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**Is habitat important? Morphology and genome size of *Epipactis helleborine* (L.) Crantz
(Orchidaceae) 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* (L.) Crantz 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, Northeast Poland, through Central Poland, to the Lower Silesia Province, Southwest 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 programme (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 plants growing in natural and anthropogenic habitats. **Discussion.** According to some researchers, the presence of phenotypic variability 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,

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

53 **Introduction**

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

71 Observations of the authors as well as data from the literature suggest that in habitats
72 changed by humans *E. helleborine* populations are characterised by high morphological
73 variability of ramets. Moreover, higher and more massive shoots compared to the populations
74 in natural habitats are observed in these populations, which can suggest differences in their
75 genome size (Rogalska et al., 2005; Stefaniak et al., 2011; Adamowski, Stefaniak &

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

84 The family Orchidaceae is characterised by high levels of phenotypic plasticity.
85 Heywood (1974) claims that the phenotype modification is a response of the genotype to the
86 surrounding environment, where changes frequently occur on the genetic level, including
87 changes in the genome size (Rogalska, Małuszyńska & Olszewska, 2005). Considering the
88 genome size of the Orchidaceae, high variation is observed, with the genome size ranging
89 168-fold, from 0.66 to 110.8 pg/2C (Leitch et al., 1974). There is no doubt that the huge range
90 of variation in DNA content has a significant effect on their phenotype. Therefore, the
91 determination of C-value is an important feature for biology and biodiversity of the
92 Orchidaceae (Bennet, Bhandol & Leitch, 2000). Earlier studies revealed a relationship
93 between the genome size and latitude, altitude at sea level, temperature or precipitation, but
94 there is no consensus as to whether the correlation is negative or positive (Knight & Ackerly,
95 2002; Bogunic et al., 2007). Vinogradov & Selfish (2003) have indicated that the species with
96 larger genomes possess less adaptability to adverse environmental conditions, and at the same
97 time, the risk of their extinction is much higher than that of the species with small genomes.
98 Within the orchid family polyploidy was also detected (Jacquemyn et al., 2016). Polyploids
99 are characterised by a large size and vigour of cells, leaves, flowers, and fruits compared to
100 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).

The 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

Studied species

The genus *Epipactis* includes 50-80 species (Kreutz & Fateryga, 2012, Jakubska-Busse et al., 2017) and systematics of this genus is complicated mainly due to similar morphology. Jakubska-Busse (2008), Jakubska-Busse et al., (2016) have 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 transition between cross- and self-pollination (Tałałaj & Brzosko, 2008). This genus has very asymmetric and very complex karyotypes, which 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, mixed 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 in 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, Northeast Poland, through Central Poland, to the Province of Lower Silesia, Southwest Poland (Fig. 1). The identified investigated habitats were separated into two categories: the populations found in anthropogenic habitats such as roadsides and in natural habitats such as mixed forests (Tab. 1). Experimental studies and material sampling were done with the consent of the Regional Director for Environmental Protection (permit WPN6400.74.2013.MW).

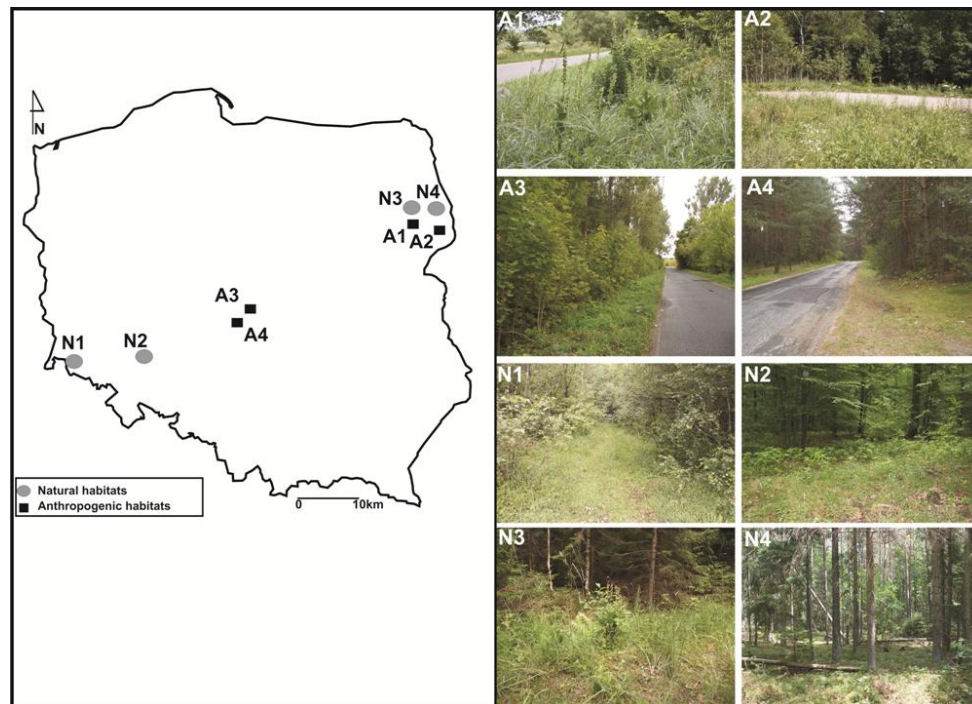


Figure 1. Distribution of studied populations of *Epipactis helleborine* (left). The studied species in their native habitats (right); Abbreviations according to Table 1. Figure 1 was generated by CorelDraw X3 software.

Table 1. List of localities of the studied populations of *Epipactis helleborine*.

Population code	Locality (habitat type)	Population size (m ²)	Number of shoots	GPS coordinates
A1	roadside (Guszczerwina)	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.

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 natural size of flowers for further research. The flowers were characterised by 25 biometric features (Table. S1, Fig. 2 B, C) and measured using a Nikon SMZ 800 binocular, microscopic Moticam-1SP cameras and the MIPlus07 programme (Conbest Co.).

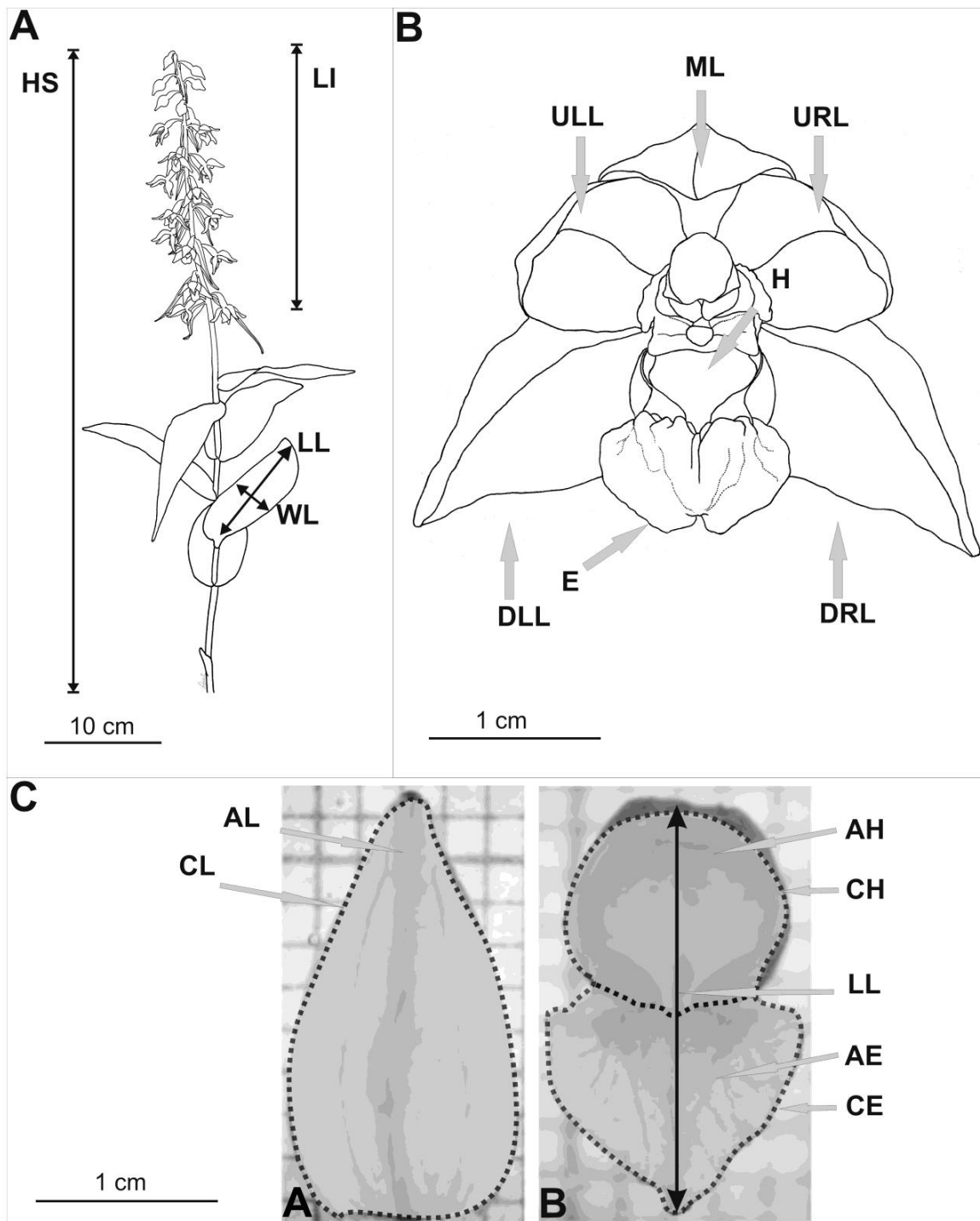


Figure 2. Illustration of locations of features measured in *E. helleborine*: A) Ramet (drawn by Z. Łobas), B) Flower (drawn by Z. Łobas), C) Measurement pattern (Abbreviations are listed in Supplementary Table S1). Figure 2 was generated by CorelDraw X3 software.

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 Jędrzejczyk & Śliwińska (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 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 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 test to determine possible differences in the nuclear DNA content among all the analysed populations of *E. helleborine*.

Results

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

248 **Table 2.** Habitat features of *E. helleborine*. Abbreviations as in Table S1.

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 (1.7-7.9)	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	

249

250

251 **Morphological variability of flowers.** The mean values of the measured elements of
 252 the analysed flowers indicated that the flowers from the studied anthropogenic habitats were
 253 bigger than the flowers from the natural habitats (t-Student's test, $P < 0.05$; Table 3).

254

Table 3. Variation of morphological features of *E. helleborine* shoots in the analysed populations. Homogeneous letters indicate homogeneous groups (Kruskal-Wallis Test $P=0.05$). Characters abbreviated as in Table S1.

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, CRLL, CH, CE, LLi; Table 3). The biggest differences were observed in the surface area of their perianth leaves. The plants in the studied 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 mm² and 42.9 mm² in the analysed anthropogenic habitats, while in the natural habitats

– 34.6 and 35.3 mm² 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 the analysed habitats. Abbreviations as in Table S1 and Table 1. (* p<0.05 – significance level, ns – non-significant, CV – coefficient of variation (%).

Feature (mm ²)	A1	A2	A3	A4	x	N1	N2	N3	N4	x	F	pP
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
CRLL	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. The conducted multiple regression analysis revealed a strong correlation between the perimeter of epichile and the labellum length ($r^2 = 0.73$) in the studied anthropogenic populations. In the flowers from the studied natural habitats, the correlations between the length of labellum and epichile area, the upper left, 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 of good quality with mean CVs under 5% for the target species (Table 5, Fig. 3).

Table 5. Biometric characteristics of trains of *E. helleborine* flower in the analysed populations. ($\bar{x}$ – arithmetic mean, F – value of F test, P – significance level). Abbreviations as in Table 1.

	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 a different letter are significantly different at $P < 0.5$ (Duncan's test)

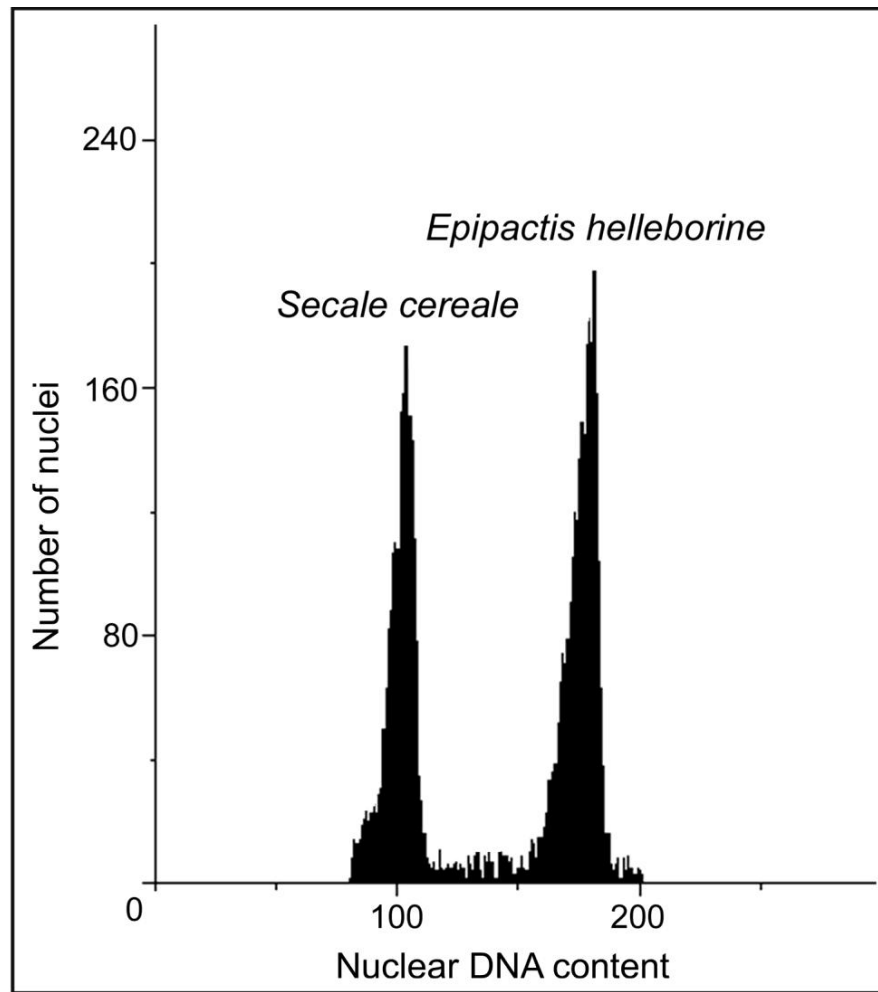


Figure 3. Histograms of nuclear DNA contents of *E. helleborine*.

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 (Rewicz et al., 2017). 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. In contrast, the features most vulnerable to environmental changes are the height of shoots, the leaf size, the colour and size of flower and the length of flowering period (Ehlers et al., 2002; Sultan, 1995; Stace, 1993; Jakubska-Busse, 2008). The shoot length, the length of inflorescence and the leaf size have proven to be the most variable characteristics, and therefore the results support the view that such features are the most susceptible to environmental changes (Heywood, 1974; Heslop-Harrison, 1953). The literature data on the length of the *E. helleborine* shoot earlier reported indicate it was within the range of 18.0-100.0 cm (Hegi, 1925; Delforge, 2001; Bernardos, Amich & Crespi, 2003; Harrap & Harrap, 2010). The mean length of generative shoots for the examined anthropogenic populations ranged from 59.3 to 62.1 cm, while for the populations from the studied natural habitats from 50.6 to 57.5 cm. Overall, the average length of shoots from the populations in the analysed anthropogenic habitats was higher than in the natural populations. Moreover, studies of other authors confirm considerable variability of this particular feature (Adamowski, 2006; Bîtea et al., 2011). The maximum and minimum length of *E. helleborine* shoots in the examined anthropogenic habitats ranged from 17.0 to 149.0 cm, while in the natural populations from 4.4 to 95.0 cm. Keller & Schlechter found shoots from 30.0 to 125.0 cm long, while Adamowski (2006) reported 130.0 cm long shoots growing on a poplar plantation. Also, Solarz (1994) found 120.0 cm long shoots on a narrow-gauge railway embankment and 103.0 cm long shoots in the population growing in a pine forest. It is believed that light is one of the most vital environmental stimuli determining phenotypic plasticity (Herman & Sultan, 2011). The highest shoots with the longest inflorescence and the largest leaves were recorded in the anthropogenic populations in Guszczewina (A1) and Hajnówka (A2). The shoots in those populations grew in the full sun, without shade from trees and bushes. The remaining anthropogenic populations grew in partial shade. Part of the Sulejów 1 (A3) population grew 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 to 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 are certainly 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* Carruth. and Pandey & Nagar (2002) on the phenotypic variability of *Valeriana jatamansi* Jones ex Roxb.

Perianth features. Flowers from the studied anthropogenic habitats were also higher than those from the analysed natural habitats. Therefore, the conclusion might be drawn that the labellum is a part of the perianth which is not "sufficiently" resistant to environmental changes, 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 are 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* flower 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 indicated 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 a 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 on 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 into 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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