

**Is habitat important? Morphology and genome size of *Epipactis helleborine* (L.) Crantz
growing in anthropogenic and natural habitats**

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Abstract: **Background.** The process of apophytism or spreading native species to human-made habitats is one of the main elements in the creation of plant cover in anthropogenic areas. Lately, an increase of anthropogenic localities with valuable flora (rare and law protected species) has been observed. Apophytes are also members of the family Orchidaceae, especially from the genus *Epipactis*. The aim of the study was to i) determine and compare the phenotypic variation of *E. helleborine* plants in anthropogenic and natural habitats, ii) compare the genome size estimation of plants growing in natural and anthropogenic habitats. The results reported in this study may indicate that a habitat influences morphological characteristics of plant species. **Methods.** The research was carried out in Poland from 2011 to 2013. The study sites were located in three different geographical regions: from the Białowieża Primeval Forest, Northern East Poland, through Central Poland, to the Lower Silesia Province, Southern West Poland. We investigated eight populations of *E. helleborine*: four from natural habitats and four from anthropogenic habitats. Biometrical analyses were performed on shoots and flowers. The flowers were characterised by 25 biometric features and measured using a Nikon SMZ 800 binocular, microscopic Moticam-1SP cameras and the MIPlus07 program (Conbest Co.). The nuclear DNA content was determined in fresh and young leaves of *E. helleborine*, collected from eight populations. **Results.** We observed that in anthropogenic populations: i) shoots were higher than shoots from natural populations, ii) flowers differed significantly in terms of ten biometric features between habitats, iii) the genome size differed significantly between the plants growing in natural and anthropogenic habitats. **Discussion.** According to some researchers, the presence of phenotypic plasticity and the occurrence of ecotypes are adaptation strategies of plants to environmental changes. In our opinion, in the case of the studied anthropogenic habitats (roadside) in which the *E. helleborine* populations grew, we can talk about ecofen due to the often repeated set of characteristic features. i.e.: high shoots, long inflorescence and long,

broad leaves. We agree, however, that it is difficult to isolate a taxonomic unit for ecofen due to the lack of experimental research.

Introduction

The family Orchidaceae comprises estimated 20,000 to 30,000 species, making it the largest and the most important family of flowering plants (Delforge, 2001). Orchids are considered to be ubiquitous, since they occur on all vegetated continents and even some Antarctic islands (Dressler, 1994). Their distribution and abundance vary between continents and regions, however, the most orchid-rich areas include South America, Madagascar, Sumatra and Borneo for mostly epiphytic species, Indochina for both epiphytic and terrestrial species, and Western Australia as a centre of terrestrial orchid richness (McCormick, Whigham & O'Neill, 2004). In Europe, there are approximately 230 species (Delforge, 2001). Despite a great number of orchid species, many are rare or even threatened with extinction (Dressler, 1994). It is observed that orchid species disappear in their natural habitats and penetrate anthropogenic environments (Dressler, 1981; Reinikka, 2008). The first report about the appearance of orchids in anthropogenic areas came from the 19th century, when those plants were observed at railway embankments in Great Britain (Procházka & Velisek, 1983). Adamowski (2006) reported that in disturbed habitats 53 species from 300 European taxa might be encountered. *Dactylorhiza majalis* and *Epipactis helleborine* are species which most frequently occupy anthropogenic habitats (Light & MacConaill 2005, 2006; Wittig & Wittig, 2007; Bítea et al., 2011, Rewicz at al., 2017).

Observations of the authors as well as data from the literature suggest that in habitats changed by humans *E. helleborine* populations are characterised by high variability of ramets. Moreover, higher and more massive shoots compared to the populations in natural habitats are observed in these populations, which can suggest changes in their genome size (Rogalska et

al., 2005; Stefaniak et al., 2011; Adamowski, Stefaniak & Święczkowska, 2012). The somatic chromosome numbers reported for this species range from $2n=20$ for diploid (Weijer, 1952) to $2n=60$ for hexaploid cytotype (Averyanov, Averyanova & Lavrenko, 1982; Meili-Frei, 1965), however, other numbers, such as $2n=36$, 38, 39, 40, were also reported (Silvestre, 1983). Jakubska-Busse (2008), Jakubska-Busse et al. (2016) observes that *E. helleborine* is a morphologically changeable species, which can be a result of several ecological factors or somatic mutations occurring in ramets within one genet. This species has more phenotypic plasticity, which allows it to more easily adapt to changes in the environment.

The Orchidaceae is characterised by their high phenotypic plasticity. Heywood (1974) claims that the phenotype modification is a response of the genotype to the surrounding environment, where changes frequently occur on the genetic level, including changes in the genome size (Rogalska, Małuszyńska & Olszewska, 2005). Considering the genome size of the Orchidaceae, high variation is observed, with the genome size ranging 168-fold, from 0.66 to 110.8 pg/2C (Leitch et al., 1974). There is no doubt that the huge range of variation in DNA content has a significant effect on their phenotype. Therefore, the determination of C-value is an important feature for biology and biodiversity of the Orchidaceae (Bennet, Bhandol & Leitch, 2000). Earlier studies revealed a relationship between the genome size and latitude, altitude at sea level, temperature or precipitation, but there is no consensus as to whether the correlation is negative or positive (Knight & Ackerly, 2002; Bogunic et al., 2007). Vinogradov & Selfish (2003) has indicated that the species with larger genomes possess less adaptability to adverse environmental conditions, and at the same time, the risk of their extinction is much higher than that of the species with small genomes. Within the Orchidaceae, polyploidy was also detected (Jacquemyn et al., 2016). Polyploids are characterised by a large size and vigour of cells, leaves, flowers, and fruits, compared to the diploid individuals (Tamayo-Ordóñez et al., 2016). They are also more tolerant to changing environmental conditions and have more

chance for expansion to new areas. This is probably related to an increased degree of heterozygosity, which can be an essential factor for growth, development and adaptability of polyploids (Tamayo-Ordóñez et al., 2016). Since chromosomes of many orchids are small and often numerous, ploidy estimation by chromosome counts is difficult. In addition, microscopic chromosome counting is time-consuming and limited to a few tissues. Therefore, flow cytometry (FCM) is a more convenient alternative for establishing the ploidy/genome size of the Orchidaceae species. The genome size is, next to morphological and anatomical descriptions, a good taxonomic marker useful for identifying many problematic taxa (Wang et al., 2016).

Objectives of this study were to: i) determine and compare the phenotypic variation of the *E. helleborine* plants from anthropogenic and natural habitats, ii) compare the genome size estimation of plants growing in natural and anthropogenic habitats.

Materials and methods

Study species

The genus *Epipactis* includes 60-80 species (Kreutz & Fateryga, 2012) and systematics of this genus is complicated mainly due to similar morphology. Jakubska-Busse (2008), Jakubska-Busse et al., (2016) has also observed some morphological adaptations to local environments in this genus. One of such adaptations is the change in floral architecture and the possibility of the transition between cross- and self-pollination (Tałałaj & Brzosko, 2008). This genus has very asymmetric and very complex karyotypes. This causes a variation in the number of chromosomes between the *Epipactis* species during the differentiation process. Verlaque, Seidenbinder & Reynaud (1987) suggest that the basic chromosome number is $x=10$.

Epipactis helleborine is a clonal taxon, growing in broadleaved and coniferous (also secondary) forests, on forest edges and also in anthropogenic habitats, such as rural and urban roadsides, railway embankments, post-mining sites, tracks, quarries, poplar plantations, parks, sandy beaches, lawns (Hollingsworth & Dickson, 1997; Wittig & Wittig, 2007; Akhalkatsi, Arabuli & Lorenz, 2014) and, furthermore, also cities (Stešević & Jovanović, 2008; Milović & Mitić, 2012; Rewicz et al., 2017). This species is rather indifferent in terms of habitat and behaves as a pioneer (Delforge, 2001). It grows on moderately wet, acidic to neutral humus soils and sometimes on substrates rich in calcium carbonate (Robatsch, 1983).

Study sites. The research was carried out in Poland from 2011 to 2013. The study sites were located in three different geographical regions: from the Białowieża Primeval Forest, Northern East Poland, through Central Poland, to the Lower Silesia Province, Southern West Poland (Fig. 1, Regional Directorate of Environmental Protection in Białystok permission no WPN6400.74.2013.MW.). The identified investigated habitats were separated into two categories: the populations found in anthropogenic habitats such as roadsides and natural as a mixed forest (Tab. 1).

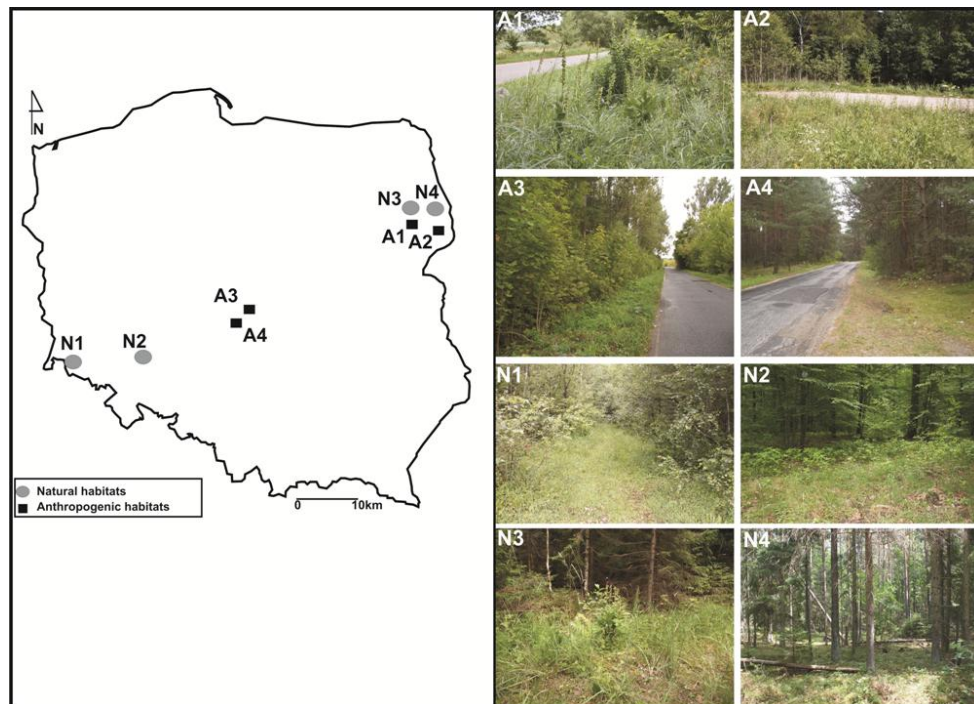


Fig. 1. Location of study populations of *Epipactis helleborine* (the left), habitats of *Epipactis helleborine*; Abbreviations according to Table 1 Figure was generated by CorelDraw X3 software.

Tab. 1 Studied populations of *Epipactis helleborine*

Population code	locality	Population size (m ²)	Number of shoots	Coordinates
A1	roadside (Guszczewina)	36	127	N 52.831600 E 23.794836
A2	roadside (Hajnówka)	108	102	N 52.734217 E 23.603314
A3	roadside (Sulejów)	460	80	N 51.353793 E 19.883155
A4	roadside (Sulejów)	46	152	N 51.349757 E 19.882484
N1	mixed forest (Kotowice)	100	300	N 50.963255

				E 15.963255
N2	mixed forest (Kaczawskie Mts)	40	150	N 51.041241 E 17.176701
N3	mixed forest (Białowieża Primeval Forest)	120	34	N 52.828706 E 23.797095
N4	mixed forest (Białowieża Primeval Forest)	400	41	N 52.832427 E 23.763069

A1 – Guszczewina, A2 – Hajnówka, A3 – Sulejów 1, A4 – Sulejów 2, N1 – Góry Kaczawskie, N2 – Siechnice, N3 – Białowieża Primeval Forest 1, N4 – Białowieża Primeval Forest 2 (the right).

Biometric analysis. Biometrical analyses were performed on shoots and flowers of *E. helleborine* (Table S1, Fig. 2 I). Live measurements were taken using a measure tape rounded up to the nearest 1 mm. A study on the variability of metric features of *E. helleborine* flowers was carried out in August 2013, taking at random a sample of 15 flowers from each population. The flowers were inserted into the preservative Kew Mixture (composition for 1 litre: 530 ml 96% EtOH, 50 ml formaldehyde, 50 ml of glycerol, 370 ml of distilled water), which allowed to maintain the shape and the natural size of flowers for the further research. The flowers were characterised by 25 biometric features (Table. S1, Fig. 2 II, III) and measured using a Nikon SMZ 800 binocular, microscopic Moticam-1SP cameras and the MIPlus07 program (Conbest Co.).

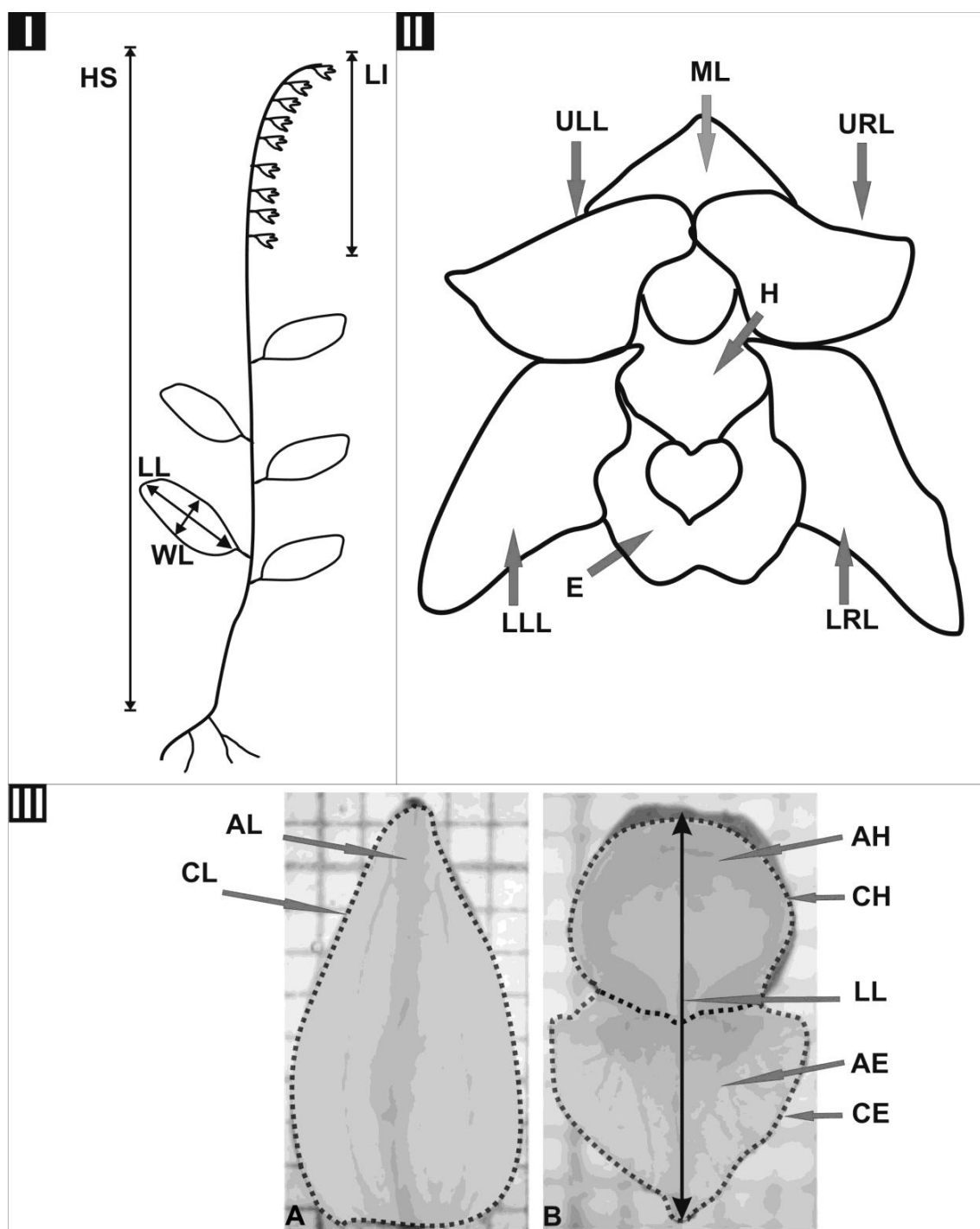


Fig. 2. Diagram of seed measurements. I) Shoots of *Epipactis helleborine*, II) Flower, III) patterns (see the supplementary Table S1). Figure was generated by CorelDraw X3 software.

Genome size estimation. The nuclear DNA content was determined in fresh and young leaves of *E. helleborine*, collected from eight populations. The leaves of *Secale cereale* ‘Dankowskie’ (2C=16.2 pg) (Doležel & Bartoš, 2005) were used as an internal standard. The studied samples were prepared according to Jedrzejczyk & Sliwinska (2010). The plant material was chopped with a sharp razor blade in a plastic Petri dish containing 1 ml of nucleus-isolation buffer (0.1 M Tris, 2.5 mM MgCl₂×6H₂O, 85 mM NaCl, 0.1% (v/v) Triton X-100; pH 7.0) supplemented with propidium iodide (PI, 50 µg/mL) and ribonuclease A (50 µg/mL). Nuclei suspension was passed through a 50 µm mesh nylon filter. For each sample, measurements of fluorescence intensities were performed in at least 7000 nuclei using a CyFlow SL Green (Partec GmbH, Münster, Germany) flow cytometer equipped with a laser with green light emissions at 532 nm. The analyses were replicated five times for each population. Histograms were analysed using the FloMax software (Partec GmbH, Münster, Germany). The nuclear genome size of *E. helleborine* was calculated using the linear relationship between the ratio of the target species and *S. cereale* 2C peak positions on the histogram of fluorescence intensities. The mean coefficients of variation (CV) of the 2C nuclei were estimated for all the samples of *E. helleborine*. The 2C genome sizes were obtained after the conversions of values in picograms into base-pair numbers using the factor 1 pg = 978 Mbp (Doležel & Bartoš, 2005).

Statistical analysis. Biometric data were statistically analysed using STATISTICA ver. 10.0 and Canoco ver. 4.5. The following basic characteristics were calculated: the arithmetic mean (x), minimum and maximum value, standard deviation (SD) and coefficient of variation (CV). The compatibility of the studied morphological features with the standard distribution was checked by means of the Shapiro-Wilk and Kolmogorov-Smirnov tests. For samples accordant with the standard spatial distribution, the ANOVA test was applied (for many groups) and the Student's test (for two groups). In the majority of cases in which the data did

not show compliance with the standard distribution, the non-parametric Kruskal-Wallis test was used. A multiple comparison of average ranks for all the samples or the Duncan test were applied as a *post hoc* test. To compare two independent groups, the U'Mann-Whitney test was used, while for the two dependent groups the Wilcoxon test was used. Differences amounting to $P < 0.05$ were considered statistically significant.

The correlation between variables was tested by means of Spearman's correlation coefficient and multiple regression (van Emden, 2008). In order to demonstrate statistical differences between the genome size for the examined populations, the one-way analysis of variance ANOVA and the *post-hoc* Duncan test were carried out.

Statistical analyses were performed using the single factor analysis of variance and the Duncan's test to determine possible differences in the nuclear DNA content among all the analysed populations of *E. helleborine*.

Results

Variability of shoots. The height of *E. helleborine* shoots ranged from 17.0 to 149.0 cm for the anthropogenic populations and from 4.4 to 95.0 cm for the natural populations. The highest shoot length was 149.0 cm, recorded in the A1 (Guszczewina) population, and the shortest was 4.4 cm, recorded in the N1 population (Góry Kaczawskie; Table 2). In the case of the anthropogenic populations, *E. helleborine* shoots were longer than shoots from the natural populations. In contrast, the mean values of the remaining parameters (i.e. inflorescence length, leaf width and length) were higher in the populations from natural habitats (Table 2). Intra-population variability was demonstrated in terms of all *E. helleborine* features; however, the arrangement of the homogeneous populations investigated during the first period of observation was not repeated in the next period. The greatest intra-population diversity was indicated for the length of shoots, where four homogeneous groups were observed (Table 2). The length of shoots (LS) in the anthropogenic populations demonstrated insignificant

225 variation, ranging from 21.0 to 39.7%, while for the natural populations it ranged from 21.4 to
 226 51.9% (Table 2). The length of inflorescence (LI) demonstrated the highest variation both for
 227 the anthropogenic and natural populations. For intra-habitat variation, statically significant
 228 differences in the two investigated periods were demonstrated for the length of shoots,
 229 inflorescence and leaves . The longest vegetative shoots were found in two anthropogenic
 230 populations in the A2 (Hajnówka – 31.0 cm) and the A3 (Guszczerwina – 26.7 cm)
 231 populations in 2012, while the shortest in the N3 and N4 populations (Białowieża Primeval
 232 Forest). The populations A1 and A2 differ significantly from the others in terms of vegetative
 233 shoots.

Table 2. Features group of the habitats of *E. helleborine*.

Population	HS (cm)		CV%	LI (cm)		CV%	WL (cm)		CV%	LL (cm)		CV%
2011												
A1	84.8 (42.0-149.0)	a	28.8	20.9 (2.0-36.0)	a	43.2	4.8 (1.0-7.0)	a	27.4	8.8 (4.0-13.0)	c	27.4
A2	54.1 (31.0-90.0)	c	29.3	14.2 (2.0-33.0)	a	51.1	4.6 (1.5-8.0)	b	34.0	9.0 (3.0-13.0)	a	31.1
A3	56.3 (40.0-80.0)	c	21.0	11.5 (3.0-27.0)	b	46.4	3.1 (1.2-7.0)	b	42.8	6.1 (2.5-11.0)	b	39.1
A4	42.0 (20.0-80.0)	c	30.1	7.0 (1.0-22.0)	b	69.0	3.6 (1.5-5.9)	c	29.4	7.8 (5.0-12.0)	b	23.7
Mean	59.3			13.4			4.0			8.9		
N1	62.0 (32.0-90.0)	a	28.9	15.8 (5.0-37.0)	c	54.0	6.0 (2.8-13.0)	a	38.6	11.6 (7.2-15.5)	a	19.7
N2	57.4 (17.0-99.0)	b	27.9	15.8 (4.0-31.0)	b	41.1	5.0 (1.5-8.5)	b	32.0	9.8 (5.6-13.5)	b	19.8
N3	34.5 (4.4-60.0)	d	44.2	11.7 (2.0-27.5)	c	53.2	5.2 (1.7-9.0)	b	32.2	4.2 (1.7-10.5)	a	63.1
N4	48.4 (16.0-95.0)	b	48.8	17.0 (4.0-40.0)	a	53.3	3.8 (1.8-7.4)	b	37.6	6.6 (3.5-12.6)	a	33.5
Mean	50.6			15.1			5.0			8.1		
2012												
A1	87.7 (30.0-129.0)	a	25.7	19.3 (6.0-41.0)	a	39.5	6.0 (3.3-11.0)	b	25.1	13.2 (8.0-18.0)	a	17.7
A2	64.3 (26.0-107.0)	b	30.3	18.5 (2.0-36.0)	b	51.6	5.8 (3.0-10.0)	b	28.0	10.0 (6.0-15.0)	b	20.6
A3	56.0 (21.0-90.0)	c	34.1	10.2 (2.0-21.0)	c	55.2	3.3 (1.4-5.7)	c	45.6	7.7 (3.2-11.5)	c	34.9
A4	40.2 (17.0-78.0)	d	39.7	7.9 (1.0-20.0)	c	50.4	3.6 (2.0-5.9)	c	26.5	7.7 (5.0-12.0)	c	22.9
Mean	62.1			14.0			6.8			7.5		
N1	66.7 (42.0-85.0)	b	20.4	24.5 (4.5-35.0)	b	35.3	5.7 (2.5-7.9)	c	27.0	11.5 (6.5-17.5)	a	27.0
N2	62.1 (34.0-90.0)	b	26.7	16.2 (5.0-37.0)	b	52.0	6.0 (2.8-13.0)	a	39.3	11.7 (7.2-15.5)	b	19.8
N3	55.2 (9.4-85.0)	d	36.8	19.6 (4.0-35.0)	c	50.4	5.2 (1.7-9.0)	c	35.6	10.0 (5.0-17.5)	a	32.9
N4	46.1 (7.6-95.0)	d	51.9	17.1 (4.0-40.0)	a	53.6	3.8 (1.8-7.6)	c	37.4	6.6 (3.5-12.6)	c	33.5
Mean	57.5			19.4			5.2			10.0		

Variability of flowers. The mean values of the measured elements of the analysed flowers indicated that the flowers from the studied anthropogenic habitats were bigger than the flowers from the natural habitats (t-Student's test, $p < 0.05$; Table 3).

Table 3. Variation of morphological features of *E. helleborine* shoots in analyzed populations.

Homogeneous letters indicate homogeneous groups (Kruskale-Wallis test $P = 0.05$).

Characters abbreviated as in the Table 1.

Feature	Anthropogenic habitat	CV (%)	Natural habitat	CV (%)	t-Student's test ($P < 0.05$)
(mm ²)					
AULL	36.8	19.4	31.9	28.3	ns
AML	41.7	26.6	36.0	28.9	ns
AURL	36.0	19.8	32.5	27.2	ns
ALLL	43.2	20.2	34.9	27.2	* (t=2.741, p=0.007)
ARLL	42.9	19.9	35.3	29.8	* (t=3.317, p=0.001)
AH	14.9	23.9	12.4	32.8	ns
AE	16.4	19.2	13.5	24.6	* (t=3.060, p=0.003)
(mm)					
CULL	28.2	8.8	25.3	13.3	* (t=3.504, p=0.000)
CML	29.8	11.3	27.8	13.9	ns
CURL	27.5	9.2	25.9	11.5	* (t=2.060, p=0.003)
CLLL	32.3	9.5	28.3	14.1	* (t=4.138, p=0.000)
CRLL	31.9	8.4	28.3	16.5	* (t=3.230, p=0.001)
CH	15.4	11.0	13.3	16.3	* (t=4.102, p=0.000)
CE	19.5	9.0	15.9	12.6	* (t=3.350, p=0.001)
(mm)					
LLi	8.1	12.0	7.1	13.0	* (t=4.90, p=0.000)
LULL	10.4	10.4	9.3	13.0	ns
LML	11.1	12.9	10.9	14.7	ns
LURL	10.1	12.0	9.5	11.9	ns
LLLL	12.1	13.6	10.6	17.1	ns
LRLL	11.7	10.7	10.7	15.6	ns
(mm)					
WULL	5.2	13.3	5.2	14.1	ns
WML	5.1	14.7	4.8	17.6	ns
WURLL	5.3	18.1	5.3	15.6	ns
WLLL	5.4	22.0	4.8	17.0	ns
WRLL	5.3	11.6	5.0	14.4	ns

The analysed flowers differed significantly (t-test, $P < 0.05$) in terms of ten biometric features (ALLL, ARLL, AE, CULL, CURL, CLLL, CRL, CH, CE, LLi; Table 3). The biggest differences were observed in the surface area of their perianth leaves. The plants in the anthropogenic habitats had evolved flowers in which the surface of the upper left lobe and the right lower lobe was greater than for the flowers from the studied natural habitats (43.3 mm^2 and 42.9 mm^2 in the anthropogenic habitats, while in the natural habitats – 34.6 and 35.3 mm^2 respectively). The coefficient of variation was higher for the parts of flowers in the analysed anthropogenic habitats (Table 4). The highest value of the coefficient of variation for both habitats was connected with the area of the measured elements, while the smallest value with the perianth perimeter, as well as the perimeter and the length of labellum.

Table 4. Biometric characteristics of *E. helleborine* flower in analyzed habitats. Abbreviations as in Table 1. (* $p < 0.05$ – significance level, ns – non-significant, CV – coefficient of variation (%).

Feature	A1	A2	A3	A4	\bar{x}	N1	N2	N3	N4	\bar{x}	F	P
(mm^2)												
AULL	35.6	33.7	38.4	39.6	36.8	31.9	32.9	27.0	35.6	31.9	1.8	0.0996
AML	38.1	40.0	38.7	49.7	41.7	34.7	39.0	30.5	40.0	36.0	1.4	0.2198
AURL	33.7	32.9	39.4	37.9	36.0	33.2	34.1	27.7	34.9	32.5	1.5	0.1753
ALLL	42.3	42.0	44.0	44.8	43.3	37.8	35.1	29.7	36.8	34.8	2.4	0.0288
ARLL	39.8	42.5	42.3	47.0	42.9	36.5	36.8	30.6	37.3	35.3	1.9	0.0918
AH	13.1	14.0	15.8	16.8	14.9	10.0	18.1	11.3	10.4	12.4	0.8	0.6301
AE	14.4	15.5	17.7	17.8	16.4	13.9	14.7	12.3	13.1	13.5	2.7	0.0185
(mm)												
CULL	27.7	27.2	28.8	28.9	28.2	25.6	25.7	23.7	26.3	25.3	2.6	0.0212
CML	28.7	29.3	29.4	31.7	29.8	28.6	28.7	25.3	28.7	27.8	1.8	0.1014
CURL	27.5	26.4	28.6	27.8	27.5	26.4	26.3	24.1	26.7	25.9	1.8	0.0974
CLLL	31.3	32.0	32.9	33.3	32.3	29.6	28.2	26.1	29.4	28.3	3.7	0.0024
CRL	29.9	31.9	32.3	33.3	31.7	29.1	28.8	26.6	28.9	28.4	2.2	0.044
CH	14.1	15.1	16.0	16.4	15.4	12.8	13.5	13.9	13.0	13.3	3.7	0.0023
CE	16.4	17.0	18.7	18.3	17.6	16.2	16.2	15.3	16.0	15.9	2.9	0.0108
LLi	7.7	8.0	8.3	8.5	8.1	6.9	7.5	6.9	7.1	7.1	3.2	0.0063
LULL	10.4	10.2	10.6	10.6	10.5	8.5	9.4	9.3	9.4	9.2	1.3	0.2808
LML	5.2	5.0	5.4	5.6	5.3	4.7	5.6	5.1	5.1	5.1	0.6	0.7426
LURL	11.1	11.0	11.4	11.7	11.3	9.7	11.0	11.0	11.3	10.7	1.4	0.2208

LLLL	4.9	5.2	4.8	6.0	5.2	4.5	5.0	4.9	4.8	4.8	0.6	0.7848
LRLL	10.4	9.8	10.2	10.3	10.2	8.6	9.8	9.4	9.9	9.5	0.9	0.5069
WULL	5.5	5.0	5.6	5.5	5.4	4.8	5.4	5.5	5.1	5.2	0.8	0.5561
WML	11.9	12.0	12.3	12.2	12.1	9.4	11.0	11.0	10.4	10.5	2.5	0.0244
WURLL	5.5	5.3	5.3	5.5	5.4	4.7	4.8	5.0	4.8	4.8	1.2	0.3090
WLLL	11.4	11.9	11.6	12.6	11.9	9.8	11.0	10.5	10.9	10.6	1.5	0.1840
WRLL	5.1	5.3	5.3	5.7	5.3	4.7	4.9	5.2	4.8	4.9	0.6	0.7912

The correlation analysis of the metric features of the flowers in both types of habitats pointed to a strong correlation ($r > 0.90$) between the surface and the perimeter of perianth leaves (Supplementary Table 1, 2). The flowers from the studied anthropogenic habitats demonstrated a very strong correlation between the labellum length and the surface perimeter of hypochile and epichile. However, the correlation between the same features in the flowers from the studied natural habitats varied from moderate to strong. The correlations between the epichile and hypochile features for the flowers of the studied anthropogenic habitats were strong or very strong, while for the flowers in the analysed natural habitats weak to moderate. A multiple regression analysis revealed a strong correlation between the perimeter of epichile and the labellum length ($r^2 = 0.73$) in the anthropogenic populations. In the flowers from the studied natural habitats, the correlations between the length of labellum and epichile area, the upper left and upper right and lower left leaf, as well as the perimeter of left and right top leaves were found.

A similarity dendrogram revealed two separated groups (Supplementary Fig. 1), where one group contains the populations from the studied anthropogenic habitats (A1, A2, A3, A4), while the other branches represent the populations from the analysed natural habitats (N1, N2, N3, N4).

Genome size estimation. The mean genome size estimated for the studied anthropogenic and natural populations of *E. helleborine* was 27.71 and 27.48 pg/2C DNA, which corresponds to 27100 and 26878 Mbp, respectively. The conducted statistical analysis indicated differences in the genome size among the populations. The DNA content values of

the analysed accessions from natural habitats ranged from 27.32 (N2 and N3) to 27.89 pg/2C for the N4 population. The anthropogenic populations resulted in the DNA content range from 27.49 (A4) to 28.39 pg/2C (A3). The obtained histograms were good quality with mean CVs under 5% for the target species (Table 5, Fig. 3).

Table 5. Biometric characteristic of trains of *E. helleborine* flower in analyzed populations. (\bar{x} – arithmetic mean, F – value of F test, P – significance level). Characters abbreviation as in Table 1.

Population	2C DNA/ pg	
A1	27.57	c*
A2	27.49	c
A3	28.39	a
A4	27.39	c
Mean	27.71	
N1	27.42	c
N2	27.32	c
N3	27.32	c
N4	27.89	b
Mean	27.49	

*values followed by the different letter are significant different at $p < 0.5$ (Duncan's test)

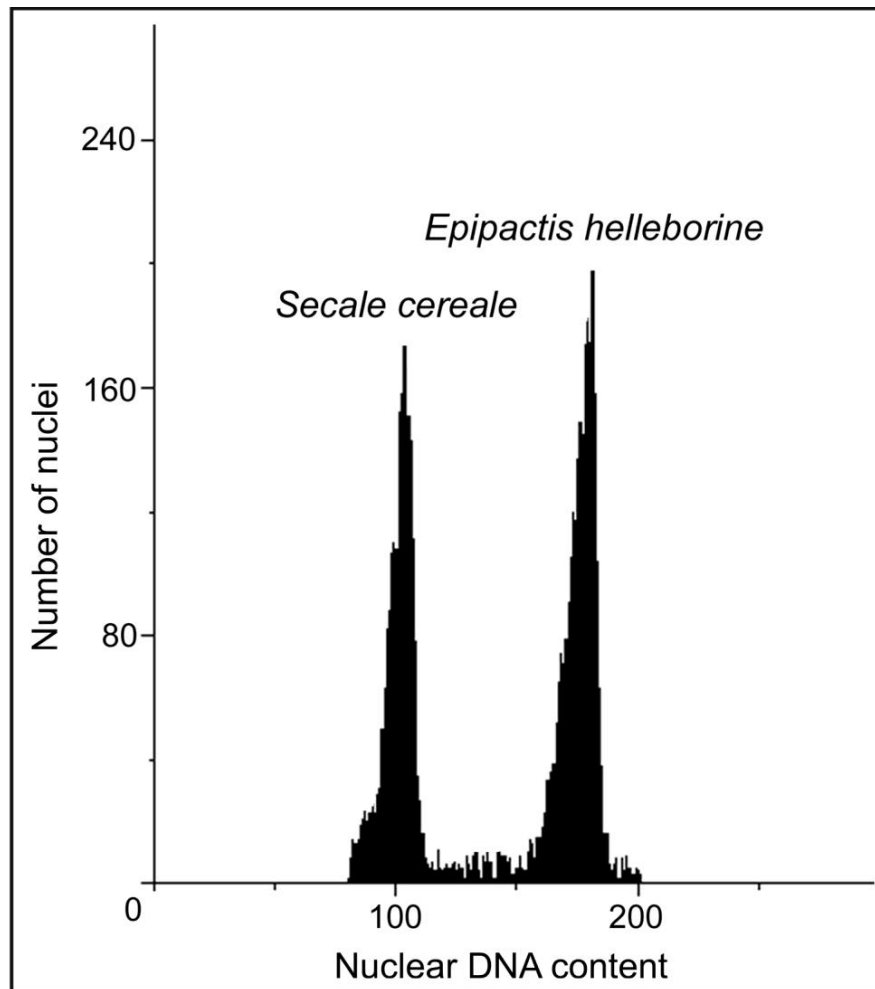


Fig. 3. Histograms of nuclear DNA contents of *E. helleborine*.

Discussion

The height of shoots. Phenotypic plasticity of the species is an expression of its potential abilities to colonise areas which differ in terms of many habitat features (Sultan, 1995; Sultan, 2000; Sultan, 2001). A response of a plant to environmental conditions can manifest itself in its morphological variability, physiological responses or reproductive potential.

Species from the genus *Epipactis* belong to a group with highly variable phenotype features. Flower and seed features, as well as the arrangement of leaves, are the elements least susceptible to environmental changes (Heywood, 1974), and therefore are used in taxonomy.

308 In contrast, the features most vulnerable to environmental changes are the height of shoots,
 309 the leaf size, the colour and size of flower and the length of flowering period (Ehlers et al.,
 310 2002; Sultan, 1995; Stace, 1993; Jakubska-Busse, 2008). The shoot length, the length of
 311 inflorescence and the leaf size have proven to be the most variable characteristics, and
 312 therefore the results support the view that such features are the most susceptible to
 313 environmental changes (Heywood, 1974; Heslop-Harrison, 1953). The literature data on the
 314 length of the *E. helleborine* shoot earlier reported indicate it was within the range of 18.0-
 315 100.0 cm (Hegi, 1925; Delforge, 2001; Bernardos, Amich & Crespi, 2003; Harrap & Harrap,
 316 2010). The mean length of generative shoots for the examined anthropogenic populations
 317 ranged from 59.3 to 62.1 cm, while for the populations from the studied natural habitats from
 318 50.6 to 57.5 cm. Overall, the average length of shoots from the populations in the
 319 anthropogenic habitats was higher than in the natural populations. Moreover, studies of other
 320 authors confirm considerable variability of this particular feature (Adamowski, 2006; Bîtea et
 321 al., 2011). The maximum and minimum length of *E. helleborine* shoots in the examined
 322 anthropogenic habitats ranged from 17.0 to 149.0 cm, while in the natural populations from
 323 4.4 to 95.0 cm. Keller & Schlechter found shoots from 30.0 to 125.0 cm long, while
 324 Adamowski (2006) reported 130.0 cm long shoots growing on a poplar plantation. Also,
 325 Solarz (1994) found 120.0 cm long shoots on a narrow-gauge railway embankment and 103.0
 326 cm long shoots in the population growing in a pine forest. It is believed that light is one of the
 327 most vital environmental stimuli determining phenotypic plasticity (Herman & Sultan, 2011).
 328 The highest shoots with the longest inflorescence and the largest leaves were recorded in the
 329 anthropogenic populations in Guszczewina (A1) and Hajnówka (A2). The shoots in those
 330 populations grew in the full sun, without shade from trees and bushes. The remaining
 331 anthropogenic populations grew in partial shade. Part of the Sulejów 1 (A3) population grew
 332 in the pine forest of *Peucedano-Pinetum*, while the Sulejów 2 (A4) population in the ruderal

poplar thicket of *Populus* sp. *Acer platanoides* and *Robinia pseudoacacia* saplings. In those populations, generative shoots were shorter in comparison to the shoots from the A1 and A2 populations. On the other hand, all the *E. helleborine* shoots from the populations in the studied natural habitats grew under the canopy of trees and the height of their generative shoots ranged from 34.5 (the N3 population) to 66.7 cm (the N1 population).

According to Harper (1986), plants at new sites often achieve considerable sizes in accordance with the strategy of "race to the sun". This study has also confirmed that in the examined anthropogenic habitats the "plant-plant" interaction occurred. Therefore, the shoots in close vicinity are similar in terms of height and shape as the ones observed particularly in the A1 and A2 populations. Adamowski (2006) suggests that the occurrence of high *E. helleborine* ramet is also influenced by the presence of species from the genus *Populus* sp. This is connected with the phenomenon of mycorrhiza occurring between fungus poplar and *E. helleborine* (McCormick et al., 2004). Our results have not confirmed unequivocally the correlation between *E. helleborine* and poplar since in the A1 and A2 populations with the highest shoots such trees were not present. However, in the A4 population, where *E. helleborine* grew among *Populus* × *canadensis*, the shoots were shorter in comparison to those from the A1 and A2 populations.

The length of *E. helleborine* inflorescence and the leaf size differ significantly both between the populations and between the habitats. The previous data indicated the inflorescence length from 8.0 to 57.0 cm (Delforge, 2001; Bîtea et al., 2011).

In the case of leaf size in both habitats, the leaf length ranged from 1.7 cm to 18.0 cm and the leaf width from 1.5 to 13.0 cm. The results obtained in this study differ significantly from values found in the literature, where *E. helleborine* leaf length ranged from 4.0 to 13.0 cm, while the leaf width from 2.0 to 7.0 cm (Delforge, 2001; Bîtea et al., 2011). High

variability of leaf size has confirmed the findings of other authors (Navas & Garniere, 2002; Guo et al., 2007; Jakubska-Busse et al., 2016) that the leaf size is affected by environmental stresses to which plants react by changing the size of their leaves. Populations growing in anthropogenic habitats certainly are subjected to constant and rapid environmental changes. The variability of leaf width and length dependent on environmental conditions, particularly their correlation with light, is confirmed by studies carried out by Xu et al., (2008) on *Quercus acutissima* and Pandey & Nagar (2002) on the phenotypic variability of *Valerian jatansi*.

Perianth features. Flowers from the studied anthropogenic habitats were also higher than those from the natural habitats. Therefore, the conclusion might be drawn that the labellum is a part of the perianth which is not "sufficiently" resistant to environmental change, as shown by statistically insignificant differences in the elements recorded in the analysed habitats. This is also confirmed by the results of Ehlers et al., (2002) who revealed high variability of the epichile length, as well as the length and width of perianth petals (Hegi, 1925; Delforge, 2001). Also, there is no data in the literature concerning the surface and the perimeter of the *E. helleborine* perianth.

The differences which were revealed in morphology of *E. helleborine* flowers as well as the data concerning leaf morphology have enhanced and complemented the number of features influencing the phenotypic plasticity of the taxon. These differences do not support Falińska's (1974) opinion that modifications of morphological characteristics of the shoot-ground, as a sign of adaptation to the particular environmental parameters where the plant exists are usually not revealed in the structure of its generative organs.

Despite the indicating high phenotypic plasticity of the species, the plants growing on roadsides in the examined anthropogenic habitats were not polyploids. This is confirmed by

studies carried out by other researchers, for instance, Bernardos et al. (2003), who revealed that *E. helleborine* growing in different habitats were diploid.

A broad range of morphological variability (manifested by particularly splendid ramets in the absence of polyploid specimens) of the examined specimens observed in all the studied populations may be the result of implementing the epistasis model (Scheiner, 1993). It assumes that plasticity is evoked by genes determining the amount of phenotypic response to the impact of environment. In our opinion, in the case of *E. helleborine* and the anthropogenic habitats occupied by it (e.g.: roadsides), the appearance of polyploid specimens might be a matter of time. This is influenced by dynamic changes of habitat conditions, such as shading water conditions or the composition of accompanying specimens (Doust & Doust, 1988).

Despite demonstrating differences in the genome size within the studied populations, we cannot point to genetic differences between the populations. In our opinion, the obtained results may form a basis for a new study distinguishing *E. helleborine* ecofens, which is particularly well-grounded in the case of very large *E. helleborine* specimens occupying anthropogenic habitats. Additionally, the genome size of *E. helleborine* enriched the DNA C-value database with new data concerning orchids. This is the first report of estimating the genome size of *E. helleborine* and the first report for *Epipactis* genus. According to Soltis et al., (2003), *E. helleborine* can be classified in the group of plants with intermediate genomes (<14 pg/1C; mean for all populations).

According to some researchers, the presence of phenotypic plasticity and the occurrence of ecotypes are adaptation strategies of plants to environmental changes (Stace, 1993). In our opinion, in the case of the studied anthropogenic habitats (roadside) in which the *E. helleborine* populations grew, we can talk about ecofen due to the often repeated set of characteristic features. i.e.: high shoots, long inflorescence and long, broad leaves. We agree,

however, that it is difficult to isolate a taxonomic unit for ecofen due to the lack of experimental research.

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