Exploring patterns of variation within the central-European *Tephroseris longifolia* agg.: karyological and morphological study

Karyologická a morfologická variabilita v rámci *Tephroseris longifolia* agg.

Katarína Olšavská[1], Barbora Šingliarová[1], Judita Kochjarová[1,3], Zuzana Labdíková[2], Iveta Škodová[1], Katarína Hegedüšová[1] & Monika Janišová[1]

[1]*Institute of* Botany, *Slovak Academy of Sciences, Dúbravská cesta 9, SK-84523 Bratislava, Slovakia, e-mail: katarina.olsavska@savba.sk;* [2]*Faculty of Natural Sciences, University of Matej Bel, Tajovského 40, SK-97401 Banská Bystrica, Slovakia;* [3]*Comenius University, Bratislava, Botanical Garden – detached unit, SK-03815 Blatnica, Slovakia*

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Tephroseris longifolia agg. is an intricate complex of perennial outcrossing herbaceous plants. Recently, five subspecies with rather separate distributions and different geographic patterns were assigned to the aggregate: *T. longifolia* subsp. *longifolia*, subsp. *pseudocrispa* and subsp. *gaudinii* predominate in the Eastern Alps; the distribution of subsp. *brachychaeta* is confined to the northern and central Apennines and subsp. *moravica* is endemic in the Western Carpathians. Carpathian taxon *T. l.* subsp. *moravica* is known only from nine localities in Slovakia and the Czech Republic and is treated as an endangered taxon of European importance (according to Natura 2000 network). As the taxonomy of this aggregate is not comprehensively elaborated the aim of this study was to detect variability within the *Tephroseris longifolia* agg. using methods of plant systematics (multivariate morphometrics of 525 individuals/33 populations based on 49 characters, DAPI flow cytometry of 98 individuals/33 populations). The relative DNA content at the homoploid level (2n ~ 6x ~ 48) varied by 25.8% and significant taxa-specific differences were confirmed among plants of *T. l.* subsp. *pseudocrispa*, subsp. *gaudinii*, subsp. *brachychaeta* and a group consisting of *T. l.* subsp. *moravica* and subsp. *longifolia*. The morphometric study indicated six morphotypes roughly corresponding to the previously distinguished subspecies. The exceptions were populations traditionally assigned to *T. l.* subsp. *longifolia*, for which two distinct morphotypes with different geographic origins were identified: Alpine morphotype and Pannonian morphotype. In general, the differences in DNA content and morphology argue for a classification at the species level for plants of *T. l.* subsp. *brachychaeta*, while differences among other morphotypes fit a subspecific level. Surprisingly, Pannonian populations of *T. l.* subsp. *longifolia* are morphologically closer to populations of the Western-Carpathian endemic subsp. *moravica* than to Alpine populations of nominate subspecies. Based on this, the taxonomic position of Pannonian morphotype and subsequently the endemic status of *T. l.* subsp. *moravica* require further study. A key for identifying the taxa and morphotypes of *Tephroseris longifolia* agg. in central Europe is presented.

K e y w o r d s: Alps, *Asteraceae*, *Compositae*, endemics, flow cytometry, multivariate morphometrics, taxonomy, *Tephroseris*, Western Carpathians

Introduction

The genus *Tephroseris* (Rchb.) Rchb. of the sunflower family (*Asteraceae*) comprises 15 (Cufodontis 1933) to 50 (Nordenstam 2007) species with Eurasian and North American distributions. Most species occur in the temperate and boreal zone of Europe and Asia,

with a few species in the north-western part of North America (Golden et al. 2001, Wang et al. 2009, Nordestam & Pelser 2011). Previously, the preference of some authors was to include all the species in the genus *Senecio* L. [also as *Senecio* sect. *Tephroseris* (Rchb.) Hallier & Wohlf.] because they are morphologically similar (e.g. Cufodontis 1933, Chater & Walters 1976, Wagenitz 1987, Meusel & Jäger 1992). Recently, the concept of two separate genera taking into account different basic chromosome numbers (Holub 1973, 1979) and phylogenetic relationships (Bremer 1994, Pelser et al. 2007, Wang et al. 2009) has become widely accepted. Molecular markers support the recognition of *Tephroseris* as a separate genus in the subtribe *Tussilagininae* (Cass.) Dumort., while *Senecio* s.s. is nested within the subtribe *Senecioninae* (Cass.) Dumort. As the taxonomy of the genus *Tephroseris* is yet to be fully established, we follow the taxonomical concept of the Euro+Med PlantBase Checklist (Euro+Med 2006–2014) in this study.

Tephroseris longifolia agg. is an intricate complex distributed throughout central Europe. Main area of its distribution extends from the Central and Eastern Alps to the Pannonian Basin and the Western Carpathians, and also reaches the Apennines and Dinarides (Meusel & Jäger 1992, Euro+Med 2006–2014). Members of this aggregate are short-lived perennials with presumably an outcrossing breeding system (Janišová et al. 2012b). To differentiate them from related genera/groups, the following combination of morphological characters can be used: dark-coloured rhizome; unbranched stem; exclusively yellow flowers; petioles of basal leaves of the same size or longer than the leaf blade; blade of basal leaves lanceolate, ovate-lanceolate or ovate, with narrowed or rounded, less commonly cordate base, dentate margins to blades; stem and leaves hairy to arachnoid especially in the young stages; pappus up to double the length of an achene (Chater & Walters 1976, Pignatti 1982, Aeschimann et al. 2004, Fischer et al. 2008).

The taxonomy of the *T. longifolia* agg. has not been comprehensively elaborated other than in the old monograph on the genus *Tephroseris* (Cufodontis 1933), which is based on descriptive and comparative morphology (Chater & Walters 1976). Scattered information can be found in national determination keys (e.g. Hess et al. 1972, Pignatti 1982, Adler et al. 1994, Kochjarová & Hrouda 2004, Fischer et al. 2008) and several publications devoted to selected subspecies (e.g. Kochjarová 1997). In summary, there are no studies using biosystematic methods on the *T. longifolia* agg. Recently, five subspecies were assigned to this aggregate (Euro+Med 2006–2014): *Tephroseris longifolia* (Jacq.) Griseb. et Schenk subsp. *longifolia*, *T. l.* subsp. *moravica* Holub, *T. l.* subsp. *gaudinii* (Gremli) Kerguélen, *T. l.* subsp. *pseudocrispa* (Fiori) Greuter, and *T. l.* subsp. *brachychaeta* Greuter. These taxa were described mainly based on characters of the indumentum of the achenes, stem and leaves. In addition, *T. l.* subsp. *moravica* and subsp. *pseudocrispa* are supposed to differ in the shapes of their basal leaves and blade bases (Electronic Appendix 1; Fiori 1925–1929, Holub 1979, Kochjarová 1995, 1997, 1998). Great morphological variation and minute morphological differences within the *T. longifolia* agg., however, complicate the clear delimitation of particular subspecies.

Taxa of the *T. longifolia* agg. occur from colline to subalpine regions (300–2000 m a. s. l.; Wagenitz 1987) and populations of this aggregate grow in open semi-dry and mesic grasslands, light broad-leaved forests, forest margins and tall-herbaceous subalpine plant communities. They are also frequently present in man-influenced and disturbed secondary habitats. The ecological requirements, altitudinal range of occurrence and preference for specific plant communities is rather specific for particular subspecies (Pignatti 1982,

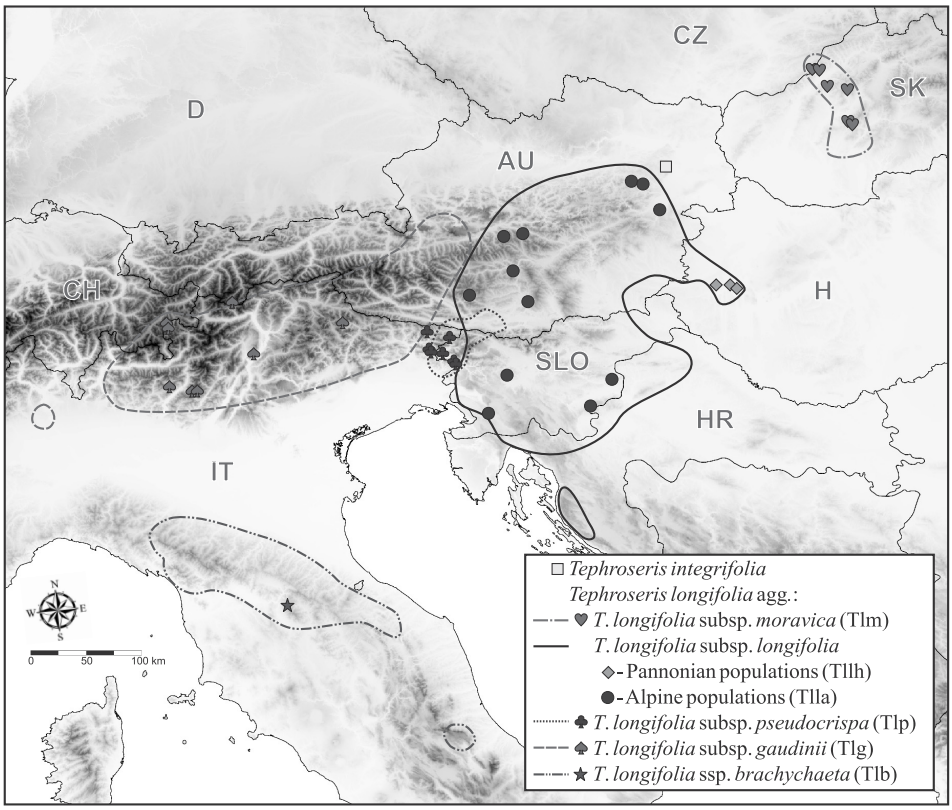

Fig. 1. – Geographical distribution of the *Tephroseris integrifolia* (■) and *T. longifolia* agg. populations analysed in the present study: *T. longifolia* subsp. *moravica* (Tlm, ♥), *T. l.* subsp. *longifolia* – Pannonian morphotype (Tllh, ◆), *T. l.* subsp. *longifolia* – Alpine morphotype (Tlla, ●), *T. l.* subsp. *gaudinii* (Tlg, ♣), *T. l.* subsp. *pseudocrispa* (Tlp, ♠) and *T. l.* subsp. *brachychaeta* (Tlb, ★). For details of the sample sites see Table 1. General distribution of *T. longifolia* subspecies is marked by lines (modified from Pignatti 1982, Welten & Sutter 1982, Rakonczay 1989, Hartl et al. 1992, Martinčič et al. 1999, Wohlgemuth et al. 1999–2001, Forenbacher 2001, Jogan 2001, Flora Croatica Database 2009, Niklfeld 2009, Hegedüšová et al. 2013).

Wagenitz 1987, Aeschimann et al. 2004, Hegedüšová et al. 2013). Similarly, the subspecies of *T. longifolia* have more or less separate distributions and different geographic ranges. On the one hand, there are three predominantly Alpine species: *T. l.* subsp. *longifolia* and subsp. *gaudinii* with wide distributions (subsp. *gaudinii* predominates in the eastern part of the Eastern Alps, subsp. *longifolia* in western part of the Eastern Alps occurring also in the Hrvatsko Zagorie region and Velebit Mts and in the western part of the Pannonian Basin) and subsp. *pseudocrispa* restricted to a small area on the borders of Italy, Austria and Slovenia (Southern limestone Alps) (Fig. 1) (Pignatti 1982, Welten & Sutter 1982, Rakonczay 1989, Hartl et al. 1992, Martinčič et al. 1999, Wohlgemuth et al. 1999–2001, Forenbacher 2001, Jogan 2001, Flora Croatica Database 2009, Niklfeld 2009). On the other hand, the distribution of *T. l.* subsp. *brachychaeta* is confined to the northern and central Apennines (Tondi & Plini 1995, Alessandrini et al. 2010, Viciani et

al. 2010) and subsp. *moravica* is a stenoendemic taxon in the Western Carpathians (Kliment 1999, Hegedüšová et al. 2013). Within the aggregate, *T. l.* subsp. *moravica* has the narrowest range and is currently known from only nine localities in Slovakia and the Czech Republic (Holub 1999, Mereďa & Hodálová 2011, Janišová et al. 2012a). It is legally protected in both countries (Decree of the Ministry of the Environment of the Czech Republic Nr. 395/1992; Decree of the Ministry of the Environment of the Slovak Republic Nr. 24/2003) and is an endangered taxon of national (Feráková et al. 2001, Grulich 2012) and European importance (Natura 2000 network, Directive 92/43/EEC Anex II; Bilz et al. 2011). This taxon is vulnerable because of its restriction to secondary, man-made habitats that are currently endangered by changes in land use. Therefore, these populations are under long-term monitoring (Janišová et al. 2005, Chmelová 2007, Gbelcová 2010) and various aspects of their ecology, sociology and demography (Kochjarová 1995, 1998, Janišová et al. 2005, 2012a, b, Hegedüšová et al. 2013) are being studied in order to determine the optimal management of the communities in which this taxon occurs. In addition, *T. l.* subsp. *longifolia* and subsp. *gaudinii* are endangered in Switzerland (Moser et al. 2002), Austria (Niklfeld & Schratt-Ehrendorfer 1999) and Hungary (Király 2007). Hungarian populations of *T. l.* subsp. *longifolia* are also legally protected (Decree of the Ministry of the Environment and Water of Hungary Nr. 23/2005).

Currently there are many studies that document the importance of taxonomical clarification for identifying conservation priorities such as legal protection and restoration of local endemics and/or endangered populations (e.g. Kolarčik et al. 2010, Španiel et al. 2011a, b, Kučera et al. 2013, Petrova et al. 2014). In spite of considerable efforts to conserve members of the *T. longifolia* agg., their taxonomic delimitations and relationships within the aggregate remain controversial. Because of this, our study aims to detect variability within the *T. longifolia* agg. using multivariate morphometrics and flow cytometry to reveal the evolutionary relationships within the aggregate.

Three major questions were addressed in this study: (i) Is the variation in morphology and DNA content within the aggregate supportive of the traditional taxonomic concept? (ii) What are the relationships within the *T. longifolia* agg. based on morphological and karyological data? (iii) Does the variation in the morphology and DNA content in the aggregate support the endemic status of *T. l.* subsp. *moravica*?

Material and methods

Plant material

Plants for the morphometric study were collected from natural habitats during 2011–2012 (May/June) throughout the Alpine, Pannonian and Western Carpathian distribution of the aggregate. In addition, material from one population of *T. l.* subsp. *brachychaeta* in the central Apennines was collected. Altogether 33 populations were sampled (Table 1, Fig. 1). If possible, samples from known type localities or adjacent areas (Electronic Appendix 1) of traditionally recognized taxa were also included in our study. Population samples ranged from 2 to 20 individuals (altogether 525 plants). The number of individuals collected depended on the population size at a particular locality. In the case of small or highly protected populations the characters of flowers and stems were measured or scored directly

Table 1. – Details of the localities studied, including geographical coordinates, altitude, date and collectors of the plants of *Tephroseris longifolia* agg. and *T. integrifolia* from Europe investigated for their morphological variation in stem, stem leaves, synflorescences (MORF) and seeds (SEED) and/or with DAPI-stained flow cytometry (FCM). Collector abbreviations: KH – K. Hegedüšová, MJ – M. Janišová, JK – J. Kochjarová, BŠ – B. Šingliarová, IŠ – I. Škodová, KO – K. Olšavská, AC – A. Čarni, NJ – N. Juvan, KD – K. Devánová, ZL – Z. Labdíková, LB – L. Borsukiewicz.

Taxon	Population code	Locality details	MORF/ SEED	FCM
T. longifolia subsp. *moravica* Holub (Tlm)	CAV	Slovakia; Strážovské vrchy Mts, Čavoj village; 48°52'56.6" N, 18°29'25.8" E; 560–585 m; 20.5.2011; KH, MJ, JK & IŠ	20/10	3
	RAD	Slovakia; Tríbeč Mts, Radobica village; 48°34'27.2" N, 18°29'54.6" E; 480–560 m; 18.5.2011, KH, MJ, JK, IŠ & KO	20/10	3
	HOD	Czech Republic; Bílé Karpaty Mts, Hodňov village; 49°04'57.0" N, 18°03'24.3" E; 480–560 m; 26.5.2011, KH, MJ, JK & IŠ	20/0	0
	LYS	Slovakia; Biele Karpaty Mts, Vršatecké Podhradie village, Lysá meadow; 49°04'17.0" N, 18°08'41.4" E; 740–780 m; 8.6.2012, MJ, KD, JK, IŠ & ZL	12/10	3
	OMS	Slovakia; Strážovské vrchy Mts, Omšenie village; 48°54'52.4" N, 18°14'36.4" E; 570–670 m; 7.6.2012; MJ, KD, JK, IŠ & ZL	7/10	3
	STR	Slovakia; Vtáčnik Mts, Mt. Stráž; 48°32'53.6" N, 18°32'40.4" E; 770–780 m; 6.6.2012; MJ, KD, JK, IŠ & ZL	5/10	3
T. longifolia (Jacq.) Griseb. et Schenk subsp. *longifolia* – Pannonian morphotype (Tllh)	GOS	Hungaria; Veszprém, Gösfa village, Mt. Göshegy; 46°58'08.0" N, 16°52'13.0" E; 210–230 m; 3.5.2011; KH, MJ, JK, BŠ, IŠ & KO	16/10	3
	HUS	Hungaria; Zala, Huszonya village; 46°55'57.0" N, 17°07'33.0" E; 160–170 m; 3.5.2011; KH, MJ, JK, BŠ, IŠ & KO	2/0	0
	ZAL	Hungaria; Zala, Zalabér village, Bagóvölgy valley; 46°58'05.0" N, 17°02'49.0" E; 210–220 m; 3.5.2011; KH, MJ, JK, BŠ, IŠ & KO	10/10	3
T. longifolia (Jacq.) Griseb. et Schenk subsp. *longifolia* – Alpine morphotype (Tlla)	EBE	Austria; Lavantater Alpen Mts, Kärnten, Eberstein village; 46°47'51.0" N, 14°33'07.0" E; 570–622 m; 14.5.2012, KH, MJ, JK & IŠ	20/10	3
	FAL	Austria; Kärnten, Ebene Reichenau village, Falkersee Lake; 46°51'45.4" N, 13°49'36.8" E; 1855–1890 m; 21.6.2012, MJ, KD, LB & IŠ	21/10	3
	FUR	Austria; Niederöstereich, Furth an der Triesting village; 47°57'35.2" N, 15°57'49.8" E; 413 m; 29.5.2011; 29.5.2012, MJ, KD & IŠ	0/0	3
	HIR	Austria; Karawanken Mts, Ebriach village, part Hirskeuche; 46°28'14.0" N, 14°29'25.0" E; 740–775 m; 14.5.2012, KH, MJ, JK & IŠ	20/10	3
	JAK	Slovenia; Polhov Gradec town, Mt. Sv. Jakob; 46°06'19.0" N, 14°22'11.0" E; 780–790 m; 15.5.2012, KH, MJ, JK, IŠ & AC	19/10	3
	LOI	Austria; Karawanken Mts, Loiblpass saddle; 46°26'41.0" N, 14°15'28.0" E; 990–1005 m; 29.5.2012, MJ, KD & IŠ	21/5	3

Taxon	Population code	Locality details	MORF/ SEED	FCM
	LOR	Slovenia; Polhov Gradec town, Mt. Sv. Lorenz; 46°04'18.0" N, 14°17'59.0" E; 780–790 m; 15.5.2012, KH, MJ, JK, IŠ & AC	20/0	3
	MAR	Austria; Niederöstereich, Ramsau bei Hainfeld village, Mariental valley; 47°59'04.9" N, 15°49'49.4" E; 510–525 m; 29.5.2012, MJ, KD & IŠ	4/0	3
	PIT	Austria; Niederöstereich, Rosalien Gebirge Mts, Pitten village; 47°42'28.0" N, 16°10'53.0" E; 320–340 m; 4.5.2011, KH, MJ, JK, BŠ, IŠ & KO	20/10	3
	POD	Slovenia; Kozje, Podsreda village; 46°01'34.0" N, 15°35'11.0" E; 470–480 m; 18.5.2012, KH, MJ, JK & IŠ	9/9	2
	TRD	Slovenia; Gorjanci Mts, Mt. Trdinov vrh; 45°45'35.0" N, 15°19'22.4" E; 1135–1185 m; 3.6.2012, KO	15/0	0
	VRE	Slovenia; Senožeče village, Mt. Vremščica; 45°41'15.5" N, 14°03'52.3" E; 1004 m; 30.5.2012, MJ, KD, AC & IŠ	15/0	2
T. longifolia subsp. *pseudocrispa* (Fiori) Greuter (Tlp)	GNI	Italy; Julian Alps, Gniviza village; 46°19'55.8" N, 13°19'32.6" E; 1066–1075 m; 31.5.2012, MJ, KD & IŠ	13/10	3
	KAM	Italy; Julian Alps, Kamno village; 46°12'36.7" N, 13°37'49.2" E; 194–210 m; 17.5.2012, KH, MJ, JK, IŠ & NJ	20/10	3
	KOL	Italy; Julian Alps, Kolovrat saddle; 46°11'21.7" N, 13°38'34.0" E; 1062–1115 m; 31.5.2012, MJ, KD & IŠ	20/10	3
	LAG	Italy; Julian Alps, Valle del Lago valley; 46°27'00.0" N, 13°34'31.0" E; 880–907 m; 16.5.2012, KH, MJ, JK, & IŠ	20/10	3
	PON	Italy; Julian Alps, Pontebba village; 46°30'28.0" N, 13°18'04.0" E; 615–625 m; 16.5.2012, KH, MJ, JK, & IŠ	20/10	3
	TAN	Italy; Julian Alps, Passo Tanemea saddle; 46°18'06.8" N, 13°20'17.1" E; 793–828 m; 31.5.2012, MJ, KD & IŠ	19/10	3
	ZAG	Slovenia; Julian Alps, Žaga village; 46°17'48.9" N, 13°29'25.5" E; 325–340 m; 17.5.2012, KH, MJ, JK, IŠ & NJ	20/10	3
T. longifolia subsp. *gaudinii* (Gremli) Kerguélen (Tlg)	BAL	Italy; Rhaetian Alps, Monte Baldo Mts; Mt. Altissimo; 45°48'12.6" N, 10°53'26.3" E; 1800–1850 m; 20.6.2012, MJ, KD, LB & IŠ	20/10	3
	BAZ	Italy; Rhaetian Alps, Breno town; Bazena saddle; 45°55'10.5" N, 10°23'52.9" E; 1869–1923 m; 19.6.2012, MJ, KD, LB & IŠ	20/10	3
	CHAS	Switzerland; Rhaetian Alps, loco dicto Alp Trupchun; 46°35'35.5" N, 10°04'52.0" E; 2098 m; 1.7.2012, BŠ	10/10	0
	DOS	Italy; Rhaetian Alps, Darfo-Boario; Dosso village; 45°57'52.1" N, 10°06'59.7" E; 1020–1050 m; 18.6.2012, MJ, KD, LB & IŠ	0/0	3
	FED	Italy; Rhaetian Alps, Val Federia valley; 46°32'57" N, 10°05'39" E; 2030 m; 2.7.2012, BŠ	0/0	3
	FEN	Italy; Rhaetian Alps, Trento town; Mt. Fenner Joch; 46°17'29.1" N, 11°09'20.1" E; 1650–1680 m; 11.7.2011; MJ, IŠ, KD & KH	0/0	3
	GAV	Italy; Rhaetian Alps, Bagolino village; Siltar de Gaver valley; 45°55'19.0" N, 10°27'34.7" E; 1400–1563 m; 19.6.2012, MJ, KD, LB & IŠ	19/10	3

Taxon	Population code	Locality details	MORF/ SEED	FCM
	MIS	Italy; Dolomity Mts, Auronzo Di Cadore; Missurina Lake; 46°35'24.0" N, 12°15'30.0" E; 1750–1770 m; 20.6.2012, MJ, KD, LB & IŠ	13/10	3
T. longifolia subsp. *brachychaeta* Greuter (Tlb)	VAL	Italy; Secciata Mts, Mt. Vallombrosa; 43°44'22.2" N, 11°34'29.2" E; 1230–1325 m; 17.6.2011, KO	15/10	5
T. integrifolia (L.) Holub	INT	Austria; Wiener Wald Mts, Perchtoldsdorfer Heide; 48°08'00.0" N, 16°15'00.0" E; 315 m; 31.5.2012, MJ		3

on living individuals and only the lower and middle leaves, terminal and three lateral capitula with pedicels were collected for further measurements. In order to minimize the effect of phenological plasticity, plant material was collected during the short period of flowering, when the terminal and at least three lateral capitula were flowering. Mature achenes (usually 10 per population, altogether 264) were collected only from selected populations (27, Table 1) at repeated visits after flowering. Achenes were collected randomly from several plants in each of the populations sampled. Voucher herbarium specimens were deposited in the herbarium of the Slovak Academy of Sciences (SAV).

Plant material for measuring the relative DNA content originated from the same populations as that used in the morphometric analyses. From 2 to 5 plants per population were subjected to flow cytometry. Plants were either transferred from natural populations or grown from collected seeds (in the case of highly protected populations). Plants were afterwards cultivated for 1–3 years under uniform conditions in an experimental garden in Banská Bystrica (48°45'08.9"N, 19°09'29.0"E, 390 m a.s.l., central Slovakia).

Karyological analyses

The relative nuclear DNA content was determined for 98 individuals from 33 populations of the *T. longifolia* agg. (Table 2) using DAPI flow cytometry. Moreover, 3 plants from a population of the closely related species *T. integrifolia* (L.) Holub were included. DAPI flow cytometry was chosen because absolute DNA content, estimated by intercalating PI, and the relative DNA content, estimated using AT-selective DAPI dye, is highly correlated and DAPI flow cytometry is more accurate and particularly useful for detecting rather small differences in genome size (Marhold et al. 2010, Suda et al. 2010, Olšavská et al. 2012). To ensure the accuracy of the estimates of relative DNA content, we used fresh leaf material and each plant was analysed separately. Further, fluorescence of at least 5000 particles was recorded and only histograms with a symetrical peak and a coefficient of variance (CV) of the standard and sample G1 peaks below 3% were considered.

Flow cytometric analyses were done in November 2012 at the Institute of Botany, Slovak Academy of Sciences, Bratislava, using a Partec Cyflow ML instrument (Partec GmbH, Münster, Germany) equipped with an HBO-100 mercury arc lamp as an excitation source. An AT-specific 4',6-diamidino-2-phenylindole (DAPI) was used as a flourochrome and *Bellis perennis* L. (2C = 3.38 pg; Schönswetter et al. 2007) as an internal standard in flow cytometric analyses.

Table 2. – Relative DNA content expressed as the ratio of G1 peak of standard (*Bellis perennis* L.; 2C = 3.38 pg; Schönswetter et al. 2007) and G1 peak of the sample (RSS of investigated taxa and populations of *Tephroseris longifolia* and *T. integrifolia*); N – number of investigated plants; SD – standard deviation. Populations for which chromosome counts 2n = 48 have been published by Kochjarová (1997) are marked by asterisk. See Table 1 for abbreviations of taxon codes.

Taxon	Intrataxonomic variation of RSS			Intrapopulation variation of RSS		
	Taxon code (N)	Mean±SD (pg)	Variation (%)	Population code (N)	Mean±SD (pg)	Variation (%)
Tephroseris longifolia agg.	Tlm (15)	2.8±0.06	0.34	CAV (3)	2.82±0.04	0.13
				RAD* (3)	2.80±0.06	0.38
				LYS (3)	2.86±0.06	0.31
				OMS (3)	2.78±0.01	0.01
				STR* (3)	2.73±0.02	0.03
	Tllh (6)	2.74±0.02	0.03	GOS (3)	2.74±0.02	0.04
				ZAL (3)	2.74±0.02	0.02
	Tlla (31)	2.79±0.04	0.14	EBE (3)	2.82±0.01	0.01
				FAL (3)	2.86±0.02	0.05
				FUR (3)	2.74±0.04	0.12
				HIR (3)	2.80±0.02	0.05
				JAK (3)	2.77±0.04	0.16
				LOI (3)	2.81±0.04	0.16
				LOR (3)	2.79±0.02	0.05
				MAR (3)	2.76±0.02	0.03
				PIT (3)	2.78 ±0.01	0.02
				POD (2)	2.79±0.01	0.02
				VRE (2)	2.78±0.01	0.01
	Tlp (20)	2.92±0.04	0.13	GNI (3)	2.92±0.06	0.35
				KAM (3)	2.92±0.02	0.04
				KOL (3)	2.93±0.05	0.22
				LAG (3)	2.91±0.02	0.03
				PON (3)	2.91±0.01	0.01
				TAN (3)	2.91±0.01	0.02
				ZAG (3)	2.93±0.07	0.49
	Tlg (21)	3.07±0.04	0.14	BAL (3)	3.10±0.06	0.35
				BAZ (3)	3.05±0.02	0.06
				DOS (3)	3.05±0.01	0.01
				FED (3)	3.05±0.02	0.05
				FEN (3)	3.10±0.02	0.05
				GAV (3)	3.09±0.00	0.00
				MIS (3)	3.04±0.04	0.19
	Tlb			VAL (5)	3.36±0.03	0.07
Tephroseris integrifolia				INT (3)	2.33±0.03	0.09

Nuclei isolation and staining procedure followed the two-step protocol (Doležel et al. 2007) with some modifications. Intact leaf tissue of the plant analysed was chopped together with an internal standard in 1 ml of ice-cold Otto I buffer (0.1 M citric acid, 0.5% Tween 20). The crude nuclear suspension was filtered through 42-μm nylon mesh. For staining, 1 ml of a solution containing Otto II buffer (0.4 M $Na_2HPO_4 \cdot 12H_2O$), 2-mercaptoethanol (2 μl/ml) and DAPI (4 μg/ml) was added to the flow-through fraction. Samples were analysed after 10 min incubation at room temperature. Flow cytometric histograms were evaluated using Partec FloMax software (v. 2.7d; Partec GmbH Münster, Germany). Simultaneous analyses of samples from the *T. longifolia* agg. differing by

more than 5% in DNA content were done to confirm the reliability of the estimated values. Relationship between chromosome number and relative DNA content was verified using previously published chromosome counts (Table 2; Kochjarová 1997). Relative DNA content was calculated as the ratio of G1 peak of standard and G1 peak of sample (RSS).

The Tukey-Kramer test for unequal sample sizes was used to test for differences in relative DNA content (RSS values) among the taxa studied [*T. longifolia* subsp. *moravica* (Tlm), *T. l.* subsp. *longifolia* – Pannonian morphotype (Tllh), *T. l.* subsp. *longifolia* – Alpine morphotype (Tlla), *T. l.* subsp. *pseudocrispa* (Tlp), *T. l.* subsp. *gaudinii* (Tlg), *T. l.* subsp. *brachychaeta* (Tlb); see results of morphological analyses]. The Spearman rank correlation coefficient was used to test whether relative DNA content of populations is related to their geographic origin (longitude, latitude and altitude). All analyses, including box-and-whiskers plots, were carried out using Statistica 7.0 (StatSoft Inc. 2006).

Morphological analyses

Morphological variability was studied using multivariate morphometric analyses based on 525 individuals originating from 33 populations of the *T. longifolia* agg. (Fig. 1). The characters of achenes were scored or measured on smaller samples (27 populations and 264 seeds, Table 1) and as they did not come from the same individuals as those used in the morphometric study, achene characters were analysed separately.

Altogether 31 quantitative and nine qualitative characters were measured or scored on fresh and/or herbarium material (Fig. 2). Subsequently nine ratios were computed (Table 3). Characters of synflorescences were measured only on fresh material; separate measurements were made for terminal and three different lateral capitula of the same synflorescence, and only mean values of three measurements of the lateral capitula were used in all morphometric analyses. Qualitative characters were scored as two (LUSB, LLSB) or three (SLI, SUI, BI, LLUSI, LLLSI, LMLSI, AI) binary characters/stages, from which only one or two stages were included in the analyses.

Several datasets were used in the morphometric analyses. Foremost, Pearson (parametric) and Spearman (non-parametric) correlation coefficients were computed for all data matrices in order to determine the relationships between particular variables. Some pairs of characters were strongly correlated, which potentially distorted further computations (more than 0.97) and therefore one character from a pair was always excluded (matrix 2: SIU1, BI1, SLUB, LLD, CLD; matrix 3–5: SLUB, see below).

Five datasets were used in further analyses: (i) a dataset consisting of 27 populations of the *T. longifolia* agg. (Table 1) characterized by mean values of four characters measured or scored on achenes (matrix 1); (ii) a dataset with 33 population samples of the *T. longifolia* agg. from the whole distribution area (Table 1) characterized by mean values of all 46 characters measured or scored on stem, leaves and synflorescences as OTUs (operational taxonomic units) (matrix 2); (iii) a complete, pooled dataset including 525 individual plants from 33 populations of the *T. longifolia* agg. and 50 characters measured as OTUs (matrix 3); (iv) a dataset including 398 individuals from Austria, Italy (excluding population VAL) and Slovenia and 50 characters measured (matrix 4); (v) a dataset of 296 individuals from Austria, Czech Republic, Hungary, Slovakia and Slovenia (except ZAG) and 50 characters measured (matrix 5).

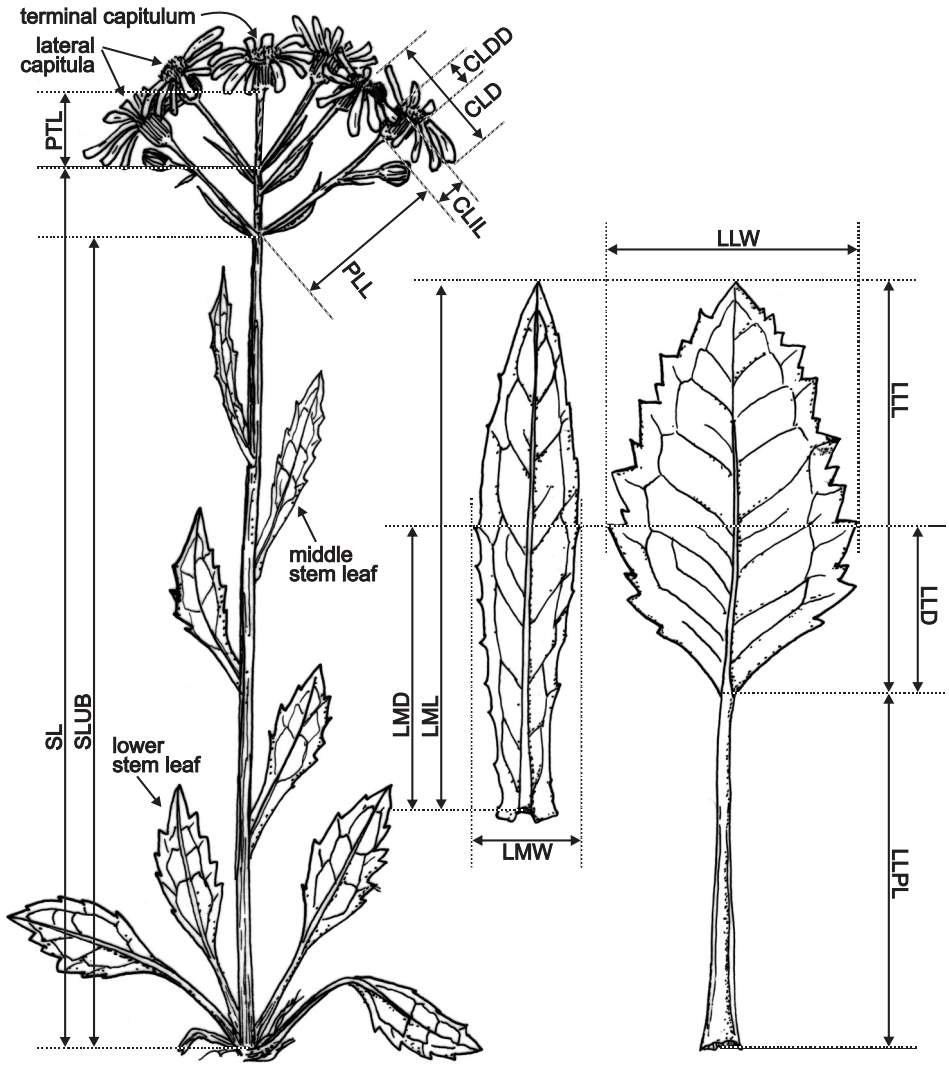

Fig. 2. – Illustrations of selected morphological characters of the *Tephroseris longifolia* agg. For character abbreviations see Table 3.

We performed both a hierarchical and non-hierarchical multivariate evaluation of the morphological data in the following steps: (i) Cluster analysis (Everitt 1986), UPGMA (unweighted pair-group method using arithmetic averages), was carried out on matrix 1 in order to infer potential morphological differentiation of achenes within the *T. longifolia* agg. (ii) To obtain an insight into the phenetic relationships among all the populations of the *T. longifolia* agg. studied a principal coordinate analysis (PCoA) was undertaken, using a Gower's coefficient for mixed data (Legendre & Legendre 2012) because the number of OTUs exceeded the number of characters, based on matrix 2.

Table 3. – List of characters scored or measured for the morphometric analyses of the *Tephroseris longifolia* agg. Characters marked with an asterisk are represented by mean values of three measurements from three different lateral capitula of the same synflorescence; characters marked with a circle were used only for calculating ratios and were not included in the morphometric analyses.

Plant part	Character	Description	Character explanation/measurement unit
Stem	NL	number of leaves	–
	SLI	indument of lower part of stem	0 – glabrous/glabrescent 1 – sparsely to moderately hairy (SLI 1) 2 – densely hairy to arachnoid (SLI 2)
	SUI	indument of upper part of stem	0 – glabrous/glabrescent 1 – sparsely to moderately hairy (SUI 1) 2 – densely hairy to arachnoid (SUI 2)
	PH	plant height (stem and synflorescence rachis length)	mm
	SLUB°	stem length up to branching	mm
	ratio: SLUB/PH		
Synflorescence	BI	indument of involucral bracts	0 – glabrous/glabrescent 1 – sparsely to moderately hairy (BI 1) 2 – densely hairy to arachnoid (BI 2)
	NPC	number of primary capitula	–
	NSC	number of secondary capitula	–
	CTD	terminal capitulum diameter	mm
	CTDD	terminal capitulum disc diameter	mm
	CTIL	terminal capitulum involucre length	mm
	CTIW°	terminal capitulum involucre width	mm
	PTL	length of pedicel of terminal capitulum	mm
	CLD*	lateral capitulum diameter	mm
	CLDD*	lateral capitulum disc diameter	mm
	CLIL*	lateral capitulum involucre length	mm
	CLIW*	lateral capitulum involucre width	mm
	PLL*	length of pedicel of lateral capitulum	mm
	ratios: CTIL/CTIW, CLIL/CLIW		
Stem leaves	LLUSI	indument of upper surface of lower stem leaf	0 – glabrous/glabrescent 1 – sparsely to moderately hairy (LLUSI 1) 2 – densely hairy to arachnoid (LLUSI 2)
	LLLSI	indument of lower surface of lower stem leaf	0 – glabrous/glabrescent 1 – sparsely to moderately hairy (LLLSI 1) 2 – densely hairy to arachnoid (LLLSI 2)
	LMLSI	indument of lower surface of middle stem leaf	0 – glabrous/glabrescent 1 – sparsely to moderately hairy (LMLSI 1) 2 – densely hairy to arachnoid (LMLSI 2)
	LUSB	presence of persistent hair's bases on upper surface of leaves	0 – no persistent base of hairs 1 – persistent base of hairs presented
	LLSB	presence of persistent hair's bases on lower surface of leaves	0 – no persistent base of hairs 1 – persistent base of hairs presented
	LLNT	number of teeth of lower stem leaf	–
	LMNT	number of teeth of middle stem leaf	–
	LLL	length of blade of lower stem leaf	mm
	LLW°	width of blade of lower stem leaf	mm
	LLD	distance of widest part of blade of lower stem leaf (from leaf base)	mm
	LLPL	length of petiole of lower stem leaf	mm
	LLBA	angle of base of blade of lower stem leaf	°

Plant part	Character	Description	Character explanation/measurement unit
	LLTD	depth of maximum tooth of lower stem leaf	mm
	LML°	length of blade of middle stem leaf	mm
	LMW	width of blade of middle stem leaf	mm
	LMD	distance of middle part of blade of lower stem leaf (from leaf base)	mm
	LMBA	angle of base of blade of middle stem leaf	°
	LMTW	width of maximum tooth of middle stem leaf	mm
	LMTD	depth of maximum tooth of middle stem leaf	mm
	ratios: LLW/LLL, LLD/LLL, LLPL/LLL, LMW/LML, LMD/LML		
Achenes	AI	indument of achenes	0 – glabrous/glabrescent 1 – hairy in the lower part (AI 1) 2 – hairy (AI 2)
	AL	achenes length	mm
	AW	achenes width	mm
	ratio: AW/AL		

In order to test the morphological differentiation indicated by PCoA and UPGMA and identify the characters most responsible for the differentiation among taxa we ran several canonical discriminate analyses (CDA). In addition to the PCoA and UPGMA results, also variation in DNA content, geographic origin and traditional taxonomic designation of particular populations was taken into account as criteria for the predefinition of groups (see Results). The following CDA analyses were performed: (iii) CDA1 based on matrix 3 [six groups predefined: *T. longifolia* subsp. *moravica* (Tlm), *T. l.* subsp. *longifolia* – Pannonian morphotype (Tllh), *T. l.* subsp. *longifolia* - Alpine morphotype (Tlla), *T. l.* subsp. *pseudocrispa* (Tlp), *T. l.* subsp. *gaudinii* (Tlg), *T. l.* subsp. *brachychaeta* (Tlb)], (iv) CDA2 based on matrix 4 (three groups predefined: Tlla, Tlp, Tlg) and (v) CDA3 based on matrix 5 (three groups predefined: Tlm, Tllh, Tlla). (vi) Parametric and non-parametric (with k = 12) classificatory discriminate analyses (Klecka 1980, Krzanowski 1990) based on matrix 3 were used to assess the percentage of plants correctly assigned to the predetermined groups. (vii) Descriptive data analysis (univariate statistics) was used to obtain basic statistics of quantitative characters and ratios (minimum, mean, percentile 5%, 10%, 90% and 95%, maximum and standard deviation) for each taxon revealed. For semi-quantitative and binary characters the frequencies of particular states are presented. For illustrating the variation in the characters selected box-and-whiskers plots were used.

PCoA analysis was performed using Canoco 5.0 (ter Braak & Šmilauer 2012), CDA analyses were carried out using SAS v.9.3 (SAS Institute Inc. 2011); cluster analysis was computed in SYN-TAX 2000 (Podani 2001) and box-and-whiskers plots were constructed in Statistica 7.0 (StatSoft Inc. 2006).

Results

Karyological analyses

Flow cytometric analyses of relative DNA content resulted in high-resolution histograms with mean CVs of G1 peaks from 1.08 to 2.91% (mean 1.58%) and from 1.37 to 2.97% (mean 1.75%) for samples of the *T. longifolia* agg. and internal reference standard, respectively (Table 2).

The relative DNA content (RSS values) within the *T. longifolia* agg. varied by 25.8% from 2.71 (for population FUR) to 3.36 (for population VAL). It is highly likely that these estimates correspond to a hexaploid ploidy level 2n = 6x = 48 (Kochjarová 1997 for *T. l.* subsp. *moravica* from populations RAD and STR). The RSS value (2.33±0.03) obtained for one of the populations of *T. integrifolia* studied indicate a lower relative DNA content than recorded for the *T. longifolia* agg. Results indicate only low intrapopulation variation in relative DNA content (RSS varied up to 0.49%).

Box-and-whisker plots (Fig. 3) of relative DNA content (depicted as RSS values) indicated differences among the groups defined based on traditional assignment as well as the morphotypes revealed by multivariate morphometrics (see below). Results of the Tukey-Kramer test confirmed significant differences (at $P < 0.001$) among *T. l.* subsp.

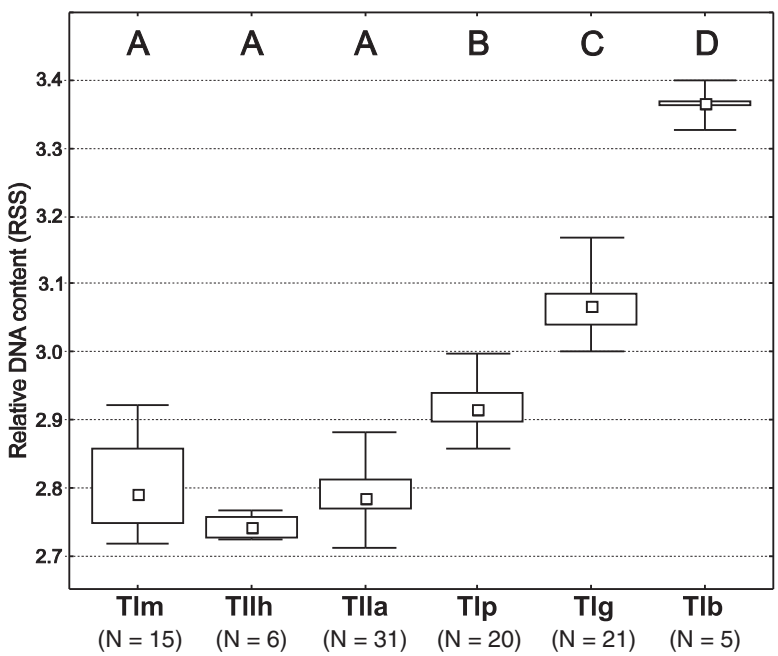

Fig. 3. – Relative DNA content represented by the ratio of G1 peak of standard (*Bellis perennis* L.; 2C = 3.38 pg; Schönswetter et al. 2007) and G1 peak of sample (RSS) of particular taxa of *Tephroseris longifolia* agg. (N = number of individuals). Boxes define 25th and 75th percentiles, squares show median values, whiskers extend from the minimum to the maximum. Same letters indicate groups of taxa that are not significantly different at $P < 0.001$ (Tukey-Kramer test).

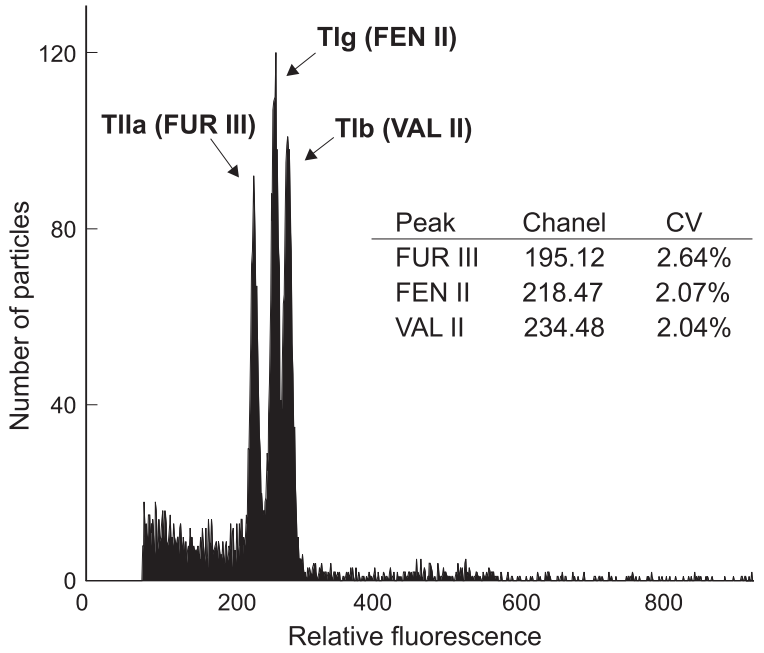

Fig. 4. – Flow cytometric histogram of the relative DNA content of DAPI-stained nuclei in a simultaneous analysis of individuals belonging to *Tephroseris longifolia* subsp. *longifolia* – Alpine morphotype (Tlla), *T. l.* subsp. *gaudinii* (Tlg) and *T. l.* subsp. *brachychaeta* (Tlb). Nuclei of plants were isolated, stained with DAPI and analysed simultaneously.

pseudocrispa (Tlp), *T. l.* subsp. *gaudinii* (Tlg), *T. l.* subsp. *brachychaeta* (Tlb) and a group consisting of *T. l.* subsp. *moravica* and *T. l.* subsp. *longifolia* (Tlm+Tllh+Tlla), this indicates that DNA content may be used as a supportive taxonomic marker within the aggregate (Fig. 3). The differences in DNA content among taxa were confirmed in simultaneous flow cytometric analyses (Fig. 4). Variation in DNA content within groups was rather low (RSS varied up to 0.34%).

The relative DNA content (RSS) recorded for the populations studied was positively correlated with altitude ($sr = 0.65$, $P < 0.0001$), and negatively correlated with longitude ($sr = –0.84$, $P < 0.0001$) and latitude ($sr = –0.57$, $P < 0.0001$).

Morphological analyses

Cluster analyses (UPGMA) based on achene characters resulted in the division of the populations of the *T. longifolia* agg. into two main clusters (Fig. 5). The populations originally assigned to *T. l.* subsp. *pseudocrispa* (Tlp), *T. l.* subsp. *gaudinii* (Tlg) and *T. l.* subsp. *brachychaeta* (Tlb) form the left cluster, while those assigned to *T. l.* subsp. *longifolia* and *T. l.* subsp. *moravica* (Tlm) form the right cluster. The only exception was population LOI of *T. l.* subsp. *longifolia*, which is included in the left cluster.

PCoA of 33 populations of the *T. longifolia* agg. based on characters of the stem, leaves and synflorescences showed clear separation of the VAL population (Tlb) along

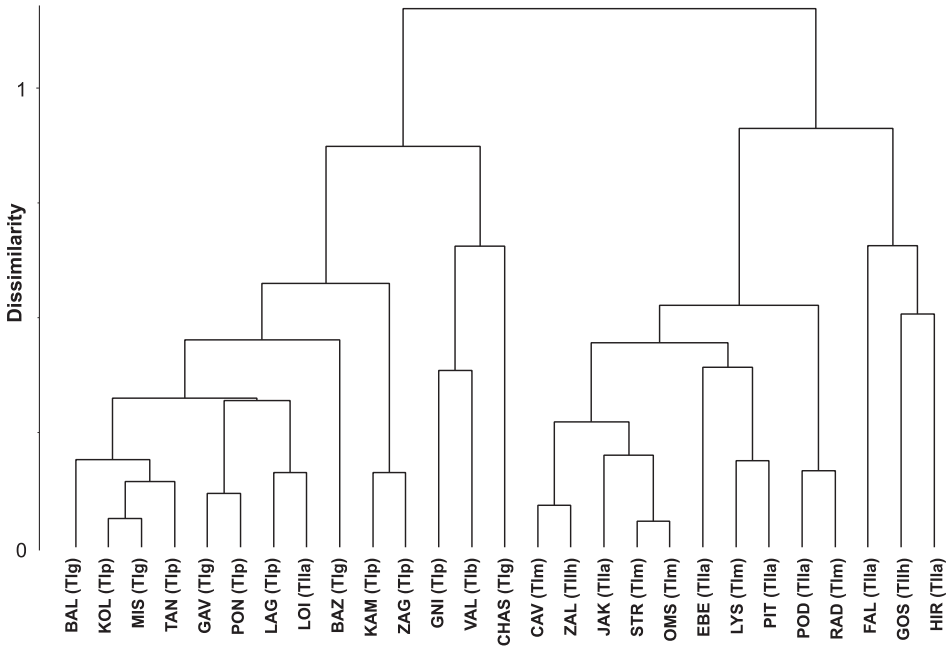

Fig. 5. – Cluster analysis (UPGMA) based on four characters measured or scored on achenes from 27 populations of *Tephroseris longifolia* agg.: *T. l.* subsp. *moravica* (Tlm), *T. l.* subsp. *longifolia* – Pannonian morphotype (Tllh), *T. l.* subsp. *longifolia* – Alpine morphotype (Tlla), *T. l.* subsp. *gaudinii* (Tlg), *T. l.* subsp. *pseudocrispa* (Tlp) and *T. l.* subsp. *brachychaeta* (Tlb). For population codes see Table 1.

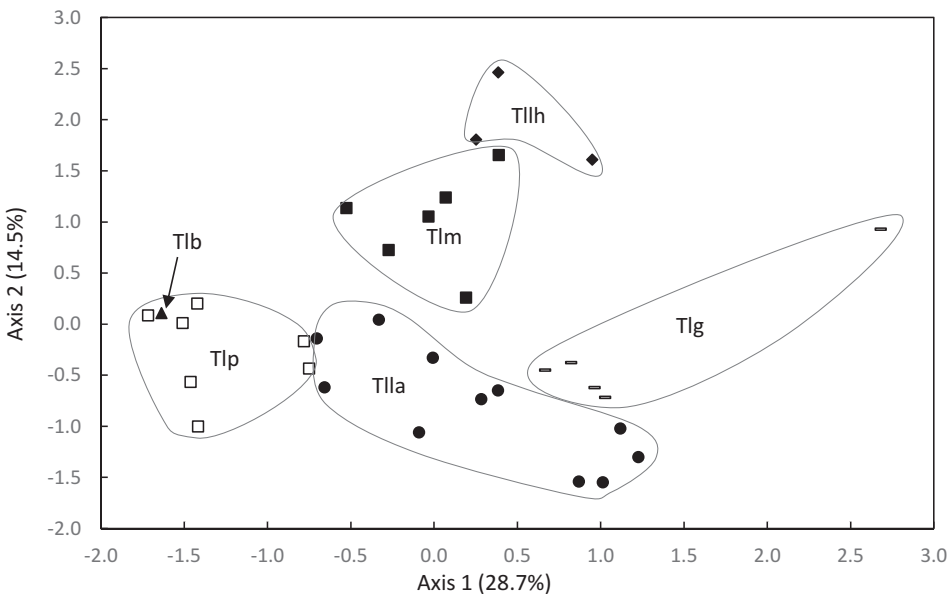

Fig. 6. – Principal coordinate analysis based on 46 morphological characters and 33 populations of *Tephroseris longifolia* agg.: *T. l.* subsp. *moravica* (Tlm, ■), *T. l.* subsp. *longifolia* – Pannonian morphotype (Tllh, ◆), *T. l.* subsp. *longifolia* – Alpine morphotype (Tlla, ●), *T. l.* subsp. *gaudinii* (Tlg, ▬), *T. l.* subsp. *pseudocrispa* (Tlp, □) and *T. l.* subsp. *brachychaeta* (Tlb, ▲).

the third axis (diagram not shown). Remaining populations formed five groups (Fig. 6), three of them corresponding broadly to previously recognized subspecies: Tlm, Tlp and Tlg. Populations traditionally assigned to *T. longifolia* subsp. *longifolia* formed two separate groups according to their geographic origin with Alpine populations (Alpine morphotype; Tlla) situated in the centre of the lower part of the diagram and Pannonian populations (Pannonian morphotype; Tllh) forming a group in the centre of the upper part close to the population of Tlm.

To test the plausibility of dividing the *T. longifolia* agg. into the six groups predicted by PCoA and UPGMA analyses (Tlm, Tllh, Tlla, Tlg, Tlp and Tlb) and at the same time identify the morphological characters suitable for distinguishing the groups, several CDA analyses and a DA analysis based on individuals and 50 characters were performed.

CDA1 indicated that individuals of Tlb tend to separate mainly along the third axis correlated with plant height (PH), shape of involucrum (CLIL, CLIL/CLIW) and length of middle stem leaf (LML) (Electronic Appendix 2B, Table 4). Almost all the leaf characters measured contributed equally to the separation of Tlp, Tlla, Tlm, Tllh and Tlg along the first axis of the CDA1 (Electronic Appendix 2b, Table 4). On the other hand, characteristics of the indumentum of the stem (SUI), involucral bracts (BI) and leaves (LLLSI, LMLSI) were strongly associated with the second axis along which groups Tlp+Tlm+Tllh and Tlla+Tlg were differentiated (Electronic Appendix 2A, Table 4).

In order to obtain a better picture of the position of samples, in further analyses well-separated samples of Tlb were omitted and remainders were reanalysed in two subsets of the results of morphological analyses (UPGMA, PCoA, CDA1), geographic origin and differences in DNA content. In the CDA2 all Alpine samples (Tlla, Tlp, Tlg) were included whereas in the CDA3 samples of Tlla and two morphologicaly similar and geographically neighbouring Tllh and Tlm populations were included. Tlla samples were included in both analyses as it occupies a somewhat central position in the PCoA and CDA1 diagrams (Fig. 6, Electronic Appendix 2A).

CDA2 indicates a clear separation of Tlla, Tlp and Tlg with minor overlaps (Fig. 7A, Table 4). Characters such as the indumentum on the stem (SLI), involucral bracts (BI) and leaves (LLLSI, LMLSI) and characters related to shape of the lower leaves (LLL, LLD, LLPL/LLL) predominantly contributed to their separation along the first axis while characters of the capitulum (CTD, CLD, PTD) contributed to their separation along the second axis.

CDA3 indicates separation of three groups corresponding to Tlla, Tllh and Tlm. Characters related to plant height (NL, PH), shape of involucrum (CLIL, CLIL/CLIW) and lower leaves (LLW/LLL) as well as the indumentum on the leaves (LLLSI, LUSB) played role in their separation along the first axis. Characteristics of their synflorescences (PLL, CTIL/CTIW, CLIL/CLIW) largely contributed to the separation along the second axis (Fig. 7B, Table 4).

Parametric and non-parametric discriminant analyses of six morphotypes identified by the ordination analyses, Tlm, Tllh, Tlla, Tlp, Tlg and Tlb, revealed that more than 78% of the individuals could be correctly classified. The largest morphological overlap was between the groups Tlla and Tlg (13.6% misclassified individuals; Table 5).

In accordance with the CDA1, univariate statistics (Electronic Appendix 3) and box-and-whisker plots (Fig. 8) of quantitative characters of all six morphotypes revealed by the multivariate morphometric analysis the plants of Tlb differ in the majority of the characters

Table 4. – Total canonical structure expressing correlations of characters with canonical axes (CDA1, CDA2, CDA3; the values exceeding the level of 0.4 are in bold). The values were retrieved from CDA analyses based on 50 morphological characters and individuals as OTUs: CDA1 based on 525 individuals with six groups predefined: *Tephroseris longifolia* subsp. *moravica* (Tlm), *T. l.* subsp. *longifolia*, Pannonian morphotype (Tllh), *T. l.* subsp. *longifolia*, Alpine morphotype (Tlla), *T. l.* subsp. *pseudocrispa* (Tlp), *T. l.* subsp. *gaudinii* (Tlg) and *T. l.* subsp. *brachychaeta* (Tlb); CDA2 based on 398 individuals with three groups predefined: Tlla, Tlp and Tlg; CDA3 based on 296 individuals with three groups predefined: Tlm, Tllh and Tlla. For character explanations see Table 3.

Character	CDA1			CDA2		CDA3	
	Axis1	Axis 2	Axis 3	Axis 1	Axis 2	Axis 1	Axis 2
NL	–0.207	0.236	0.152	–0.041	–0.283	**–0.442**	–0.060
SLI1	0.246	0.342	0.107	0.398	–0.211	–0.211	–0.019
SLI2	–0.271	**–0.548**	–0.060	**–0.568**	0.297	0.363	0.331
SUI1	0.178	0.397	0.153	**0.407**	–0.138	–0.300	0.092
SUI2	–0.177	**–0.405**	–0.149	**–0.413**	0.144	0.307	–0.076
PH	–0.006	0.378	**0.509**	0.128	–0.138	**–0.519**	–0.082
SLUB/PH	–0.064	0.034	0.072	–0.079	–0.321	–0.157	–0.297
BI1	0.234	0.502	–0.159	**0.598**	–0.058	–0.224	–0.100
BI2	–0.251	**–0.586**	0.188	**–0.677**	0.097	0.285	0.083
NPC	–0.110	0.150	0.205	–0.036	–0.008	–0.254	–0.047
NSC	–0.126	0.283	0.287	0.068	0.083	–0.331	0.044
CTD	0.273	0.240	0.252	0.345	**0.508**	0.071	–0.045
CTDD	–0.182	0.164	0.065	–0.018	0.180	–0.222	–0.058
CTIL	–0.071	–0.032	**0.527**	–0.263	–0.247	–0.323	0.220
CTIW	0.103	–0.145	0.061	–0.091	–0.351	0.086	–0.358
PTL	–0.070	0.188	–0.062	0.155	**0.441**	–0.064	0.249
CLD	0.234	0.261	0.267	0.323	**0.444**	–0.001	–0.031
CLDD	–0.097	0.310	0.142	0.123	–0.007	–0.365	–0.034
CLIL	–0.190	0.063	**0.532**	–0.272	–0.065	**–0.425**	0.044
CLIW	0.012	–0.100	–0.027	–0.079	–0.100	0.081	–0.387
PLL	0.099	0.301	0.148	0.295	0.253	–0.164	**0.437**
CTIL/CTIW	–0.168	0.101	**0.482**	–0.190	0.042	–0.331	**0.486**
CLIL/CLIW	–0.224	0.162	**0.572**	–0.233	0.018	**–0.489**	**0.405**
LLUSI1	0.245	0.173	0.085	0.275	–0.118	–0.033	–0.024
LLUSI2	–0.271	–0.399	–0.013	**–0.474**	0.282	0.227	0.122
LLLSI1	–0.039	–0.028	–0.203	0.021	0.116	0.088	–0.058
LLLSI2	–0.014	**–0.551**	**0.410**	**–0.521**	0.261	**0.402**	0.250
LMLSI1	0.172	0.139	–0.029	0.253	0.212	0.077	–0.042
LMLSI2	–0.241	**–0.550**	0.172	**–0.617**	0.047	0.249	0.280
LUSB	–0.092	0.376	–0.015	0.216	–0.174	**–0.418**	–0.125
LLSB	0.105	**0.404**	0.022	0.327	–0.121	–0.297	–0.204
LLNT	0.314	–0.096	–0.148	0.219	0.146	0.364	–0.144
LMNT	**0.488**	0.025	–0.044	0.387	–0.002	0.335	–0.213
LLL	**–0.518**	–0.070	0.204	**–0.515**	0.227	–0.283	–0.217
LLW	**0.538**	0.125	0.303	**0.412**	0.059	0.251	–0.083
LLD	**–0.587**	–0.048	0.128	**–0.533**	0.283	–0.319	–0.221
LLPL	**0.400**	0.111	0.247	0.275	–0.335	0.024	–0.150
LLBA	0.147	–0.127	–0.060	0.054	0.342	0.317	0.082
LLTD	**0.539**	0.051	0.121	0.395	–0.097	0.314	–0.095
LML	0.181	–0.064	**0.424**	–0.068	–0.091	0.046	–0.202
LMW	**0.556**	0.085	0.387	0.373	0.025	0.283	–0.066
LMD	0.218	–0.088	0.226	0.012	0.014	0.180	–0.281
LMBA	–0.039	0.122	0.084	0.044	0.086	–0.112	–0.142
LMTW	**0.415**	–0.068	0.162	0.205	0.054	0.317	–0.143
LMTD	**0.518**	–0.003	0.251	0.320	–0.035	0.356	–0.053
LLW/LLL	**0.806**	0.226	–0.017	**0.763**	–0.204	**0.425**	0.079
LLD/LLL	**–0.594**	–0.073	0.048	**–0.494**	0.321	–0.306	–0.132
LLPL/LLL	**0.562**	0.164	0.037	**0.486**	–0.336	0.163	–0.069
LMW/LML	**0.595**	0.164	0.075	**0.536**	0.123	0.326	0.021
LMD/LML	0.151	–0.051	–0.126	0.131	0.191	0.216	–0.239

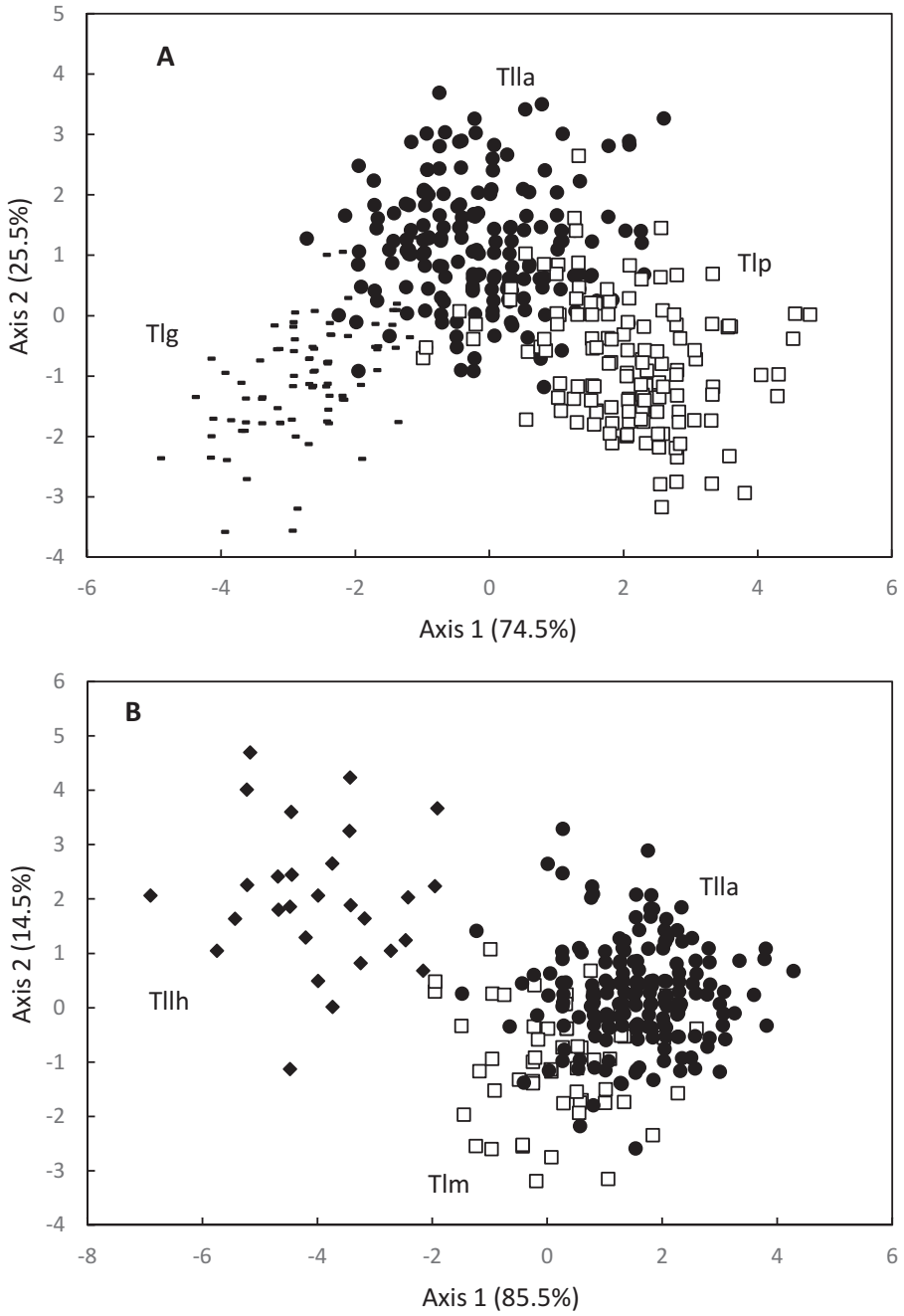

Fig. 7. – Canonical discriminant analyses based on 50 morphological characters of individuals from *Tephroseris longifolia* agg. (A) CDA2 based on 398 individuals with three groups predefined: *T. l.* subsp. *longifolia* – Alpine morphotype (Tlla, ●), *T. l.* subsp. *gaudinii* (Tlg, ▬) and *T. l.* subsp. *pseudocrispa* (Tlp, □). (B) CDA3 based on 296 individuals with three groups predefined: *T. l.* subsp. *moravica* (Tlm, □), *T. l.* subsp. *longifolia* – Pannonian morphotype (Tllh, ◆) and *T. l.* subsp. *longifolia* – Alpine morphotype (Tlla, ●).

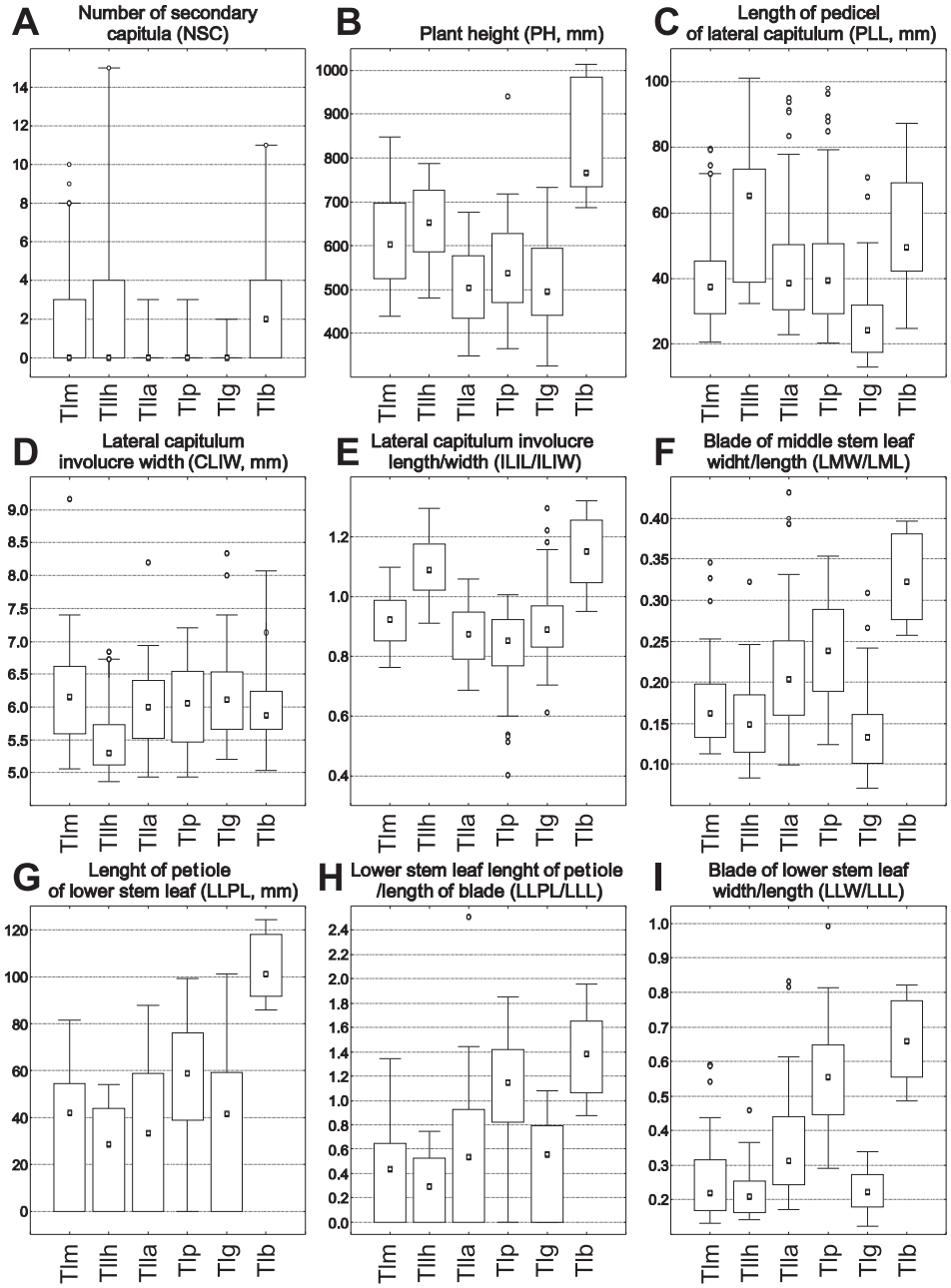

Fig. 8. – Box-and-whisker plots displaying the variation in selected morphological characters among six groups within *Tephroseris longifolia* agg.; *T. l.* subsp. *moravica* (Tlm, N = 84), *T. l.* subsp. *longifolia* – Pannonian morphotype (Tllh, N = 28), *T. l.* subsp. *longifolia* – Alpine morphotype (Tlla, N = 184), *T. l.* subsp. *gaudinii* (Tlg, N = 132), *T. l.* subsp. *pseudocrispa* (Tlp, N = 82) and *T. l.* subsp. *brachychaeta* (Tlb, N = 15). Boxes define the 25th and 75th percentiles, squares show the median, whiskers extend from the 5th to 95th percentiles and circles show outliers.

Table 5. – Results of parametric (P) and non-parametric (N; k = 12) discriminant analysis of individuals of *Tephroseris longifolia* agg. with the following six predefined groups: *T. l.* subsp. *moravica* (Tlm), *T. l.* subsp. *longifolia* – Pannonian morphotype (Tllh), *T. l.* subsp. *longifolia* – Alpine morphotype (Tlla), *T. l.* subsp. *pseudocrispa* (Tlp), *T. l.* subsp. *gaudinii* (Tlg) and *T. l.* subsp. *brachychaeta* (Tlb).

Actual group		Group membership predicted (number of observations and percentage classified into groups)					
		Tlm	Tllh	Tlla	Tlp	Tlg	Tlb
Tlm	P	**69 (82.1%)**	5 (6.0%)	3 (3.6%)	3 (3.6%)	4 (4.8%)	0 (0%)
	N	**66 (78.6%)**	2 (2.4%)	3 (3.6%)	4 (4.8%)	8 (9.5%)	0 (0%)
Tllh	P	3 (10.7%)	**25 (89.3%)**	0 (0%)	0 (0%)	0 (0%)	0 (0%)
	N	4 (14.3%)	**23 (82.1%)**	0 (0%)	0 (0%)	0 (0%)	0 (0%)
Tlla	P	9 (4.9%)	2 (1.1%)	**148 (80.4%)**	17 (9.2%)	8 (4.4%)	0 (0%)
	N	6 (3.3%)	1 (0.5%)	**156 (84.8%)**	8 (4.4%)	11 (6.0%)	0 (0%)
Tlg	P	1 (0.8%)	0 (0%)	12 (9.1%)	**116 (87.9%)**	2 (1.5%)	1 (0.8%)
	N	2 (1.5%)	0 (0%)	18 (13.6%)	**108 (81.8%)**	3 (2.3%)	1 (0.8%)
Tlp	P	1 (1.2%)	0 (0%)	3 (3.7%)	0 (0%)	**78 (95.1%)**	0 (0%)
	N	1 (1.2%)	1 (1.2%)	3 (3.7%)	0 (0%)	**77 (93.9%)**	0 (0%)
Tlb	P	0 (0%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)	**15 (100%)**
	N	0 (0%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)	**15 (100%)**

investigated: its plants were the tallest (PH, Fig. 8B) with the widest capitula (CTD, CLD) and widest stem leaves (LLW, LMW) (Electronic Appendix 3). The lower stem leaves of Tlb always had a petiole (LLPL, Fig. 8G), which was approximately of the same length as the blade (LLPL/LLL, Fig. 8H).

The plants of Tlm and Tllh were taller (PH, Fig. 8B) and had more leaves (NL, Electronic Appendix 3) than Tlla, Tlp and Tlg. The middle leaves of Tllh were wider (LMW) with the widest part closer to the base (LMD) than in other groups of the *T. longifolia* agg. (Electronic Appendix 3). Plants of Tllh differed from Tlm, Tlla, Tlp and Tlg also in having a taller and narrower terminal and lateral involucres (CLIW, Fig. 8D; CLIL/CLIW, Fig. 8E; CTIL, CTIW, CLIL, CLIWCTIL/CTIW, CLILCLIW, Electronic Appendix 3). Synflorescences of Tlla, Tlp and Tlg were only rarely secondarily branched (NSC, Fig. 8A). Tlp differed from Tlm, Tllh, Tlla and Tlg mainly in having broader lower leaves (widest part close to blade base) with a long petiole usually of the same length as the leaf blade (LLW/LLL, Fig. 8I; LLD, LLD/LLL, Electronic Appendix 3; LLPL, Fig. 8G; LLPL/LLL, Fig. 8H). Plants of Tlg differed from Tlm, Tllh, Tlla and Tlp in that the diameter of its capitula is smaller (CTD, CLD, Electronic Appendix 3) and pedicels of the lateral capitula shorter (PLL, Fig. 8C; PTL, Electronic Appendix 3).

Frequencies of qualitative characters (Electronic Appendix 4) revealed that the plants investigated could be divided based on the indumentum on the achenes: Tlp, Tlg and Tlb always had hairy achenes, while those of Tllh were glabrescent and those of Tlm and Tlla were predominantly glabrescent. Persistent bases of hairs were present mainly on the upper leaf surface of Tlla and Tlg (more than 70% of plants), absent on the lower leaf surface of Tlm and Tlb or were only rarely present on the lower leaf surfaces of Tllh and Tlp (less than 7% of the plants). Differences in the indumentum on the stem, leaves and involucral bract among the groups investigated were indistinct. In general, the most hairy were plants of Tlg and Tlb, while the lower surface of the leaves of Tlm and Tlp were predominantly glabrescent.

Discussion

Karyological variation

The relative DNA content data presented are the first published records for the *T. longifolia* agg. and *T. integrifolia*. Previously, genome size data were available for only four plants of *T. helenitis* s.l. from the Untersberg region (Salzburg, Austria) (Schistek, unpubl. data in Pflugbeil 2012).

As all the chromosomal records for the *T. longifolia* agg. are the same (2n = 6x = 48; Kochjarová 1997 for *T. l.* subsp. *moravica*; Afzelius 1949, Krähenbühl & Küpfer 1992, Druskovic & Lovka 1995 for *T. l.* subsp. *longifolia*) the accessions analysed for relative DNA content in this study are also hexaploids. The chromosome number 2n = 6x = 48, recorded for the *T. longifolia* agg., is also reported for other European members of the genus, e.g. *T. crispa* (Jacq.) Schur. (Skalińska et al. 1974, Krahulcová 1990, Kochjarová 1997), *T. helenitis* (L.) B. Nord. (Afzelius 1949), *T. integrifolia* subsp. *integrifolia* (Krach 1988, Kochjarová 1997, 2006), *T. papposa* (Rchb.) Schur (Kochjarová 1997, 2005, Mráz 2005) and *T. palustris* (L.) Rchb. (Lövkvist & Hultgård 1999). However, the exceptions are high polyploids with 2n = 8x = 64: *T. integrifolia* subsp. *capitata* (Wahlenb.) B. Nord. (Váchová 1970, Kochjarová 2006) and 2n = 12x = 96: *T. integrifolia* subsp. *capitata* (Favarger 1965, Kochjarová 2006), *T. integrifolia* subsp. *vindelicorum* Krach (Krach 1988) and *T. integrifolia* subsp. *aurantiaca* (Willd.) B. Nord. (Uhríková & Májovský 1980, Kochjarová 2006).

Differences in the DNA content among related taxa with the same chromosome number are nowadays widely accepted as one of the attributes of taxa (Šmarda & Bureš 2006). Thus DNA content can be used as a supporting characteristic for circumscribing taxa at various taxonomic levels and for resolving complex low-level taxonomies (reviewed in Loureiro et al. 2010, Šmarda & Bureš 2010) even at the intraspecific level (Moscone et al. 2003, Pečinka et al. 2006, Schönswetter et al. 2007, Slovák et al. 2009, Suda et al. 2010, Olšavská et al. 2012). In the present study, the significant taxa-specific differences in relative DNA content detected can be used as a supportive taxonomic marker for distinguishing morphotypes/taxa within the *T. longifolia* agg. (Fig. 3).

In some plant groups nuclear DNA content and environmental conditions and/or geographical distribution are correlated (Pečinka et al. 2006, Dušková et al. 2010). In the case of the *T. longifolia* agg. the variation in DNA content is correlated with environmental variables such as altitude and geographic location, as populations with the smallest genomes belonging to *T. l.* subsp. *moravica*, and subsp. *longifolia* are predominantly distributed at lower altitudes in the north-eastern part of the distribution of the *T. longifolia* agg., while populations with bigger genomes belonging to *T. l.* subsp. *gaudinii* grow at high altitudes in the southeastern part of the Eastern Alps. Our analyses revealed very little DNA-content variation in Alpine populations of *T. l.* subsp. *longifolia* in spite of their broad distribution (Fig. 1), altitudinal range (300–1800 m a.s.l.; Table 1) and wide ecological niche (M. Janišová et al., unpubl. results). Close phylogenetic relationships could account for the overlap in RSS values of relative DNA content of populations of *T. l.* subsp. *moravica* and subsp. *longifolia*, which are, however, distinct from the three remaining taxa. In the case of *T. l.* subsp. *gaudinii* their large genome correlates with high altitudes and a high number of frost days (M. Janišová et al., unpubl. results). This finding is contrary to the common observation of a predominance of taxa with small genomes at

high altitudes (Šmarda & Bureš 2010) and might be accounted for by greater tolerance of freezing of taxa with large cells and therefore large genomes (MacGillivray & Grime 1995). For plants growing at high altitudes, such tolerance of freezing would circumvent the limitation of low spring temperatures on mitosis and enable rapid early growth (Grime et al. 1985). A positive correlation of genome size with altitude is recorded in other plant genera in central Europe (Albach & Greilhuber 2004, Olšavská et al. 2012).

Morphological variation

While the taxonomy, ecology and population biology of some of the related taxa in the genus *Tephroseris* have been studied (*T. integrifolia*: Widén 1987, 1993, Widén & Anderson 1993, Isaksson 2009, Meindl 2011; *T. crispa*: Czarnecka 1995, 2006, 2008; *T. helenitis*: Brunerye 1969, Pflugbeil 2012), our study is the very first morphometric investigation of the *T. longifolia* agg. The results underlined morphological complexity and variability of this group previously suggested (Chater & Walters 1976).

Validity of several characters traditionally considered as useful for discriminating between taxa was not confirmed. On the other hand, some other characters seem to be relevant (see Electronic Appendix 1 for comparison of discriminate characters of subspecies/morphotypes cited in the most important floras and those revealed by our study). More specifically, the cordate base of the basal leaves is cited as the typical character of *T. l.* subsp. *pseudocrispa* (Fiori 1903, Pignati 1982, Martinčič et al. 1999). Our results indicated only small differences in the angle of blade bases of lower stem leaves, but on the other hand the shape of these leaves (longer petiole, broader blade) appeared to be important for its delimitating. In accordance with previous authors (Chater & Walters 1976, Pignatti 1982, Kochjarová & Hrouda 2004, Fischer et al. 2008), we confirmed that the character of achene indumentum can be used to discriminate between *T. l.* subsp. *moravica* and subsp. *longifolia* (glabrescent or only sparsely hairy) and other subspecies in the aggregate (densely hairy). Pflugbeil (2012) reports that genetic differentiation (AFLP markers) within *T. helenitis* is correlated with the type of indumentum on the achenes: two genetic clusters were identified at the northern fringe of the Alps, the western populations assigned to the first cluster had mainly glabrous or sparsely hairy achenes typical of *T. helenitis* subsp. *salisburgensis* (Cufod.) B. Nord. while plants of the eastern populations assigned to the second cluster had only pubescent achenes typical of the nominate subspecies.

Type of indumentum (density and distribution of hairiness) on stem, leaves and involucral bracts is often used in identification keys as discriminant characters (Chater & Walters 1976, Pignatti 1982), but our result indicate that differences among the groups investigated were not large enough and only some trends were recorded (Electronic Appendix 1, 2). Another character of indumentum frequently used in *T. longifolia* taxa discrimination is presence/absence of persistent hair's bases (Chater & Walters 1976, Holub 1979, Kochjarová & Hrouda 2004). This character is often misinterpreted by some authors as presence of glandular hairs (Chater & Walters 1976, Fischer et al. 2008). For example, *T. l.* subsp. *moravica* was described based on absence of persistent hair's bases (Holub 1979), while the upper surface of leaves of subsp. *brachychaeta* is reported as rough because of their presence. Our study indicated that hair's bases persist on upper surface of leaves of *T. l.* subsp. *pseudocrispa*, subsp. *gaudinii*, subsp. *brachychaeta* and

Alpine morphotype of subsp. *longifolia*, while they are present only rarely on leaves of *T. l.* subsp. *moravica* and Pannonian morphotype of subsp. *longifolia*.

Our preliminary investigation indicated that coloration of involucral bracts was highly variable (green and reddish) in all the populations sampled, including *T. l.* subsp. *pseudocrispa* and subsp. *gaudinii*, in which exclusively green involucral bracts were previously reported (Pignatti 1982, Wagenitz 1987, Fischer et al. 2008). Therefore, we decided not to include this character in the morphometric analyses. Because the number of involucral bracts was not counted in this study, we could not confirm or disprove a difference in the number is a useful character for distinguishing between *T. l.* subsp. *longifolia* and subsp. *gaudinii* (21 involucral bracts indicated for subsp. *longifolia* and 13 for subsp. *gaudinii*; Pignatti 1982, Wagenitz 1987, Adler et al. 1994, Fischer et al. 2008). But contrary to expectation, the data presented revealed a narrower involucrum for Pannonian and Alpine morphotypes of *T. l.* subsp. *longifolia* [3.0–9.6 mm for terminal (CTIW) and 3.7–7.1 mm for lateral (CLIW) capitula] than for subsp. *gaudinii* [4.9–10.0 mm for terminal (CTIW) and 4.8–8.3 mm for lateral (CLIW) capitula] (Electronic Appendix 1; for CLIW see also Fig. 8D).

It has to be stressed that many morphological characters of *T. longifolia* are phenologically variable (e.g. elongation of stems and pedicels, decreasing density of overall indumentum) and this should be taken into consideration when identifying taxa. Therefore, we made an effort to collect plant material at the same phenological stage (time when the terminal and at least three lateral capitula are flowering, which occurs over a short period of 3–5 days) in order to eliminate this effect on the pattern of morphological variation.

Taxonomic level and endemic status of the taxa investigated

Unstable taxonomic position of *T. longifolia* agg. taxa (for example *T. l.* subsp. *pseudocrispa* is assigned to *T. crispa* and *T. l.* subsp. *brachychaeta* included in *T. l.* subsp. *gaudinii* by some authors; Hayek 1929, Chater & Walters 1976, Adler et al. 1994) and the relatively late circumscription of the Carpathian taxon *T. l.* subsp. *moravica* (Holub 1979) is most probably due to only slight differences in morphology and high variability in this aggregate, as is also confirmed by this study.

Based on our results, the most different, in terms of morphology and DNA content, are samples of *T. l.* subsp. *brachychaeta*. Degree of detected differentiation argues for their recognition at the species level. Unfortunately only one population of this rather rare Italian taxon was studied. Therefore, whether this is a result of long-term isolation of Apennine populations or a genetic relation of other *Tephroseris* lineages should be verified by including more populations of this taxon as well as related species and using molecular markers.

In accordance with the recent concept (Euro+Med 2006–2014), the taxonomic rank of "subspecies" is justifiable for *T. l.* subsp. *moravica*, subsp. *longifolia*, subsp. *pseudocrispa*, and subsp. *gaudinii* as their distributions in general do not overlap (or only to a minor degree), they have different ecological requirements (Janišová et al. 2013) and display taxon-specific DNA content as illustrated by this study. However, these subspecies can only be distinguished morphologically based on a few characters that often differ only slightly. Moreover, these taxa are not reproductively isolated, which implies recent divergence (Šingliarová et al. 2013).

Interestingly, two morphologically distinct groups of populations were identified within the nominate subspecies, which are associated with their geographic origin: Pannonian morphotype and Alpine morphotype. Analyses showed that Pannonian populations are morphologically closer to populations of the Western-Carpathian endemic *T. l.* subsp. *moravica* than to Alpine populations of nominate subspecies. In addition, to their morphology, populations from the Carpathians and Pannonia are similar also in terms of ecological requirements and habitat preferences (Hegedüšová et al. 2013, Janišová et al. 2013). Our data suggest that Pannonian morphotype does not belong to the nominate subspecies. Whether it is distinct subspecies within the *T. longifolia* agg. and their morphological similarity is a result of parallel evolution in similar habitats or it is a part of the variation within *T. l.* subsp. *moravica*, requires further study using genetic analyses. Such a study will also conclusively clarify the status of *T. l.* subsp. *moravica* because although its separation from all Alpine populations was confirmed by this study other morphological similarities with the Panonnian morphotype of *T. l.* subsp. *longifolia* question its geographic restriction to the Western Carpathians. Several studies have revealed the endemic status of rare species thanks to comprehensive taxonomic investigations including the use of molecular markers (e.g. Schönswetter et al. 2004, Španiel et al. 2011a, b, Kučera et al. 2013). On the other hand, there are several examples of taxa, previously thought to be endemic, that were proven to represent only part of the continual variation (often peripheral populations) of a more widespread species (Kolarčik et al. 2010, Španiel et al. 2011a, Petrova et al. 2014).

Protection status

Whether the endemic status of *T. l.* subsp. *moravica* is confirmed or not, we would like to stress that the habitats of *T. longifolia* populations deserve protection and regular management as in the last few decades all types of grassland and semi-natural habitats have become rarer and increasingly more fragmented due to changes in land use practices or abandonment of traditionally used areas (Hillier et al. 1990, Gustavsson et al. 2007, Meindl 2011). Restriction to such specific and vulnerable habitats most probably underlies the decline in abundance recorded over the last 30 years for Carpathian populations of *T. l.* subsp. *moravica* (still present at nine localities, it is considered to be extinct at seven localities; Grulich 2012, Hegedüšová et al. 2013) and Pannonian populations of *T. l.* subsp. *longifolia* (present at 19 microlocalities in Zala region, it is considered to be extinct at 12 microlocalities; Soó 1970, Károlyi et al. 1975, Farkas 1999, G. Király, pers. comm.).

Detailed studies of *T. l.* subsp. *moravica* (Kochjarová 1998, Chmelová 2007, Janišová et al. 2012b) report massive seed production: average percentage of well-developed achenes per flowering shoot in particular populations varied from 54% to 85%, mean numbers of well-developed achenes per capitulum ranged from 80 to 128 and percentage germination was also relatively high, about 70%. However, the granivorous butterfly *Phycitodes albatella* Ragonot causes a severe reduction in total seed set and the emergence of seedlings represents a critical stage in the plant's population demography (Janišová et al. 2012b). In situ, number of seedlings of *T. l.* subsp. *moravica* emerging was very low (0.9–2.1%) because their recruitment from seeds is very dependent on the availability of microsites (disturbances, gaps with little competition) suitable for germination (Janišová et al. 2012b). Thus potential genetic depletion due to small population

size and isolation, which often results in reduced seed set (Aguilar et al. 2012, Morgan et al. 2013), does not seem to play a role in populations of *T. l.* subsp. *moravica*. Above data indicates that reduced fitness of seedlings (including competitive ability) is more likely to hamper establishment and persistence of new individuals and populations.

Remarks on speciation and potential biogeography of the Tephroseris longifolia agg.

Weak morphological differentiation among subspecies of the *T. longifolia* agg. might be due to recent divergence and thus a short time for speciation. Even if plants of different subspecies grow close by and there is apparently no reproductive barrier, identification of intermediate forms indicating gene flow could be difficult because of the minute morphological differences between taxa. No hybrids are reported so far either within the *T. longifolia* agg. or between *T. longifolia* agg. taxa and other closely related species. The only record of the existence of hybrids between *T. longifolia* subsp. *gaudinii* and *T. integrifolia* subsp. *capitata* in the surroundings of lake Como is mentioned by Hess et al. (1972). In addition, one population with intermediate morphology between *T. longifolia* subsp. *moravica* and *T. crispa*, which is regarded as a putative hybrid, is recorded in the Western Carpathians (J. Kochjarová, unpubl. results).

Experimental hybridization revealed no reproductive isolation among the subspecies of *T. longifolia* (Janišová et al. 2012b, Šingliarová et al. 2013) and thus divergences among these taxa have been most likely maintained mainly by geographical isolation. Distinct differences in DNA content, morphological differences (this study) coupled with different environmental requirements and habitat preferences recorded for members of the *T. longifolia* agg. (Hegedüšová et al. 2013, Janišová et al. 2013) indicate that the current pattern is a result of allopatric speciation.

Because extensive parts of the current distribution range of the *T. longifolia* agg. in the Alps were glaciated during the Wuermian (Pleistocene, last glacial maximum, about 18,000 years ago) it is likely that Alpine populations of *T. longifolia* (re)colonized this area postglacially from refugia in the vicinity of the glaciers. Similarly, there is molecular evidence indicating that the related species *T. helenitis* survived the Pleistocene glaciations in an Alpine refugium close to its current distribution at the northern fringe of the Alps (Pflugbeil 2012). Thus processes of allopatric diversification should be connected with the separation of populations of a common ancestor of the *T. longifolia* agg. into different refugia situated at the southern and northern fringes of the Alps, as proposed by Schönswetter et al. (2005). The current distributions of *T. l.* subsp. *pseudocrispa* and subsp. *gaudinii* is in accord with the subdivisions between the two major areas of glacial survival identified in the Eastern Alps as suggested by molecular data (Schönswetter et al. 2005). Outside the Alps, the refugium identified in the Alpe Apuane Mts (Médail & Diadema 2009) could account for persistence of *T. l.* subsp. *brachychaeta* and a long period of isolation from the remaining subspecies might have resulted in its morphological and karyological peculiarities. Pannonian and Carpathian population could have survived the Ice Ages in local refugia close to their current distributions as the important role of the Western Carpathians for the survival of plants during Pleistocene glaciations is repeatedly emphasized (Fér et al. 2007, Mráz et al. 2007, Olšavská et al. 2011, Kučera et al. 2013). Survival and recolonization from different refugia might trigger morphological and karyological differentiation in parallel with ecological specialization.

Because Pannonian and Alpine morphotypes of *T. l.* subsp. *longifolia* and Carpathian populations of subsp. *moravica* are closely related (same DNA content and morphologically similar as well as similar ecological requirements) another scenario needs to be considered. They might have diverged recently (in a postglacial period) and their population might consist of either more recent immigrants or remnants of a once more widely distributed common ancestor. A similar distribution connecting the Alpine, Carpathian and western-Pannonian area in central Europe is documented for other subalpine or montane taxa, e.g. *T. integrifolia* subsp. *aurantiaca*, *Arnica montana* L., *Globularia cordifolia* L., *Alnus viridis* (Chaix) DC., *Buphthalmum salicifolium* L., *Gentiana asclepiadea* L. and *Crocus vernus* subsp. *albiflorus* (Kit. ex Schult.) Ces. (Pallag 2000, Bartha et al. 2005). Peripheral populations of the *T. longifolia* agg. might have experienced strong selection pressures and the effect of genetic drift (because of reduced gene flow, small effective population size) (Barrett & Husband 1990, Eckert et al. 2008). In order to reveal the pattern of genetic variation and understand past processes, further genetic analyses of the *T. longifolia* agg. and closely related species are required.

Key for identifying the subspecies/morphotypes of the Tephroseris longifolia agg. in central Europe

For confident identification it is necessary to use a combination of characters because of the morphological overlap between some taxa in some characters. The values of the characters given in the key are rounded 10–90 percentiles (5–95 percentiles are given in brackets).

1a Stem length up to the first synflorescence branching 67–89 (–91) cm; length of middle stem leaf (100–) 102–158 (–170) mm, width of lower stem leaves (38–) 42–62 (–64) mm; petioles of lower stem leaves always present, (89–) 90–123 mm long; terminal capitulum diameter (35.3–) 38.3–48.9 (–49) mm, lateral capitulum diameter (35–) 36–41 (–43) mm (Apennines) ..... ***T. l.* subsp. *brachychaeta*** (Tlb)

1b Stem length up to the first synflorescence branching max. 72 (–76) cm; length of middle stem leaf max. 118 (–129) mm, width of lower stem leaves up to 43 (–50) mm; petioles of lower stem leaves present (up to 93 (–100 mm) or absent; terminal capitulum diameter max. 41 (–44) mm, lateral capitulum diameter max 38 (–40) mm (Alps, Carpathians, Pannonia) **2**

2a Achenes glabrescent **3**

2b Achenes hairy **5**

3a Stem length up to the first synflorescence branching (31–) 34–58 (–61) cm; lower surface of stem leaves moderate hairy to arachnoid; persistent hair's bases on upper surface of leaves usually present; synflorescence only rarely secondarily branched with 1–3 secondary capitula (Alps) ..... ***T. l.* subsp. subsp. *longifolia* – Alpine morphotype** (Tlla)

3b Stem length up to the first synflorescence branching (35–) 43–72 (–76) cm; lower surface of stem leaves glabrescent to moderately hairy; persistent hair's bases on upper surface of leaves usually absent; synflorescence only often secondarily branched with 1–13 secondary capitula (Carpathians, Pannonia) .. **4**

4a Middle stem leaf width (6.5–) 9–22 (–24.5) mm, involucrum of terminal capitulum width (6.1–) 6.4–8.5 (–9.3) mm, involucrum of lateral capitulum width (5.1–) 5.2–7.0 (–7.4) mm (Carpathians) ..... ***T. l.* subsp. *moravica*** (Tlm)

4b Middle stem leaf width (5–) 6–14 (–17) mm, involucrum of terminal capitulum width (5.4–) 5.5–7.3 (–7.5) mm, involucrum of lateral capitulum width 4.9–6.1 (–6.5) mm (Pannonia) ..... ***T. l.* subsp. subsp. *longifolia* – Pannonian morphotype** (Tllh)

5a Lamina of lower stem leaves length (36–) 39–77 (–87) mm and width (17–) 18–43 (–50) mm; lower surface of stem leaves glabrescent to moderately hairy ..... ***T. l.* subsp. *pseudocrispa*** (Tlp)

5b Lamina of lower stem leaves length (43–) 48–128 (–144) mm and width (8–) 9–37 (–43) mm; lower surface of stem leaves moderately hairy to arachnoid **6**

6a Length of pedicels of lateral capitula (23–) 25–64 (–77) mm; terminal capitulum diameter (26–) 27–41 (–44) mm, lateral capitulum diameter (24–) 25–38 (–40) mm ..... ***T. l.* subsp. subsp. *longifolia* – Alpine morphotype** (Tlla)

6b Length of pedicels of lateral capitula (13–) 16–46 (–51) mm mm); terminal capitulum diameter 21–33 (–35) mm, lateral capitulum diameter 20–31 (–32) mm ..... ***T. l.* subsp. *gaudinii*** (Tlg)

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Súhrn

Tephroseris longifolia agg. je taxonomicky spletitý komplex trvácich cudzoopelivých rastlín z čeľade *Asteraceae*. V agregáte sa v súčasnosti rozoznáva 5 poddruhov s takmer neprekrývajúcimi sa areálmi a odlišnou geografickou distribúciou: *T. longifolia* subsp. *longifolia* (Tll), subsp. *pseudocrispa* (Tlp) a subsp. *gaudinii* (Tlg) majú ťažisko rozšírenia vo východných Alpách; subsp. *brachychaeta* (Tlb) sa vyskytuje roztrúsene na severe a v centrálnej časti Apeninského polostrova. Jediný karpatský taxón, *T. longifolia* subsp. *moravica*, je známy len z 9 lokalít na Slovensku a v Českej republike a je vedený ako ohrozený taxón európskeho významu (Natura 2000). Napriek mnohým štúdiám venovaným ochrane *T. l.* subsp. *moravica*, neexistujú žiadne poznatky o jeho taxonomickom postavení v rámci agregátu a jeho vzťahoch s najbližšie príbuznými taxónmi/poddruhmi. V predkladanej štúdii sme využili základné biosystematické metódy (multivariačná morfometrika 525 jedincov z 33 populácií založená na 50 znakoch, DAPI prietoková cytometria 98 jedincov z 33 populácií) na zodpovedanie troch otázok: (1) Zodpovedá morfologická variabilita a variabilita relatívneho obsahu DNA v rámci *T. longifolia* agg. súčasnému taxonomickému konceptu? (2) Aké sú evolučné vzťahy v rámci *T. longifolia* agg. na základe morfologických a karyologických dát? (3) Podporujú získané dáta endemické postavenie *T. l.* subsp. *moravica*? Pomocou prietokovej cytometrie bola na homoploidnej úrovni (2n ~ 6x ~ 48) zaznamenaná značná variabilita (25,8%) v relatívnom obsahu DNA, keď pomer vzorky voči štandardu sa pohyboval od 2.71 do 3.36. Zistené rozdiely boli poddruhovo špecifické. Ukázalo sa, že obsah DNA môže byť použitý ako podporný taxonomický znak v rámci agregátu. Variabilita v relatívnom obsahu DNA korelovala pozitívne s nadmorskou výškou lokalít, a naopak negatívne korelovala s ich zemepisnou dĺžkou aj šírkou. Na základe výsledkov morfometrickej štúdie bolo odlíšených šesť morfotypov, ktoré zhruba zodpovedajú v súčasnosti rozlišovaným poddruhom. Výnimku predstavujú populácie tradične priraďované k nominátnemu poddruhu, v rámci ktorých boli identifikované dva odlišné morfotypy zodpovedajúce ich zemepisnému pôvodu: alpský a panónsky morfotyp. Celkovo, zistená morfologická a karyologická diferenciácia rastlín *T. l.* subsp. *brachychaeta* podporuje ich klasifikáciu na úrovni samostatného druhu. Morfologické a karyologické rozdiely medzi ostatnými morfotypmi zodpovedajú poddruhovej úrovni. Prekvapivým výsledkom je, že karpatským populáciam *T. l.* subsp. *moravica* sú morfologicky najpodobnejšie panónske populácie *T. l.* subsp. *longifolia*. To, či tieto populácie predstavujú samostatný poddruh v rámci *T. longifolia* agg. a morfologická podobnosť s *T. l.* subsp. *moravica* je výsledkom paralelnej evolúcie na podobných, človekom ovplyvnených biotopoch alebo sú súčasťou variability *T. l.* subsp. *moravica*, vyžaduje ďalšie štúdium využívajúce aj genetické analýzy.

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