) among the studied populations was performed by Mantel's test (Mantel 1967) was performed using ARLEQUIN v 3.0 (Excoffier et al. 2006) The neighbor-joining dendrogram was constructed based on the matrix of genetic distances using Mega 4 (Tamura et al. 2007).

The significance of differences in coefficient values between the studied populations was established by analysis of variance and the LSD test.

Results

Genetic diversity

Genetic diversity of Polish populations of *Chamaedaphne calyculata* was analysed based on 192 individuals representing 9 natural populations and 1 introduced (Table 1). The analysis with use of 12 primers revealed 160 loci, on average 13.3 bands per population. The highest number of bands (18) was amplified primer IS825, while the smallest number of bands (9 loci) was scored for primer ISJ6 (Table 2). Only 20 among all analysed loci were monomorphic (IS807-1, IS807-4, IS810-2, IS810-4, IS822-7, IS825-1, IS825-2, IS825-5 IS828-12, IS831-5, IS843-13, IS834-13, ISJ2-10, ISJ2-13, ISJ4-3, ISJ4-8, ISJ5-4, ISJ5-5, ISJ6-5, ISJ6-8).

The studied populations differed significantly with regard to the number of scored loci and their polymorphism. The highest number of loci, were amplified for population TU (132) and population MA (130) while for LJ population only 78 bands appeared. The most polymorphic from investigated populations was population MA with 78 polymorphic loci (48.75%), while the lowest percentage of polymorphic bands was found in KR population (8.75%). The number of polymorphic loci in the remaining populations ranged from 30 to 62 (Table 3). The mean value of P for all examined populations was 27.63%.

A similar correlation was observed during the analysis of mean number of allels per locus (A_E) and Nei's gene diversity index (H_E). The mean number of alleles per locus (A_E) revealed for the species was relatively high at 1.468. Yet at the population level, the value of coefficient A_E was lower, ranging from A_E=1.041 (population KR) to A_E=1.299 (population MA). Like the mean value of H_E at species level was high (0.290), but at population level value of this coefficient was considerably lower (0.098). Respectively the lowest level of genetic diversity (0.026) was reported in population KR, while population MA was the most genetically diversified (H_E=0.169) (Table 3). The value of the gene diversity coefficient and number of allels per locus were strongly correlated

Table 4. Hierarchical analysis of molecular variance (AMOVA) for ten populations of *Chamaedaphne calyculata* in Poland

Source of variance	d.f.	SS	Variance component	Total variation (%)	P-value[a]
Among population	9	658.07	12.52266	62.00	0.001
Within population	88	397.19	7.11318	38.00	0.001

[a]Significance tests after 1.000 permutations

Table 5. Genetic distance and geographical distance (km) between populations of *Chamaedaphne calyculata*. Genetic distance below is listed diagonal, geographic distance is listed above diagonal.

Populations	KR	CZ	GO	LJ	MA	SO	DB	KA	TU	DR
KR	–	90	100	330	140	180	400	398	426	600
CZ	0.2739	–	30	231	130	170	233	230	400	564
GO	0.3216	0.2471	–	190	180	200	210	207	430	580
LJ	0.3909	0.5083	0.6118	–	300	302	160	157	440	545
MA	0.2918	0.2242	0.1452	0.4368	–	48	250	253	277	400
SO	0.3390	0.2172	0.1285	0.5249	0.1833	–	181	184	230	360
DB	0.2872	0.2675	0.1962	0.5540	0.2108	0.2156	–	3	250	370
KA	0.2624	0.2363	0.1763	0.5270	0.1785	0.1867	0.1334	–	253	373
TU	0.3208	0.1948	0.1502	0.5140	0.1951	0.2000	0.2902	0.2247	–	140
DR	0.3277	0.1425	0.1706	0.4964	0.2502	0.2407	0.2946	0.2938	0.1416	–

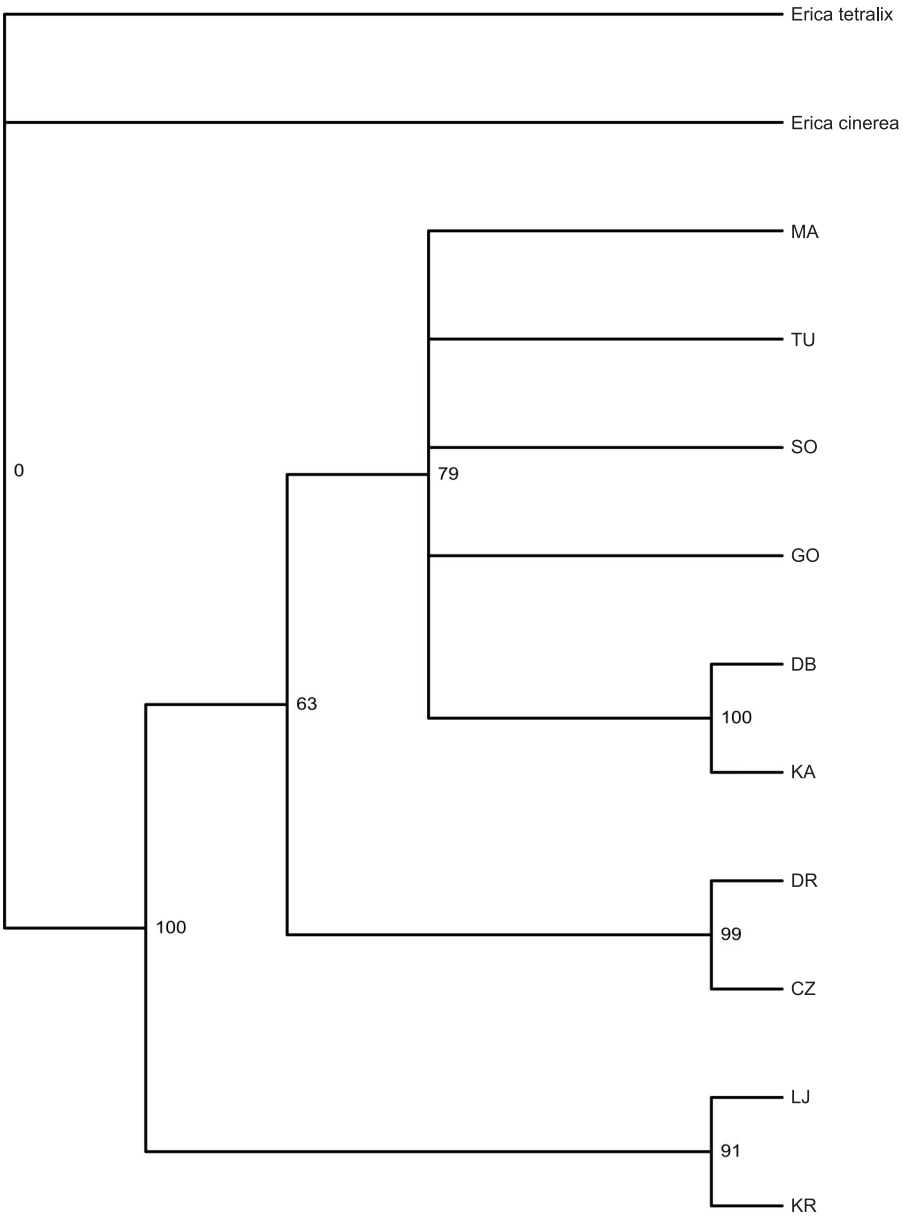

Fig. 2. NJ grouping of analyzed population of *Ch. calyculata* based on Nei (1972) genetic distances. Bootstrap values (bs) higher than 50% are indicated below branches

with population size (Spearman's rank correlation $P<0.05$). The Shannon information index (I_S) at the population level ranged from 0,040 for population KR to 0.250 for population MA reaching an average value of 0.146 (Table 3).

Genetic variation

The AMOVA analysis has shown that the most part (62%) of a genetic variation observed in *Ch. calyculata* is a consequence of interpopulation variability and only 38% of it depends on variability within populations (Table 4). This relations is also confirmed by a relatively high G_{ST} index ($G_{ST}=0.676$).

Gene flow (Nm) between the ten studied populations, determined based on the G_{ST} index, was very low at 0.239. The vast differences between *Ch. calyculata* populations are related to variations in allele frequency. Statistically significant differences in allele frequency between the studied populations were determined in 82 of 160 amplified bands, i.e. in 51.2%. But the number of private bands observed in all individuals was very low (6 bands). Private alleles were reported in only three of the ten investigated populations (CZ, KR and MA). Genetic differences between the analysed populations were validated by the low level of genetic identity index (I). The mean value of I was 0.757, and it ranged from (I=0.557) to I=0.869) for different populations.

The statistical analysis performed by Mantel's test did not reveal correlations ($P<0.05$ $r=-0.250$) between the geographical distance and genetic distance of the studied populations. The greatest genetic distance (0.611) was noted between population GO and LJ which were not separated by the greatest geographical distance (190 km). The genetic distance between the two most geographically isolated populations (600 km) was 0.327 (Table 5).

Neighbor-joining method of clustering was carried out to estimate phylogenetic relationship between ten populations using Nei' genetic distance matrices. The ten populations of *Ch. calyculata* were clustered into two main groups (Fig 2). The first group included eight from ten analysed population (MA, TU, SO, GO, DB, KA, DR, CZ) and in the second clade include only two population (LJ and KR). This two clades were well supported with bootstrap 91%.

Discussion

Rare and endangered plant species are generally characterised by a low level of genetic variability. This lack of diversity has often been associated with small size of their populations, their isolation or disjunction or alternatively to strong, directional natural selection driven by uniformity of habitats (Barret and Kohn 1991, Ellstrand and Elam 1993, Godt and Hamrick 1995).

According to Hamrick and Godt (1989) an effective measure of genetic variation is geographic range. The results of analyses based mostly on enzymatic data indicate that species with a limited geographic range showed relativley low level of genetic variation in comparison with widespread species (Hamrick and Godt 1989, Premoli et al. 2001).

As revealed ISSR and ISJ markers *Ch. calyculata* had low genetic diversity at population level ($P=27.6\%$, $A_E=1.17$, $H_E=0.098$) and it was similar to the mean values noted for other rare and endangered plant species ($P=31.1$, $A=1.4$ and $Hs=0.105$) (Gitzendanner and Soltis 2000). Unfortunately we cannot compare this level of genetic variation with other population of *Ch. calyculata* from continuous range, since no other studies of genetic variability of this species have been published. But the other species from genus Ericaceae characterised by higher genetic diversity (Zawko et al. 2001, Apte et al. 2006, Jin et al. 2006).

Analysed in this study population of *Ch. calyculata* differed significantly the level of genetic variation which increased in the following order: KR, LJ, DB, CZ, SO, KA, GO, DR, TU and MA. *Ch. calyculata* populations with a relatively small number of individuals, growing unstable habitats (KR, CZ, KA, LJ) characterised by water level fluctuations and tree species succession and non natural population (DB) were marked by a lower level of genetic variation than more abundant populations which grow in more stable habitats (SO, DR, TU, MA). The low level of genetic variation in the least abundant populations of *Ch. calyculata* is mostly due to a low population size and critical habitat conditions at those localities. Our observations have confirmed that the stochastic habitats of those populations obstruct population growth, and as regards population CZ and KR, suboptimal habitat conditions could actually be responsible for the decrease in the number of individuals.

The value of genetic diversity of analysed population of *Ch. calyculata* (A_E, P and H_E) were strongly correlated with population size and number of flowering ramet. A positive correlation between population size and the degree of genetic diversity was observed in many other plant populations, including *Trollius europaeus* (Despres et al. 2002), *Arnica montana* (Luijten 2000), *Gentianella germanica* (Fisher et. al. 1997) and *Euryngium alpinum* (Gaudeul et al. 2000).

A positive correlation between genetic diversity and population size could suggest that genetic drift has a more intense, negative impact on small populations of this species than on more abundant populations (Barret and Kohn 1991). The above underlies the observed differences in allele frequency between the analysed populations. Alleles marked by low frequency in the largest populations (TU and MA, SO) were not determined in the smallest populations (CZ, DB, KA). As a result of genetic drift, those alleles

were most likely eliminated from the least abundant populations. The loss of rare alleles due to genetic drift was also observed in the populations of other rare and endangered plant species, such as *Silene sennenii* (Lopez-Pujol et al. 2007) and *Lactoris fernandeziana* (Crawford et al. 1994).

Similarly to many other taxa with a limited geographic range, the genetic erosion of small populations of the analysed species could have been accelerated by the absence of gene flow between particular populations as well as population bottleneck and founder effect (Mitka 1995).

The low level of genetic variation in small populations of *Ch. calyculata* in Poland can also be explained by species biology. According to observations, small populations of *Ch. calyculata* in Poland are characterised by low flowering capacity, infrequent fruiting (population CZ where no flowering individuals were determined) and negligent seedling recruitment (Kruszelnicki 2001). Similarly to many other species with small, isolated populations situated on the boundaries of their respective geographic range, the genetic structure of *Ch. calyculata* is largely affected by vegetative reproduction, while generative reproduction is limited or not observed at all (Johansson 1994, Stewart and Nilsen 1995, Mitka 1995, Jones et al. 2002).

Chamadeaphne calyculata as endangered species in Poland had relatively high levels of genetic variation (P=88.7%, A_E=1.468, H_E=0.290) at species level. A similar, high level of genetic variation was determined in many other rare and endangered plant species. Jin and Li (2007) used ISSR markers to analyse the genetic structure of a rare and relict shrub species, *Sinocalycanthus chinensis*, which revealed high genetic diversity at the species level: P=73.08, I=0.350 and H=0.1987. A similarly high degree of genetic variation was revealed with the use of the AFLP analysis for the critically endangered species *Primulina tabacum* (P= 85.6%, H=0.339) (Xiawoei et al. 2006) and *Adenophorus periens* (P=80%, A=2.8, H=0.191) (Ranker 1994).

The analysed *Ch. calyculata* populations were characterised by relatively high genetic heterogeneity. Study results have shown that interpopulation variety accounts for 62% of the observed variation. The high value of index F_{ST} is indicative of a continued process of isolation between the analysed populations and suggests the absence of an equilibrium between drift and migration (Tero et al. 2003). The low level of gene flow between the analysed populations (Nm=0.268) has cumulated the effects of genetic drift and mutation, leading to their independent divergence. Numerous research studies in the area of population genetics have shown that in species marked by small and isolated populations, interpopulation variation has a higher share of the entire species variation (Xiawoei et al. 2006, Jin and Li 2007).

The noted absence of positive correlations between genetic and geographical distance could indicate that the process of colonisation in Poland did not follow the stepping stone model (Kimura and Weiss 1964). The genetic isolation of the Polish populations of *Ch. calyculata* is due to both historical and contemporary factors. In the distant past, the analysed species was probably marked by uniform geographic distribution in Poland. Yet changing climatic conditions and progressive climate warming eradicated favourable habitats for this taxon and wiped out entire populations of the species. Human activity such as land improvement also heavily contributed to the fragmentation of the geographic range and the disappearance of *Ch. calyculata* populations in Poland. In addition to geographic range, gene flow between the studied populations is also hindered by the species' reproductive system. In principle, the plant is classified as an autogamous species which is characterised by the highest number of migrants per generation, as shown by the high mean value of index G_{ST} (0.651). Yet limited gene flow of *Ch. calyculata* in Poland could be due to the fact that vegetative reproduction was predominant in the investigated populations. The high level of interpopulation variation noted for *Ch. calyculata* was confirmed by low level of genetic identity (I=0.763). Similar observations were reported in respect of many other rare plant species (Godt et al. 1995).

Implications for conservation

According to numerous studies investigating rare and endangered species (Xiawoei et al. 2006), the fragmentation and eradication of favourable habitats poses the greatest threat for those taxa. Stochastic habitat conditions lead to a drastic drop in population size which affects the species' genetic diversity. A similar situation has been observed in the Polish populations of *Ch. calyculata*. The noted low level of genetic diversity of the least abundant populations restricts their adaptive capacity due to the absence of alleles which support adaptation to constantly changing habitat conditions (Oostermeijer et al. 1998, Reed and Frankham 2003, Vergeer et al. 2003, Leimu et al. 2006). Limited gene flow between populations also hinders the increase in genetic diversity or its maintenance at a satisfactory level. For this species to be effectively conserved in the Polish flora, favourable habitat conditions and adequate population size must be preserved for this taxon. *Ex situ* protection may also offer a promising solution for the preservation of this species in the future.

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