1 **Is habitat important? Morphology and genome size of *Epipactis helleborine* (L.) Crantz**

2 **(Orchidaceae) growing in anthropogenic and natural habitats**

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26   Abstract: **Background.** The process of apophytism or spreading native species to human-

27   made habitats is one of the main elements in the creation of plant cover in anthropogenic

28   areas. Lately, an increase of anthropogenic localities with valuable flora (rare and law

29   protected species) has been observed. Apophytes are also members of the family Orchidaceae,

30   especially from the genus *Epipactis*. The aim of the study was to i) determine and compare

31   the phenotypic variation of *E. helleborine* (L.) Crantz plants in anthropogenic and natural

32   habitats, ii) compare the genome size estimation of plants growing in natural and

33   anthropogenic habitats. The results reported in this study may indicate that a habitat

34   influences morphological characteristics of plant species. **Methods.** The research was carried

35   out in Poland from 2011 to 2013. The study sites were located in three different geographical

36   regions: from the Białowieża Primeval Forest, Northeast Poland, through Central Poland, to

37   the Lower Silesia Province, Southwest Poland. We investigated eight populations of *E.*

38   *helleborine*: four from natural habitats and four from anthropogenic habitats. Biometrical

39   analyses were performed on shoots and flowers. The flowers were characterised by 25

40   biometric features and measured using a Nikon SMZ 800 binocular, microscopic Moticam-

41   1SP cameras and the MIPlus07 programme (Conbest Co.). The nuclear DNA content was

42   determined in fresh and young leaves of *E. helleborine*, collected from eight populations.

43   **Results.** We observed that in anthropogenic populations: i) shoots were higher than shoots

44   from natural populations, ii) flowers differed significantly in terms of ten biometric features

45   between habitats, iii) the genome size differed significantly between plants growing in natural

46   and anthropogenic habitats. **Discussion.** According to some researchers, the presence of

47   phenotypic variability and the occurrence of ecotypes are adaptation strategies of plants to

48   environmental changes. In our opinion, in the case of the studied anthropogenic habitats

49   (roadside) in which the *E. helleborine* populations grew, we can talk about ecofen due to the

50   often repeated set of characteristic features, i.e.: high shoots, long inflorescence and long,

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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 at 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 &

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Święczkowska, 2012). The somatic chromosome numbers reported for this species range from 2n=20 for the diploid cytotype (Weijer, 1952) to 2n=60 for the 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) report 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 displays a wide range of phenotypic variability which allows it to more easily adapt to changes in the environment.

The family Orchidaceae is characterised by high levels of 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) have 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 orchid family 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 diploid individuals (Tamayo-Ordóñez et al., 2016). They are also more tolerant to changing

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101   environmental conditions and have more chance for expansion to new areas. This is probably

102   related to an increased degree of heterozygosity, which can be an essential factor for growth,

103   development and adaptability of polyploids (Tamayo-Ordóñez et al., 2016). Since

104   chromosomes of many orchids are small and often numerous, ploidy estimation by

105   chromosome counts is difficult. In addition, microscopic chromosome counting is time-

106   consuming and limited to a few tissues. Therefore, flow cytometry (FCM) is a more

107   convenient alternative for establishing the ploidy/genome size of the Orchidaceae species.

108   The genome size is, next to morphological and anatomical descriptions, a good taxonomic

109   marker useful for identifying many problematic taxa (Wang et al., 2016).

110   The objectives of this study were to: i) determine and compare the phenotypic

111   variation of the *E. helleborine* plants from anthropogenic and natural habitats, ii) compare the

112   genome size estimation of plants growing in natural and anthropogenic habitats.

113   **Materials and methods**

114   **Studied species**

115   The genus *Epipactis* includes 50-80 species (Kreutz & Fateryga, 2012, Jakubska-

116   Busse et al., 2017) and systematics of this genus is complicated mainly due to similar

117   morphology. Jakubska-Busse (2008), Jakubska-Busse et al., (2016) have also observed some

118   morphological adaptations to local environments in this genus. One of such adaptations is the

119   change in floral architecture and the possibility of transition between cross- and self-

120   pollination (Tałałaj & Brzosko, 2008). This genus has very asymmetric and very complex

121   karyotypes, which causes a variation in the number of chromosomes between the *Epipactis*

122   species during the differentiation process. Verlaque, Seidenbinder & Reynaud (1987) suggest

123   that the basic chromosome number is x=10.

124   *Epipactis helleborine* is a clonal taxon, growing in broadleaved, mixed and coniferous

125   (also secondary) forests, on forest edges and also in anthropogenic habitats, such as rural and

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126 urban roadsides, railway embankments, post-mining sites, tracks, quarries, poplar plantations,

127 parks, sandy beaches, lawns (Hollingsworth & Dickson, 1997; Wittig & Wittig, 2007;

128 Akhalkatsi, Arabuli & Lorenz, 2014) and, furthermore, also in cities (Stešević & Jovanović,

129 2008; Milović & Mitić, 2012; Rewicz et al., 2017). This species is rather indifferent in terms

130 of habitat and behaves as a pioneer (Delforge, 2001). It grows on moderately wet, acidic to

131 neutral humus soils and sometimes on substrates rich in calcium carbonate (Robatsch, 1983).

132 **Study sites.** The research was carried out in Poland from 2011 to 2013. The study sites were

133 located in three different geographical regions: from the Białowieża Primeval Forest,

134 Northeast Poland, through Central Poland, to the Province of Lower Silesia, Southwest

135 Poland (Fig. 1). The identified investigated habitats were separated into two categories: the

136 populations found in anthropogenic habitats such as roadsides and in natural habitats such as

137 mixed forests (Tab. 1). Experimental studies and material sampling were done with the

138 consent of the Regional Director for Environmental Protection (permit

139 WPN6400.74.2013.MW).

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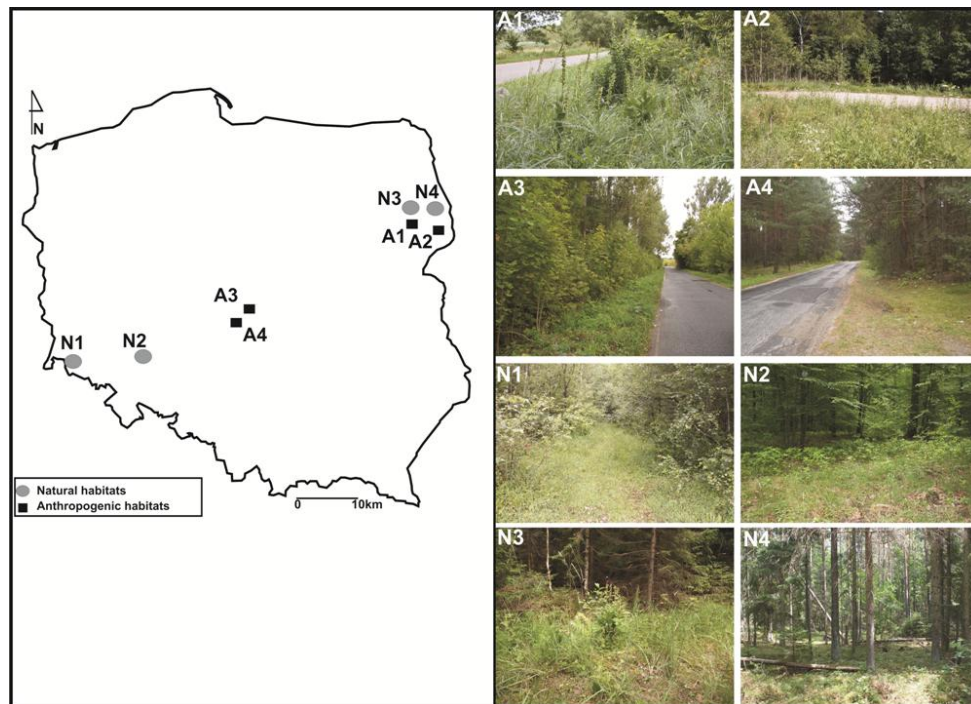
150 **Figure 1.** Distribution of studied populations of *Epipactis helleborine* (left). The studied

151 species in their native habitats (right); Abbreviations according to Table 1. Figure 1 was

152 generated by CorelDraw X3 software.

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

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Population code	Locality (habitat type)	Population size (m^2)	Number of shoots	GPS 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

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155   A1 – Guszczewina, A2 – Hajnówka, A3 – Sulejów 1, A4 – Sulejów 2, N1 – Góry

156   Kaczawskie, N2 – Siechnice, N3 – Białowieża Primeval Forest 1, N4 – Białowieża Primeval

157   Forest 2.

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159   **Biometric analysis.** Biometrical analyses were performed on shoots and flowers of *E.*

160   *helleborine* (Table S1, Fig. 2 I). Live measurements were taken using a measure tape rounded

161   up to the nearest 1 mm. A study on the variability of metric features of *E. helleborine* flowers

162   was carried out in August 2013, taking at random a sample of 15 flowers from each

163   population. The flowers were inserted into the preservative Kew Mixture (composition for 1

164   litre: 530 ml 96% EtOH, 50 ml formaldehyde, 50 ml of glycerol, 370 ml of distilled water),

165   which allowed to maintain the shape and natural size of flowers for further research. The

166   flowers were characterised by 25 biometric features (Table. S1, Fig. 2 B, C) and measured

167   using a Nikon SMZ 800 binocular, microscopic Moticam-1SP cameras and the MIPlus07

168   programme (Conbest Co.).

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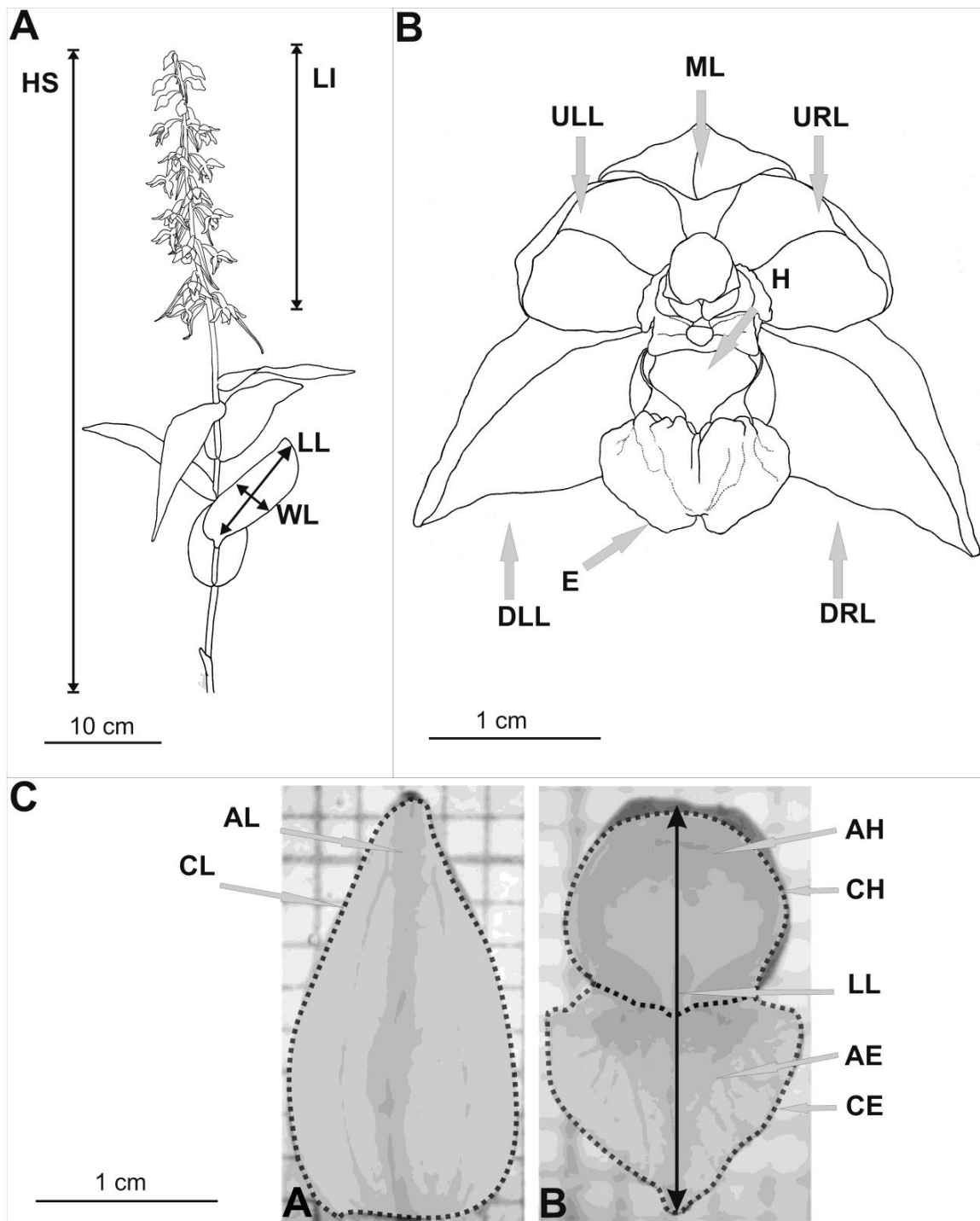
Figure 2. Illustration of locations of features measured in *E. helleborine:* A) Ramet (drawn by
Z. Łobas), B) Flower (drawn by Z. Łobas), C) Measurement pattern (Abbreviations are listed
in Supplementary Table S1). Figure 2 was generated by CorelDraw X3 software.

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174   **Genome size estimation.** The nuclear DNA content was determined in fresh and young

175   leaves of *E. helleborine*, collected from eight populations. The leaves of *Secale cereale*

176   'Dankowskie' (2C=16.2 pg) (Doležel & Bartoš, 2005) were used as an internal standard. The

177   studied samples were prepared according to Jędrzejczyk & Śliwińska (2010). The plant

178   material was chopped with a sharp razor blade in a plastic Petri dish containing 1 ml of

179   nucleus-isolation buffer (0.1 M Tris, 2.5 mM $MgCl_2 \times 6H_2O$, 85 mM NaCl, 0.1% (v/v) Triton

180   X-100; pH 7.0) supplemented with propidium iodide (PI, 50 μg/mL) and ribonuclease A (50

181   μg/mL). Nuclei suspension was passed through a 50 μm mesh nylon filter. For each sample,

182   measurements of fluorescence intensities were performed in at least 7000 nuclei using a

183   CyFlow SL Green (Partec GmbH, Münster, Germany) flow cytometer equipped with a laser

184   with green light emissions at 532 nm. The analyses were replicated five times for each

185   population. Histograms were analysed using FloMax software (Partec GmbH, Münster,

186   Germany). The nuclear genome size of *E. helleborine* was calculated using the linear

187   relationship between the ratio of the target species and *S. cereale* 2C peak positions on the

188   histogram of fluorescence intensities. The mean coefficients of variation (CV) of the 2C

189   nuclei were estimated for all the samples of *E. helleborine*. The 2C genome sizes were

190   obtained after the conversions of values in picograms into base-pair numbers using the factor

191   1 pg = 978 Mbp (Doležel & Bartoš, 2005).

192   **Statistical analysis.** Biometric data were statistically analysed using STATISTICA ver. 10.0

193   and Canoco ver. 4.5. The following basic characteristics were calculated: the arithmetic mean

194   (x), minimum and maximum value, standard deviation (SD) and coefficient of variation (CV).

195   The compatibility of the studied morphological features with the standard distribution was

196   checked by means of the Shapiro-Wilk and Kolmogorov-Smirnov tests. For samples

197   accordant with the standard spatial distribution, the ANOVA test was applied (for many

198   groups) and Student's test (for two groups). In the majority of cases in which the data did not

199   show compliance with the standard distribution, the non-parametric Kruskal-Wallis test was

200   used. A multiple comparison of average ranks for all the samples or the Duncan test were

201   applied as a *post hoc* test. To compare two independent groups, the U'Mann-Whitney test was

202   used, while for the two dependent groups the Wilcoxon test was used. Differences amounting

203   to P<0.05 were considered statistically significant.

204   The correlation between variables was tested by means of Spearman's correlation

205   coefficient and multiple regression (van Emden, 2008). In order to demonstrate statistical

206   differences between the genome size for the examined populations, the one-way analysis of

207   variance ANOVA and the *post-hoc* Duncan test were carried out.

208   Statistical analyses were performed using the single factor analysis of variance and the

209   Duncan test to determine possible differences in the nuclear DNA content among all the

210   analysed populations of *E. helleborine*.

211   **Results**

212   **Morphological variability of shoots.** The height of *E. helleborine* shoots ranged from 17.0 to

213   149.0 cm for the anthropogenic populations and from 4.4 to 95.0 cm for the natural

214   populations. The highest shoot length was 149.0 cm, recorded in the A1 (Guszczewina)

215   population, and the shortest was 4.4 cm, recorded in the N1 population (Góry Kaczawskie;

216   Table 2). In the case of the anthropogenic populations, *E. helleborine* shoots were longer than

217   shoots from the natural populations. In contrast, the mean values of the remaining parameters

218   (i.e. inflorescence length, leaf width and length) were higher in the populations from natural

219   habitats (Table 2). Intra-population variability was demonstrated in terms of all *E. helleborine*

220   features; however, the arrangement of the homogeneous populations investigated during the

221   first period of observation was not repeated in the next period. The greatest intra-population

222   diversity was indicated for the length of shoots, where four homogeneous groups were

223   observed (Table 2). The length of shoots (LS) in the anthropogenic populations demonstrated

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224   insignificant variation, ranging from 21.0 to 39.7%, while for the natural populations it ranged

225   from 21.4 to 51.9% (Table 2). The length of inflorescence (LI) demonstrated the highest

226   variation both for the anthropogenic and natural populations. For intra-habitat variation,

227   statistically significant differences in the two investigated periods were demonstrated for the

228   length of shoots, inflorescence and leaves. The longest vegetative shoots were found in two

229   anthropogenic populations in the A2 (Hajnówka – 31.0 cm) and the A3 (Guszczewina – 26.7

230   cm) populations in 2012, while the shortest in the N3 and N4 populations (Białowieża

231   Primeval Forest). The populations A1 and A2 differ significantly from the others in terms of

232   vegetative shoots.

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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 (1.77.9)	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**		

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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).

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255 **Table 3.** Variation of morphological features of *E. helleborine* shoots in the analysed

256 populations. Homogeneous letters indicate homogeneous groups (Kruskal-Wallis Test

257 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

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259 The analysed flowers differed significantly (t-test, P <0.05) in terms of ten biometric

260 features (ALLL, ARLL, AE, CULL, CURL, CLLL, CRLL, CH, CE, LLi; Table 3). The

261 biggest differences were observed in the surface area of their perianth leaves. The plants in

262 the studied anthropogenic habitats had evolved flowers in which the surface of the upper left

263 lobe and the right lower lobe was greater than for the flowers from the studied natural habitats

264 (43.3 mm^2 and 42.9 mm^2 in the analysed anthropogenic habitats, while in the natural habitats

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265 – 34.6 and 35.3 mm^2 respectively). The coefficient of variation was higher for the parts of

266 flowers in the analysed anthropogenic habitats (Table 4). The highest value of the coefficient

267 of variation for both habitats was connected with the area of the measured elements, while the

268 smallest value with the perianth perimeter, as well as the perimeter and the length of labellum.

269 **Table 4**. Biometric characteristics of *E. helleborine* flower in the analysed habitats.

270 Abbreviations as in Table S1 and Table 1. (* p<0.05 – significance level, ns – non-significant,

271 CV – coefficient of variation (%).

Feature	A1	A2	A3	A4	x	N1	N2	N3	N4	x	F	pP
(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
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

272

273 The correlation analysis of the metric features of the flowers in both types of habitats

274 pointed to a strong correlation (r>0.90) between the surface and the perimeter of perianth

275 leaves (Supplementary Table 1, 2). The flowers from the studied anthropogenic habitats

15

276   demonstrated a very strong correlation between the labellum length and the surface perimeter

277   of hypochile and epichile. However, the correlation between the same features in the flowers

278   from the studied natural habitats varied from moderate to strong. The correlations between the

279   epichile and hypochile features for the flowers of the studied anthropogenic habitats were

280   strong or very strong, while for the flowers in the analysed natural habitats weak to moderate.

281   The conducted multiple regression analysis revealed a strong correlation between the

282   perimeter of epichile and the labellum length ($r^2 = 0.73$) in the studied anthropogenic

283   populations. In the flowers from the studied natural habitats, the correlations between the

284   length of labellum and epichile area, the upper left, upper right and lower left leaf as well as

285   the perimeter of left and right top leaves were found.

286   A similarity dendrogram revealed two separated groups (Supplementary Fig. 1), where

287   one group contains the populations from the studied anthropogenic habitats (A1, A2, A3, A4),

288   while the other branches represent the populations from the analysed natural habitats (N1, N2,

289   N3, N4).

290   **Genome size estimation.** The mean genome size estimated for the studied

291   anthropogenic and natural populations of *E. helleborine* was 27.71 and 27.48 pg/2C DNA,

292   which corresponds to 27100 and 26878 Mbp, respectively. The conducted statistical analysis

293   indicated differences in the genome size among the populations. The DNA content values of

294   the analysed accessions from natural habitats ranged from 27.32 (N2 and N3) to 27.89 pg/2C

295   for the N4 population. The anthropogenic populations resulted in the DNA content range from

296   27.49 (A4) to 28.39 pg/2C (A3). The obtained histograms were of good quality with mean

297   CVs under 5% for the target species (Table 5, Fig. 3).

298

16

299   **Table 5.** Biometric characteristics of trains of *E. helleborine* flower in the analysed

300   populations. (*x* – arithmetic mean, *F* – value of *F* test, P– significance level). Abbreviations as

301   in Table 1.

302

	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**	

312   *values followed by a different letter are significantly different at P<0.5 (Duncan's test)

313

314

315
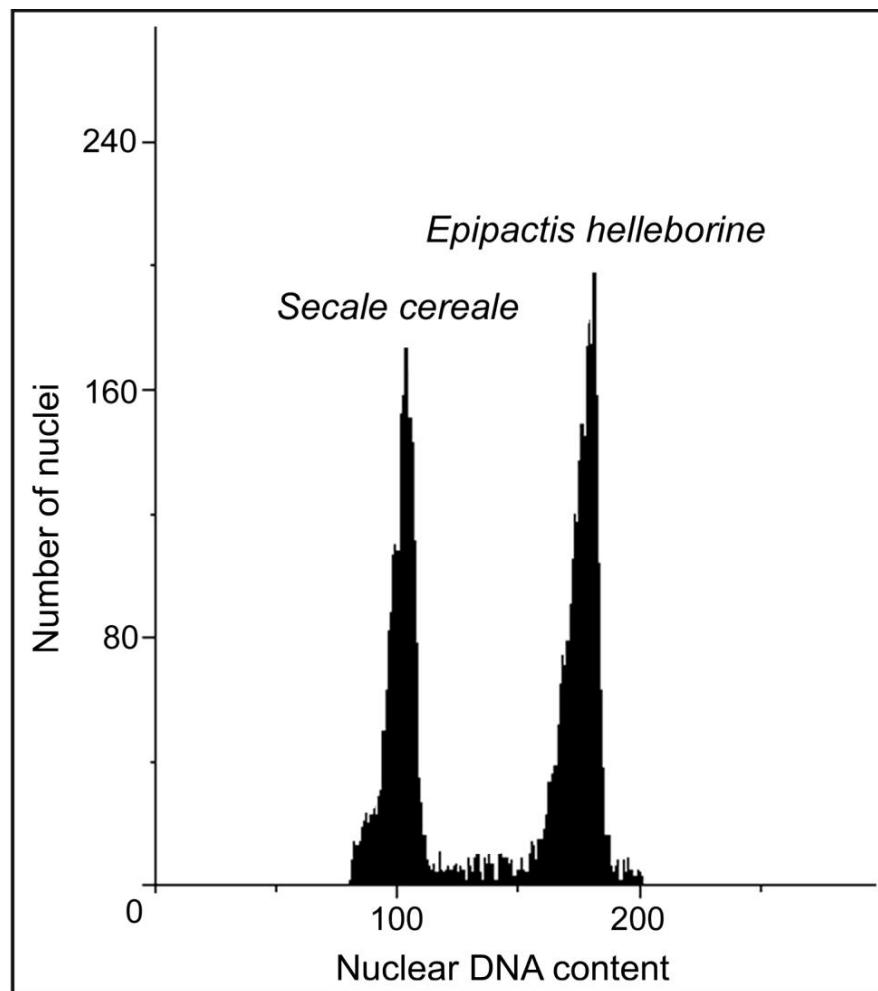
316   **Figure 3.** Histograms of nuclear DNA contents of *E. helleborine*.

317

318   **Discussion**

319   **The height of shoots.** Phenotypic plasticity of the species is an expression of its potential

320   abilities to colonise areas which differ in terms of many habitat features (Sultan, 1995; Sultan,

321   2000; Sultan, 2001). A response of a plant to environmental conditions can manifest itself in

322   its morphological variability, physiological responses or reproductive potential.

323   Species from the genus *Epipactis* belong to a group with highly variable phenotype

324   features (Rewicz et al., 2017). Flower and seed features, as well as the arrangement of leaves,

325   are the elements least susceptible to environmental changes (Heywood, 1974), and therefore

18

326   are used in taxonomy. In contrast, the features most vulnerable to environmental changes are

327   the height of shoots, the leaf size, the colour and size of flower and the length of flowering

328   period (Ehlers et al., 2002; Sultan, 1995; Stace, 1993; Jakubska-Busse, 2008). The shoot

329   length, the length of inflorescence and the leaf size have proven to be the most variable

330   characteristics, and therefore the results support the view that such features are the most

331   susceptible to environmental changes (Heywood, 1974; Heslop-Harrison, 1953). The

332   literature data on the length of the *E. helleborine* shoot earlier reported indicate it was within

333   the range of 18.0-100.0 cm (Hegi, 1925; Delforge, 2001; Bernardos, Amich & Crespi, 2003;

334   Harrap & Harrap, 2010). The mean length of generative shoots for the examined

335   anthropogenic populations ranged from 59.3 to 62.1 cm, while for the populations from the

336   studied natural habitats from 50.6 to 57.5 cm. Overall, the average length of shoots from the

337   populations in the analysed anthropogenic habitats was higher than in the natural populations.

338   Moreover, studies of other authors confirm considerable variability of this particular feature

339   (Adamowski, 2006; Bîtea et al., 2011). The maximum and minimum length of *E. helleborine*

340   shoots in the examined anthropogenic habitats ranged from 17.0 to 149.0 cm, while in the

341   natural populations from 4.4 to 95.0 cm. Keller & Schlechter found shoots from 30.0 to 125.0

342   cm long, while Adamowski (2006) reported 130.0 cm long shoots growing on a poplar

343   plantation. Also, Solarz (1994) found 120.0 cm long shoots on a narrow-gauge railway

344   embankment and 103.0 cm long shoots in the population growing in a pine forest. It is

345   believed that light is one of the most vital environmental stimuli determining phenotypic

346   plasticity (Herman & Sultan, 2011). The highest shoots with the longest inflorescence and the

347   largest leaves were recorded in the anthropogenic populations in Guszczewina (A1) and

348   Hajnówka (A2). The shoots in those populations grew in the full sun, without shade from

349   trees and bushes. The remaining anthropogenic populations grew in partial shade. Part of the

350   Sulejów 1 (A3) population grew in the pine forest of *Peucedano-Pinetum*, while the Sulejów

19

351   2 (A4) population in the ruderal poplar thicket of *Populus* sp., *Acer platanoides* and *Robinia*

352   *pseudoacacia* saplings. In those populations, generative shoots were shorter in comparison to

353   the shoots from the A1 and A2 populations. On the other hand, all the *E. helleborine* shoots

354   from the populations in the studied natural habitats grew under the canopy of trees and the

355   height of their generative shoots ranged from 34.5 (the N3 population) to 66.7 cm (the N1

356   population).

357   According to Harper (1986), plants at new sites often achieve considerable sizes in

358   accordance with the strategy of "race to the sun". This study has also confirmed that in the

359   examined anthropogenic habitats the "plant-plant" interaction occurred. Therefore, the shoots

360   in close vicinity are similar in terms of height and shape to the ones observed particularly in

361   the A1 and A2 populations. Adamowski (2006) suggests that the occurrence of high *E.*

362   *helleborine* ramet is also influenced by the presence of species from the genus *Populus* sp.

363   This is connected with the phenomenon of mycorrhiza occurring between fungus poplar and

364   *E. helleborine* (McCormick et al., 2004). Our results have not confirmed unequivocally the

365   correlation between *E. helleborine* and poplar since in the A1 and A2 populations with the

366   highest shoots such trees were not present. However, in the A4 population, where *E.*

367   *helleborine* grew among *Populus ×canadensis*, the shoots were shorter in comparison to those

368   from the A1 and A2 populations.

369   The length of *E. helleborine* inflorescence and the leaf size differ significantly both

370   between the populations and between the habitats. The previous data indicated the

371   inflorescence length from 8.0 to 57.0 cm (Delforge, 2001; Bîtea et al., 2011).

372   In the case of leaf size in both habitats, the leaf length ranged from 1.7 cm to 18.0 cm

373   and the leaf width from 1.5 to 13.0 cm. The results obtained in this study differ significantly

374   from values found in the literature, where *E. helleborine* leaf length ranged from 4.0 to 13.0

375   cm, while the leaf width from 2.0 to 7.0 cm (Delforge, 2001; Bîtea et al., 2011). High

20

376   variability of leaf size has confirmed the findings of other authors (Navas & Garniere, 2002;

377   Guo et al., 2007; Jakubska-Busse et al., 2016) that the leaf size is affected by environmental

378   stresses to which plants react by changing the size of their leaves. Populations growing in

379   anthropogenic habitats are certainly subjected to constant and rapid environmental changes.

380   The variability of leaf width and length dependent on environmental conditions, particularly

381   their correlation with light, is confirmed by studies carried out by Xu et al., (2008) on

382   *Quercus acutissima* Carruth. and Pandey & Nagar (2002) on the phenotypic variability of

383   *Valeriana jatamansi* Jones ex Roxb.

384   **Perianth features**. Flowers from the studied anthropogenic habitats were also higher than

385   those from the analysed natural habitats. Therefore, the conclusion might be drawn that the

386   labellum is a part of the perianth which is not "sufficiently" resistant to environmental

387   changes, as shown by statistically insignificant differences in the elements recorded in the

388   analysed habitats. This is also confirmed by the results of Ehlers et al. (2002) who revealed

389   high variability of the epichile length, as well as the length and width of perianth petals (Hegi,

390   1925; Delforge, 2001). Also, there are no data in the literature concerning the surface and the

391   perimeter of the *E. helleborine* perianth.

392   The differences which were revealed in morphology of *E. helleborine* flower as well

393   as the data concerning leaf morphology have enhanced and complemented the number of

394   features influencing the phenotypic plasticity of the taxon. These differences do not support

395   Falińska's (1974) opinion that modifications of morphological characteristics of the shoot-

396   ground, as a sign of adaptation to the particular environmental parameters where the plant

397   exists, are usually not revealed in the structure of its generative organs.

398   Despite the indicated high phenotypic plasticity of the species, the plants growing on

399   roadsides in the examined anthropogenic habitats were not polyploids. This is confirmed by

21

400 studies carried out by other researchers, for instance, Bernardos et al. (2003), who revealed

401 that *E. helleborine* growing in different habitats were diploid.

402 A broad range of morphological variability (manifested by particularly splendid ramets in the

403 absence of polyploid specimens) of the examined specimens observed in all the studied

404 populations may be a result of implementing the epistasis model (Scheiner, 1993). It assumes

405 that plasticity is evoked by genes determining the amount of phenotypic response to the

406 impact of environment. In our opinion, in the case of *E. helleborine* and the anthropogenic

407 habitats occupied by it (e.g.: roadsides), the appearance of polyploid specimens might be a

408 matter of time. This is influenced by dynamic changes of habitat conditions, such as shading

409 water conditions or the composition of accompanying specimens (Doust & Doust, 1988).

410 Despite demonstrating differences in the genome size within the studied populations,

411 we cannot point to genetic differences between the populations. In our opinion, the obtained

412 results may form a basis for a new study distinguishing *E. helleborine* ecofens, which is

413 particularly well-grounded in the case of very large *E. helleborine* specimens occupying

414 anthropogenic habitats. Additionally, the genome size of *E. helleborine* enriched the DNA C-

415 value database with new data concerning orchids. This is the first report on estimating the

416 genome size of *E. helleborine* and the first report for *Epipactis* genus. According to Soltis et

417 al. (2003), *E. helleborine* can be classified into the group of plants with intermediate genomes

418 (<14 pg/1C; mean for all populations).

419 According to some researchers, the presence of phenotypic plasticity and the

420 occurrence of ecotypes are adaptation strategies of plants to environmental changes (Stace,

421 1993). In our opinion, in the case of the studied anthropogenic habitats (roadside) in which

422 the *E. helleborine* populations grew, we can talk about ecofen due to the often repeated set of

423 characteristic features, i.e.: high shoots, long inflorescence and long, broad leaves. We agree,

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

425   experimental research.

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