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Original Research

Chemical Composition and Morphology of Basal Leaves of *Trollius europaeus* L. and *T. altissimus* Crantz (Ranunculaceae)

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Abstract

We studied characteristics of basal leaves of *Trollius europaeus* L. and *T. altissimus* Crantz, including blade morphology, phenolic acids and flavonoids. We also verified the influence of site conditions on leaf structure. Co-chromatography showed mostly quantitative differences in the contents of particular phenolic acids and flavonoids in leaves. Similarly, there were no important morphological differences between the species and with respect to some traits a significant distinction appeared within *T. altissimus* populations. Additionally, site conditions had an effect on leaf morphology of both examined species. Therefore, based on our results, the separation of two *Trollius* species is questionable. The study supported the statement that *T. europaeus* and *T. altissimus* are not separated species, thus *T. europaeus* should be divided into two lower taxa in the rank of variety or subspecies.

Keywords: *Trollius europaeus*, *T. altissimus*, phenolic compounds, flavonoids, leaf morphology

Introduction

The genus *Trollius* (Ranunculaceae) includes about 30 species connected with temperate and arctic regions of the northern hemisphere [1]. Most of the described species originate from Asia. On the basis of the molecular phylogenetic analysis, Després et al. [2] determined southern China to be the centre of origin of species from the genus *Trollius*. Only three species are known from North America [3]. According to Tutin [4] and Doroszewska [1] two species naturally occur in Europe: *T. europaeus* and *T. asiaticus*. Asiatic globeflower mainly occupies extensive areas of northwestern and central Asia. European globeflower is found in almost all areas of the continent, from the polar zone to the Mediterranean region. In northern and central parts of Europe it usually occurs at low altitudes and its populations are rather large and more or less continuously distributed. Towards the South the populations of the species are less frequent and are irregularly and sparsely distributed in the mountains up to the alpine level.

Due to the wide range and significant variability of the species, *T. europaeus* has been divided into some lower

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taxa in the rank of variety or subspecies. Doroszewska [1] distinguished two varieties of *T. europaeus*: *europaeus* – characteristic through almost the whole range of the species and *transsilvanicus* – known from central Europe and growing mainly in the mountains such as the Carpathians, the Sudeten Mountains, and the Eastern and Dinaric Alps. The cited author took into account the style length and the beak length of follicle (both longer at *T. europaeus* var. *europaeus*), as relatively constant features, closely connected with particular taxa and their geographical ranges. According to Tutin [4] *T. europaeus* should be divided into two subspecies: *europaeus* (being equivalent of Doroszewska's var. *europaeus*) and *transsilvanicus* (being equivalent of Doroszewska's var. *transsilvanicus*, but restricted only to the Carpathians). On the basis of the correlation between some morphological features (mainly length of the style) of globeflower populations and their geographical position, Chrtek and Chrtkova [5] recognized the above-mentioned subspecies to be separate taxa: *T. europaeus* and *T. altissimus*. Recently the same opinion was stated by Piękoś-Mirkowa and Mirek [6], who distinguished these species in the flora of Poland. Their study showed that populations of *T. europaeus* are scattered all over the country, with the exception of higher mountain levels. Thus, the range of *T. altissimus* in Poland includes mountain regions, especially above the forest zone. However, the distribution of both species in the country has not been investigated sufficiently.

According to Doroszewska [1] almost all *Trollius* species have highly variable foliage and generative organs, even within a given population. Additionally, many species of this genus (such as *T. europaeus* and *T. altissimus*) are not markedly separate from each other, and their ranges (at least partly) overlap. Significant morphological differentiation of *T. europaeus* populations from north-western Poland was confirmed by some investigations [7-9]. Similarly, those populations were distinctly differentiated in relation to the content of flavonoids and phenolic acids [9]. High genetic variability of European globeflower in the main regions of its whole distribution (the Alps, Pyrenees and Fennoscandia) also has been revealed [10].

Until now, studies referring to the morphology of *T. altissimus* have been carried out to distinguish this taxon from *T. europaeus* [1, 5]. The length of the style of the pistil as well as the length and shape of the beak of the follicle are regarded as the basic diagnostic features, making it possible to separate the species. Thus the question of possible differences in leaf morphology between these taxa is still unsolved. However, leaves of *T. altissimus* are larger, with narrower and more distinctly dentate segments [5]. There has been no investigation of phytochemical variability of *T. altissimus*.

The aim of this study was to compare phytochemical properties (phenolic acids and flavonoids) and morphological features of leaves of *T. europaeus* and *T. altissimus* and to investigate their utility in the taxonomy of both examined species. Additionally, we wanted to examine the influence of site conditions on leaf morphology of the investigated species.

Materials and Methods

Basal leaves of one population of *T. europaeus* and two populations of *T. altissimus* were investigated in this study. Samples were collected in June of 2003 from natural localities in Poland (Tab. 1). At the same time, some individuals from all populations were transplanted into a common garden experiment. They were planted in the garden of the Agricultural University in Poznań (52°25'N, 16°54'E), ensuring similar site conditions. In June of the next year basal leaves were collected from the cultivated plants.

One batch of leaves from the cultivated plants was used in the phytochemical analyses of content of phenolic acids and flavonoid compounds. The second one, as well as leaves gathered from the natural localities, was used to analyze blade morphology. This made it possible to verify whether site conditions modified the leaf structure of globeflowers.

During extract preparation for chromatography, air-dried and powdered blades (10 g for each sample) were extracted three times with boiling methanol, then with 70% methanol. The extraction time was one hour in each case. The combined extracts were concentrated, treated with hot water and filtered. The filtrate was extracted successively with petroleum ether and diethyl ether. As a result, etheric extracts and the remaining aqueous phases

Table 1. The origin of analyzed populations of *Trollius* species and the number of basal leaves used form morphology measurements.

Species	Origin in Poland	Latitude and longitude	Altitude (m a.s.l.)	Number of basal leaves analyzed in biometry according to the origin of sample			
				Sample	From natural locality	Sample	From cultivation
T. altissimus	Bieszczady Mts – Połonina Wetlińska	49°09'N 22°33'E	1255	b-n	79	b-c	34
T. altissimus	Tatra Mts – Bobrowiec	49°15'N 19°47'E	1665	t-n	18	t-c	30
T. europaeus	Wielkopolska Province –Uścikowo	52°37'N 16°45'E	75	u-n	66	u-c	32

were obtained. Etheric extracts were evaporated to dryness, then dissolved in 2 ml methanol. The water layer was transferred into a volumetric flask and supplemented to 20 ml with water.

In the phenolic acid analyses thin layer chromatography (cellulose plates, DCAlufolien, Merck Art. 5552) was used with the 1st direction: toluene:acetic acid:water (7:8:3) and the 2nd direction: acetic acid:water (15:85). The 10 µl of etheric extracts were applied onto cellulose plates. Ten phenolic acid standards (caffeic, chlorogenic, γ-resorcylic, p-coumaric, synapic, vanillic, p-hydroxybenzoic, ferulic, syringic, p-hydroxyphenylacetic) were also subjected to chromatographic analyses. After running and drying, chromatograms were observed under UV light at λ=254 and 366 nm, treated with a mixture of diazotized sulphanilic acid and 20% aqueous Na_2CO_3 and then observed under daylight.

Paper chromatography (Whatman No. 1) was used in the observation of flavonoid compounds – A: acetic acid:water (15:85) and B: ethyl acetate:formic acid:water (10:2:3 upper layer). The 20 µl of the water layer were analyzed by co-chromatography against flavonoid compounds isolated and identified from *T. europaeus* leaves as: isoorientin, orientin, vitexin, orientin 2”-*O*-xylopyranoside, orientin 2”-*O*-glucopyranoside, vitexin 2”-*O*-arabinopyranoside and genkwanin 4’-*O*-rhamnopyranosido (1→ 2) xylopyranoside [9]. The chromatograms were viewed under UV light at λ=366 nm before and after spraying with 1% methanolic $AlCl_3$ and 1% methanolic solution of Naturstoffreagenz A (diphenylboric acid 2-aminoethyl ester).

In the morphological experiment of the presented study the biometry of basal leaves was measured using the computer program WinFolia™ 2003a,b (Régent Instruments Inc., Quebec, Canada; http://www.regentinstruments.com/). The number of leaves analyzed varied among locations due to the availability of leaves for collection. Sample sizes for each location are shown in Table 1.

Properly developed 5-sected basal leaves were selected. Subsequently, each was divided into particular segments with a scalpel and the following features were measured for each segment:

1. length (cm),
2. blade maximum width measured perpendicular to length (cm),
3. position where maximum blade width perpendicular to length was measured (% of blade length),
4. blade width at 25% and 50% of blade length, perpendicular to length (cm),
5. blade projected area (cm^2),
6. width-to-length ratio.

Then the segments of leaves were dried (65°C, 72 h) to constant weight in a forced circulation drier (ULE 600, Memmert GmbH+Co.KG, Germany). Dry segments of leaves were weighed separately and specific leaf area (SLA, cm^2g^{-1}) for each one was calculated.

The data obtained were analyzed statistically. For each investigated feature, separately for each blade segment, one-factor variance analysis (ANOVA) was applied to show critical differences among the samples. Independent comparisons for the leaves from natural localities (samples b-n, t-n, u-n) and for the leaves from cultivated plants (b-c, t-c, u-c) were made (see Table 1). If critical differences were noted, multiple comparison was carried out based on Tukey’s test for unequal samples.

Table 2. The occurrence of phenolic acids in leaves of investigated samples of *T. altissimus* (b-c from Bieszczady Mts., t-c from Tatra Mts.) and *T. europaeus* (u-c from Uścikowo). The analyzed material was collected from cultivation.

Phenolic acids	Rf values[a]	Rf values[b]	b-c	t-c	u-c
Caffeic	0.04	0.37	+	+	+
Chlorogenic	0.00	0.43	++	+	+
γ-resorcylic	0.00	0.72	+	++	++
p-coumaric	0.22	0.40	++	++	++
Synapic	0.75	0.37	–	–	trace
Vanillic	0.68	0.58	+	++	++
p-hydroxybenzoic	0.22	0.65	+	+	+
Ferulic	0.75	0.32	++	++	++
X	0.98	0.40	++	–	+
Syringic	0.90	0.65	++	++	++
Y	0.55	0.85	++	+++	–
p-hydroxyphenylacetic	0.40	0.83	++	–	+++

Explanations: [a] cellulose plates with acetic acid-water (85:15) as mobile phase; [b] cellulose plates with toluene-acetic acid-water (8:7:3) as mobile phase; spot intensity: + – weak, ++ – medium, +++ – strong, – – not detected

Characteristics of leaves of plants growing in cultivation vs. natural locations were compared for each population (i.e. b-n samples were compared to b-c samples). Ward's hierarchical clustering method was used to compute cluster groups of *Trollius* species based on analyzed leaf features. All statistical analyses were performed using the program Statistica 6.0 (StatSoft Polska, www.statsoft.pl) and JMP 5.5.1.2. (SAS Institute Inc., Cary, NC, USA, www.sas.com).

Results

Phytochemical Analysis

Co-chromatography with standard substances revealed the presence of eight phenolic acids: caffeic, chlorogenic, γ-resorcylic, p-coumaric, vanillic, p-hydroxybenzoic, ferulic and syringic in all fractions of analyzed samples (b-c, t-c and u-c), but in different proportions in individual fractions. Additionally, on chromatograms of samples b-c and u-c the spots corresponding to p-hydroxyphenylacetic acid and spots corresponding to an unidentified phenolic compound X were revealed, whereas in the chromatograms of samples b-c and t-c there was a spot corresponding to a phenolic compound Y different from the spots of the reference standards applied. Synapic acid was found in trace amounts in samples u-c only (Table 2).

Co-chromatography with the spots corresponding to *C*- and *O*-glycosylflavones, previously separated and identified in *T. europaeus*, i.e. isoorientin, orientin, vitexin, orientin 2"-*O*-xylopyranoside, orientin 2"-*O*-glucopyranoside, vitexin 2"- *O*-arabinopyranoside and genkwanin 4'-*O*-rhamnopyranosido (1→2) xylopyranoside proved the presence of the flavonoids in all the samples studied. The presence of the unidentified flavonoid compounds, X, in samples b-c, and Y in samples t-c was not observed. Analysis of spot intensity showed that the studied samples exhibited only quantitative differences in the contents of particular flavonoids (Table 3).

Morphological Analysis

Comparison of Leaves from Natural Localities

Statistically significant differences were noted for most of the investigated features. Only the means of the width/length ratio for 4 blade segments, the means of maximum width position for 3 segments, and width at 25% of blade length for 2 segments were not considerably different (Table 4).

Tukey's test showed a strong similarity between the populations of *T. europaeus* from Uścikowo (u-n) and of *T. altissimus* from the Bieszczady Mts. (b-n) with reference to the blade length and maximum width. At the same time these features markedly separated the population of *T. altissimus* from the Tatra Mts. (t-n).

However, the differences among the means of width at 25% and at 50% of blade length and the position of maximum width were statistically significant (depending on the feature for all or at least for 2-3 blade segments), they did not make it possible to unambiguously classify the investigated samples. The specific leaf area (SLA) of leaves from each locality significantly differed from those of the other localities.

Table 3. The occurrence of flavonoids in leaves of investigated samples of *T. altissimus* (b-c from Bieszczady Mts., t-c from Tatra Mts.) and *T. europaeus* (u-c from Uścikowo). The analyzed material was collected from cultivation.

Flavonoid	Rf values[a]	b-c	t-c	u-c
A	0.05	+	+	+
Orientin	0.11	++	++	++
Vitexin	0.21	+	+	+
Isoorientin	0.34	+	++	+
Genkwanin 4'-*O*-rhamnopyranoside(1→ 2) xylopyranoside	0.40	++	+	++
Z	0.45	++	++	+
Orientin 2"-*O*-glucopyranoside	0.59	++	++	++
Orientin 2"-*O*-xylopyranoside	0.63	+++	+++	+++
Vitexin 2"-*O*-arabinopyranoside	0.69	++	++	++
X	0.75	–	++	++
Y	0.83	+	–	++

Explanations: spot intensity: + – weak ++ – medium +++ – strong – – not detected; [a] PC on Whatman 1 with acetic acid-water (85:15) as mobile phase

Table 4. Mean values (± SE) of investigated leaves' features of the samples of *T. altissimus* and *T. europaeus*. ANOVAs were performed separately for the leaves collected from natural localities (n) and from cultivation (c). The same letters indicate a lack of statistically significant differences between the samples according to Tukey's a posteriori test ($p<0.05$).

Feature	No of segment	b-n			t-n			u-n			ANOVA	b-c			t-c			u-c			ANOVA
		X	SE		X	SE		X	SE		P>F	X	SE		X	SE		X	SE		P>F
(1) Length [cm]	1	3.66	(±0.10)	a	3.01	(±0.14)	b	3.80	(±0.09)	a	0.0012	4.96	(±0.15)	b	5.32	(±0.22)	ab	5.77	(±0.16)	a	0.0051
	2	3.92	(±0.11)	a	3.33	(±0.14)	b	4.06	(±0.10)	a	0.0049	5.20	(±0.13)	b	5.50	(±0.21)	b	6.07	(±0.13)	a	0.0007
	3	3.92	(±0.10)	a	3.36	(±0.14)	b	4.04	(±0.09)	a	0.0064	5.15	(±0.13)	b	5.51	(±0.23)	ab	6.07	(±0.16)	a	0.0011
	4	3.87	(±0.11)	a	3.09	(±0.13)	b	4.01	(±0.10)	a	0.0002	5.19	(±0.14)	b	5.39	(±0.20)	b	6.03	(±0.15)	a	0.0013
	5	3.67	(±0.11)	a	3.00	(±0.15)	b	3.80	(±0.09)	a	0.0014	5.01	(±0.12)	b	5.30	(±0.21)	ab	5.82	(±0.12)	a	0.0009
(2) Max. width [cm]	1	2.72	(±0.10)	a	2.12	(±0.17)	b	2.64	(±0.06)	a	0.0086	3.51	(±0.13)	b	3.56	(±0.15)	b	4.34	(±0.15)	a	<0.0001
	2	2.54	(±0.09)	a	1.83	(±0.09)	b	2.59	(±0.07)	a	<0.0001	3.34	(±0.11)	b	3.60	(±0.17)	b	4.33	(±0.14)	a	<0.0001
	3	2.49	(±0.09)	a	2.07	(±0.10)	b	2.60	(±0.06)	a	0.0172	3.38	(±0.09)	b	3.68	(±0.17)	b	4.30	(±0.14)	a	<0.0001
	4	2.48	(±0.08)	a	1.89	(±0.10)	b	2.61	(±0.06)	a	0.0002	3.38	(±0.08)	b	3.55	(±0.16)	b	4.30	(±0.13)	a	<0.0001
	5	2.72	(±0.10)	a	2.12	(±0.15)	b	2.68	(±0.08)	a	0.0080	3.55	(±0.14)	b	3.70	(±0.16)	b	4.33	(±0.15)	a	0.0009
(3) Width to length ratio	1	0.75	(±0.02)	a	0.71	(±0.04)	a	0.70	(±0.01)	a	0.1496	0.71	(±0.02)	ab	0.67	(±0.02)	b	0.76	(±0.02)	a	0.0155
	2	0.65	(±0.01)	a	0.55	(±0.02)	b	0.64	(±0.01)	a	0.0033	0.64	(±0.02)	b	0.66	(±0.02)	ab	0.71	(±0.01)	a	0.0245
	3	0.63	(±0.01)	a	0.62	(±0.03)	a	0.65	(±0.01)	a	0.7179	0.66	(±0.01)	b	0.67	(±0.02)	ab	0.71	(±0.02)	a	0.0418
	4	0.64	(±0.01)	a	0.62	(±0.03)	a	0.66	(±0.01)	a	0.6348	0.66	(±0.02)	b	0.66	(±0.01)	b	0.71	(±0.02)	a	0.0100
	5	0.75	(±0.02)	a	0.71	(±0.04)	a	0.71	(±0.02)	a	0.2052	0.71	(±0.02)	a	0.70	(±0.02)	a	0.74	(±0.02)	a	0.2772
(4) Position where max. width was measured [%]	1	56.41	(±1.27)	a	53.32	(±2.41)	a	59.59	(±1.05)	a	0.0406	57.37	(±1.96)	a	57.21	(±1.74)	a	60.37	(±1.81)	a	0.4027
	2	61.35	(±1.01)	a	61.46	(±2.34)	a	59.35	(±1.17)	a	0.3804	55.36	(±1.60)	a	59.10	(±2.21)	a	61.57	(±1.89)	a	0.0645
	3	60.79	(±1.16)	a	56.94	(±2.31)	a	58.96	(±1.19)	a	0.2825	57.82	(±1.62)	a	56.20	(±1.58)	a	59.56	(±1.70)	a	0.3641
	4	62.16	(±1.09)	a	58.82	(±1.76)	ab	57.88	(±0.99)	b	0.0104	55.65	(±1.37)	a	59.09	(±1.46)	a	60.01	(±1.58)	a	0.0841
	5	58.54	(±1.12)	a	53.32	(±2.93)	a	56.58	(±1.39)	a	0.1544	55.88	(±1.77)	a	55.45	(±1.38)	a	60.72	(±1.66)	a	0.0451
(5) Width at 25% of height of blade length [cm]	1	1.62	(±0.08)	a	1.28	(±0.15)	a	1.58	(±0.05)	a	0.0839	2.25	(±0.10)	a	2.38	(±0.11)	a	2.68	(±0.18)	a	0.0738
	2	1.28	(±0.06)	a	0.90	(±0.08)	b	1.33	(±0.04)	a	0.0018	1.97	(±0.08)	a	2.29	(±0.14)	a	2.24	(±0.08)	a	0.0621
	3	1.18	(±0.06)	b	0.98	(±0.11)	b	1.38	(±0.05)	a	0.0020	2.04	(±0.07)	b	2.38	(±0.12)	a	2.40	(±0.09)	a	0.0097
	4	1.20	(±0.06)	ab	1.01	(±0.10)	b	1.41	(±0.06)	a	0.0054	2.09	(±0.08)	b	2.18	(±0.12)	ab	2.45	(±0.11)	a	0.0344
	5	1.62	(±0.09)	a	1.39	(±0.15)	a	1.60	(±0.06)	a	0.3987	2.36	(±0.12)	a	2.40	(±0.12)	a	2.63	(±0.13)	a	0.2334

Table 4. continued

Feature	No of segment	b-n			t-n			u-n			ANOVA	b-c			t-c			u-c			ANOVA
		X	SE		X	SE		X	SE		P>F	X	SE		X	SE		X	SE		P>F
(6) Width at 50% of height of blade length [cm]	1	2.38	(±0.09)	a	1.75	(±0.10)	b	2.35	(±0.05)	a	0.0007	3.05	(±0.10)	b	3.19	(±0.14)	b	3.89	(±0.15)	a	<0.0001
	2	2.16	(±0.08)	a	1.58	(±0.09)	b	2.27	(±0.05)	a	<0.0001	2.93	(±0.10)	b	3.12	(±0.16)	b	3.73	(±0.12)	a	<0.0001
	3	2.07	(±0.08)	ab	1.76	(±0.10)	b	2.27	(±0.05)	a	0.0073	3.04	(±0.08)	b	3.32	(±0.16)	ab	3.63	(±0.14)	a	0.0050
	4	2.06	(±0.07)	b	1.65	(±0.10)	c	2.32	(±0.06)	a	<0.0001	2.98	(±0.08)	b	3.20	(±0.14)	b	3.77	(±0.13)	a	<0.0001
	5	2.32	(±0.08)	a	1.76	(±0.14)	b	2.33	(±0.07)	a	0.0036	3.17	(±0.13)	b	3.31	(±0.13)	b	3.79	(±0.15)	a	0.0050
(7) Specific leaf area (cm^2g^{-1}d.w.)	1	153.16	(±3.46)	b	111.15	(±4.12)	c	214.10	(±6.59)	a	<0.0001	155.67	(±5.09)	c	203.68	(±7.63)	a	181.17	(±5.59)	b	<0.0001
	2	150.10	(±3.34)	b	110.63	(±3.66)	c	205.59	(±5.77)	a	<0.0001	154.21	(±4.08)	c	200.15	(±7.76)	a	177.75	(±5.49)	b	<0.0001
	3	155.07	(±3.67)	b	120.89	(±3.90)	c	209.08	(±5.44)	a	<0.0001	159.07	(±4.18)	c	200.62	(±8.05)	a	181.42	(±5.81)	b	<0.0001
	4	151.45	(±3.68)	b	115.22	(±4.15)	c	210.81	(±6.06)	a	<0.0001	155.77	(±4.10)	c	197.14	(±7.86)	a	178.84	(±5.41)	b	<0.0001
	5	151.77	(±3.51)	b	113.88	(±4.17)	c	216.71	(±6.30)	a	<0.0001	155.98	(±4.57)	c	203.18	(±7.16)	a	180.47	(±5.39)	b	<0.0001

When samples from the two populations of *T. altissimus* were combined, comparisons of leaves from *T. altissimus* and *T. europaeus* showed statistically significant differences only for several measured features of blade segments (Table 5).

Comparison of Leaves from Cultivation

In cultivation, blade length, maximum width, and width at 50% of blade length of both populations of *T. altissimus* (b-c and t-c) were similar, and these samples differed from those of *T. europaeus* (u-c) in these measurements (Table 4). Statistically highly significant differences were also found for SLA, with particular blade segments showing differences in SLA between all three populations or the convergence between *T. altissimus* from the Tatra Mts (b-c) and *T. europaeus* (u-c). The other analyzed features were of no statistical significance or Tukey's test did not reveal distinct relationships among the investigated populations on their basis.

We found that leaves of *T. altissimus* (both populations jointly) and *T. europaeus* gathered from cultivation (typical garden conditions) showed statistically significant differences for most of the measured features of blade segments (Table 5). There were no differences between the species with regard to SLA.

Comparison of Leaves from the Same Origin

One-way analysis of variance showed similarity between *T. altissimus* leaves gathered from natural locations and cultivation only in terms of the width to length ratio, the position where maximum width was measured (except for two segments of sample b-c) and SLA (but only the sample from the Bieszczady Mts.; Table 6). There were no statistically important differences among the leaves of *T. europaeus* originated from nature and cultivation with regard to the maximum width position (all segments) and width to length ratio (only one segment).

The determined means of all morphological features divided the populations into two groups according to the origin of the leaves, while the species classification of particular samples seemed to be of less significance (Fig. 1). However, both populations of *T. altissimus* from cultivation (b-c and t-c) were arranged closely, although the relationships of the samples of this species from natural sites were not distinctly marked. It appeared that population b-n was similar to the population of *T. europaeus* (u-n) rather than the other one of *T. altissimus* (t-n).

Discussion and Conclusions

Phenolic compounds belong to a group of secondary metabolites most often used in plant taxonomy [11, 12]. These compounds, especially flavonoids, can be a helpful criterion in investigations of taxonomy at the species or interspecies level and of hybridization [13-16]. The envi-

Table 5. Mean values (± SE) of investigated leaves' features of the *T. altissimus* with both populations combined and of *T. europaeus*. ANOVAs were performed separately for the leaves collected from natural localities and from cultivation ($p<0.05$).

Feature	No. of segment	*T. altissimus* (nature)		*T. europaeus* (nature)		ANOVA	*T. altissimus* (cultivation)		*T. europaeus* (cultivation)		ANOVA
		X	SE	X	SE	P>F	X	SE	X	SE	P>F
(1) Length [cm]	1	3.54	(±0.09)	3.80	(±0.09)	0.0366	5.13	(±0.13)	5.77	(±0.16)	0.0037
	2	3.81	(±0.10)	4.06	(±0.10)	0.0531	5.34	(±0.12)	6.07	(±0.13)	0.0003
	3	3.81	(±0.09)	4.04	(±0.09)	0.0715	5.32	(±0.13)	6.07	(±0.16)	0.0007
	4	3.73	(±0.10)	4.01	(±0.10)	0.0244	5.28	(±0.12)	6.03	(±0.15)	0.0004
	5	3.54	(±0.10)	3.80	(±0.09)	0.0413	5.01	(±0.12)	5.82	(±0.12)	0.0005
(2) Max. width [cm]	1	2.61	(±0.09)	2.64	(±0.06)	0.8582	3.53	(±0.10)	4.34	(±0.15)	<0.0001
	2	2.41	(±0.08)	2.59	(±0.07)	0.1030	3.46	(±0.10)	4.33	(±0.14)	<0.0001
	3	2.41	(±0.08)	2.60	(±0.06)	0.1211	3.52	(±0.09)	4.30	(±0.14)	<0.0001
	4	2.37	(±0.08)	2.61	(±0.06)	0.0426	3.46	(±0.09)	4.30	(±0.13)	<0.0001
	5	2.60	(±0.09)	2.68	(±0.08)	0.6545	3.62	(±0.11)	4.33	(±0.15)	0.0002
(3) Width to length ratio	1	0.74	(±0.02)	0.70	(±0.01)	0.0894	0.69	(±0.02)	0.76	(±0.02)	0.0093
	2	0.63	(±0.01)	0.64	(±0.01)	0.5471	0.65	(±0.02)	0.71	(±0.01)	0.0080
	3	0.63	(±0.01)	0.65	(±0.01)	0.4334	0.67	(±0.01)	0.71	(±0.02)	0.0134
	4	0.64	(±0.01)	0.66	(±0.01)	0.5241	0.66	(±0.02)	0.71	(±0.02)	0.0023
	5	0.74	(±0.02)	0.71	(±0.02)	0.1219	0.70	(±0.02)	0.74	(±0.02)	0.1128
(4) Position where max. width was measured [%]	1	55.84	(±1.13)	59.59	(±1.05)	0.0245	57.29	(±1.31)	60.37	(±1.81)	0.1767
	2	61.37	(±0.93)	59.35	(±1.17)	0.1640	57.12	(±1.35)	61.57	(±1.89)	0.0588
	3	60.08	(±1.04)	58.96	(±1.19)	0.5699	57.06	(±1.13)	59.56	(±1.70)	0.2143
	4	61.53	(±0.95)	57.88	(±0.99)	0.0078	57.26	(±1.01)	60.01	(±1.58)	0.1326
	5	57.54	(±1.08)	56.58	(±1.39)	0.5783	55.68	(±1.14)	60.72	(±1.66)	0.0128
(5) Width at 25% of height of blade length [cm]	1	1.56	(±0.07)	1.58	(±0.05)	0.8676	2.31	(±0.07)	2.68	(±0.18)	0.0294
	2	1.21	(±0.06)	1.33	(±0.04)	0.0795	2.12	(±0.08)	2.24	(±0.08)	0.3620
	3	1.14	(±0.05)	1.38	(±0.05)	0.0018	2.20	(±0.07)	2.40	(±0.09)	0.1016
	4	1.17	(±0.05)	1.41	(±0.06)	0.0041	2.13	(±0.07)	2.45	(±0.11)	0.0116
	5	1.58	(±0.08)	1.60	(±0.06)	0.9774	2.38	(±0.08)	2.63	(±0.13)	0.0898
(6) Width at 50% of height of blade length [cm]	1	2.26	(±0.08)	2.35	(±0.05)	0.5159	3.12	(±0.09)	3.89	(±0.15)	<0.0001
	2	2.06	(±0.07)	2.27	(±0.05)	0.0242	2.02	(±0.09)	3.73	(±0.12)	<0.0001
	3	2.02	(±0.07)	2.27	(±0.05)	0.0170	3.17	(±0.09)	3.63	(±0.14)	0.0040
	4	1.99	(±0.06)	2.32	(±0.06)	0.0011	2.08	(±0.08)	3.77	(±0.13)	<0.0001
	5	2.21	(±0.08)	2.33	(±0.07)	0.3740	3.24	(±0.09)	3.79	(±0.15)	0.0014
(7) Specific leaf area [cm^2g^{-1}]	1	145.37	(±3.36)	214.10	(±6.59)	<0.0001	178.17	(±5.38)	181.17	(±5.59)	0.7272
	2	142.77	(±3.21)	205.59	(±5.77)	<0.0001	175.74	(±5.10)	177.75	(±5.49)	0.8067
	3	148.73	(±3.36)	209.08	(±5.44)	<0.0001	178.54	(±5.07)	181.42	(±5.81)	0.7283
	4	144.59	(±3.41)	210.81	(±6.06)	<0.0001	175.16	(±4.98)	178.84	(±5.41)	0.6474
	5	144.52	(±3.32)	216.71	(±6.30)	<0.0001	178.11	(±5.07)	180.47	(±5.39)	0.7716

Table 6. One factor analysis of the investigated segment features within the samples of the same origin.

Feature	No. of leaf segment	b-n-b-c	t-n-t-c	u-n-u-c
		ANOVA P>F	ANOVA P>F	ANOVA P>F
(1) Length [cm]	1	<0.0001	<0.0001	<0.0001
	2	<0.0001	<0.0001	<0.0001
	3	<0.0001	<0.0001	<0.0001
	4	<0.0001	<0.0001	<0.0001
	5	<0.0001	<0.0001	<0.0001
(2) Max. width [cm]	1	<0.0001	<0.0001	<0.0001
	2	<0.0001	<0.0001	<0.0001
	3	<0.0001	<0.0001	<0.0001
	4	<0.0001	<0.0001	<0.0001
	5	<0.0001	<0.0001	<0.0001
(3) Width to length ratio	1	0.2830	0.4210	0.0217
	2	0.8471	0.0020	0.0012
	3	0.1969	0.0945	0.0022
	4	0.4477	0.1876	0.0055
	5	0.2308	0.8100	0.1283
(4) Position where max. width was measured [%]	1	0.6814	0.1888	0.6181
	2	0.0017	0.4891	0.2961
	3	0.1526	0.7880	0.8577
	4	0.0007	0.9080	0.2279
	5	0.1966	*0.4631*	0.0719
(5) Width at 25% of height of blade length [cm]	1	<0.0001	<0.0001	<0.0001
	2	<0.0001	<0.0001	<0.0001
	3	<0.0001	<0.0001	<0.0001
	4	<0.0001	<0.0001	<0.0001
	5	<0.0001	<0.0001	<0.0001
(6) Width at 50% of height of blade length [cm]	1	<0.0001	<0.0001	<0.0001
	2	<0.0001	<0.0001	<0.0001
	3	<0.0001	<0.0001	<0.0001
	4	<0.0001	<0.0001	<0.0001
	5	<0.0001	<0.0001	<0.0001
(7) Specific leaf area [cm^2g^{-1}]	1	0.6888	<0.0001	0.0018
	2	0.4773	<0.0001	0.0028
	3	0.5235	<0.0001	0.0028
	4	0.4867	<0.0001	0.0008
	5	0.4894	<0.0001	0.0003

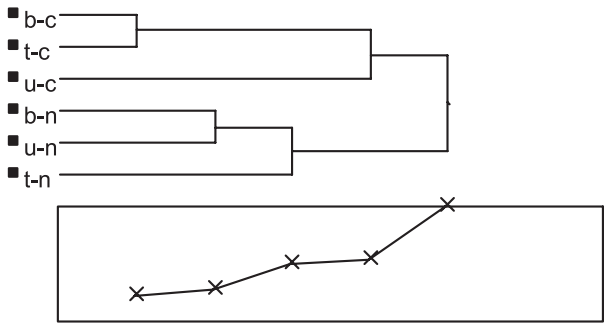

Fig. 1. Dendrogram of cluster groupings of *Trollius* from Bieszczady Mts, Tatry Mts and Uścikowo (natural locality and cultivation). The plot beneath the dendrogram presents points for each cluster. The distance and curvature between the points represent.

ronment has no qualitative effect on them, but flavonoid quantity is directly related to light accessibility [17]. However, information on the meaning of these substances in *Trollius* systematics is so far insufficient. There are only a few papers referring to the phytochemical characteristics of *T. chinensis*, *T. ledebourii* and *T. macropetalus* as medicinal plants and one study of phytochemical variability of *T. europaeus* populations from northwestern Poland [9, 18-22]. The current study showed limited meaning of phenolic compounds extracted from leaves in separation of the investigated *Trollius* species. Only the lack of unknown phenolic acid Y (u-c) distinguished *T. europaeus* and *T. altissimus*.

In the main regions of *T. europaeus* distribution, Després et al. [10] revealed that most genetic variability of the species is within its populations. Significant differentiation of specimens with reference to the vegetative and generative organs of this species was confirmed by some investigations from northwestern Poland [7-9]. Similarly, variability among the populations from that study was highly significant and connected with site conditions, especially with the acidity of soil substratum. In this study only one population of European globeflower, randomly selected from within the above-mentioned region of Poland, was analyzed. It appeared to be influenced by habitat conditions, although Uścikowo (the site of samples u-n) is not far (about 40 km) from Poznań (the site of cultivation of samples u-c). Generally the cultivated plants had larger segments of leaves than those in natural localities. Size extension after transfer of European globeflower plants from natural sites to garden conditions was also noted by Pašina [23].

Most of the *Trollius* species readily cross with another and give a fertile progeny and the hybrids between *T. europaeus* and *T. altissimus* have also been known from cultivation [1]. The distance between the 3 sites of investigated populations excludes the possibility of their interfertilization. However, the ranges of *T. europaeus* and *T. altissimus* within the mountain regions in Poland may overlap and theoretically there is possibility of cross-polination with the any other adjacent populations, due to *Chiastocheta* flies, whose larvae feed only on globeflower seeds [28-32].

The range of morphological variability of *T. altissimus* had not been recognized before. However, Chrtek and Chrtkova [5] considered this species to exhibit more plasticity in leaf morphology than *T. europaeus*. In the presented study the analyzed Polish populations of mountain globeflower distinctly differed from each other. A comparison of the leaves from natural localities and from cultivation also revealed significant differences. Cultivated plants differed in morphology compared to those growing in natural localities, exhibiting larger leaf segments.

According to Garnier et al. [24] specific leaf area (SLA) can be used for species comparisons. It is genetically encoded and it varies more than tenfold among species growing within the same habitat [25]. In this study, leaves of plants grown in natural localities exhibited statistically important differences from those grown in cultivation regardless of the species. Similarity in SLA between the pairs of samples of the same origin was noted only in the case of *T. altissimus* from the Bieszczady Mts. With respect to *T. altissimus* from the Tatra Mts., SLA of all blade segments of cultivated plants was 40-45% higher than that of plants from natural sites. Then in the case of *T. europaeus* the value of SLA was larger in plants growing naturally (but the difference was less than 17%). Such different reactions of particular samples showed the significant plasticity of SLA, which might occur even among individual plants of the same species [26]. Wright et al. [27], while considering some leaf traits simultaneously, revealed that 18% of variation was explained by climate. For example, they observed a strong correlation between leaf mass per area (LMA, inverse of SLA) and site irradiance, as well as a distinct impact of mean annual temperature (MAT) on this indicator.

Our investigation showed the limited application of phytochemical properties and morphology of leaves in *Trollius* biosystematics. Since on the basis of the investigated characters in our study, the separation of *T. altissimus* from *T. europaeus* is questionable. There were no morphologically or phytochemically important differences between the species and with respect to some traits a significant distinction appeared between the *T. altissimus* populations. Additionally, site conditions influenced leaf morphology of both species. Our study provided valuable evidence for the statement that *T. europaeus* and *T. altissimus* are not separated species and *T. europaeus* should be divided into two lower taxa in the rank of variety or subspecies.

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