The reassessment of *Taraxacum pieninicum* reveals polyploidy, agamospermy and a substantial range extension

Nové hodnocení druhu *Taraxacum pieninicum* odhalilo polyploidii, agamospermii a značně rozsáhlejší areál

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A remarkable West Carpathian endemic member of *Taraxacum* sect. *Erythrocarpa* (*Compositae*, *Crepidinae*), *Taraxacum pieninicum* Pawłowski, is reassessed from the viewpoint of its reproduction, ploidy level and taxonomy. Numerous previous experimental papers present *T. pieninicum* as a sexual diploid. On the basis of new, taxonomically verified material from the Pieniny Mts, using flow cytometry, FCSS and chromosome counting, *T. pieninicum* is shown to be a tetraploid (2n = 4x = 32) diplosporous agamospermous taxon. It proved to be conspecific with populations from western Slovakia, originally described as *T. erythrocarpum* Kirschner et Štěpánek. Thus, *Taraxacum pieninicum* is not endemic to the Pieniny Mts, but occupies a wider geographical range in the northernmost and westernmost Carpathians. It is a morphologically and taxonomically distinct member of the section *Erythrocarpa*, a section almost confined to the Mediterranean area in Europe. It is hypothesized that the progenitors of *T. pieninicum* should be sought in the northern and central Balkans and its migration or diversification probably followed the South and East Carpathian route.

Keywords: agamospermy, endemism, flow cytometry, karyology, seed screen, *Taraxacum pieninicum*, taxonomy

Introduction

Taraxacum section *Erythrocarpa* Hand.-Mazz. is a group characterized by frequent endemism, with species confined to rocky montane refugia, often calcareous cliffs, mountain summits, mostly in subalpine to alpine zones. Many species occur only at a limited number of localities and are local endemics or narrow endemics; they do not spread outside their original sites (J. Štěpánek & J. Kirschner, unpublished). In Europe, *T.* sect. *Erythrocarpa* is confined to the Mediterranean peninsulas (including the Apennine and

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Iberian Peninsulas) and southern Alps, with two notable exceptions, *T. pieninicum* Pawłowski and *T. erythrocarpum* Kirschner et Štěpánek, which occur in the northernmost and westernmost Carpathians, respectively; for an early account of *T.* sect. *Erythrocarpa* in central and south-central Europe, see Kirschner & Štěpánek (1985).

The name *Taraxacum pieninicum* Pawłowski was published in 1924, on the basis of plants collected from Mt Okrąglica in the Trzy Korony Group, a part of the Pieniny Mts, southern Poland, in 1922. Already in 1923, achenes from the original plants collected by B. Pawłowski were used to grow plants at the Jagiellonian University Botanical Garden, Kraków and other live collections in Poland (Poznań, Lublin) and abroad (Uppsala, 1931). To the best of our knowledge, the latest herbarium specimen of *T. pieninicum* was collected from the Pieniny Mts in 1954 (J. Kornaś, KRA). In the 1970s, the site on Mt Okrąglica underwent changes (a scree and rock slide), and since then *T. pieninicum* was thought to be extinct in the wild (Zarzycki 1976, Tacik 1980), and, reportedly, survived only in botanical gardens. This was considered as a significant loss because this species was regarded as the oldest Pieniny endemic and hypothesized to have evolved already in the Paleogene (Puchalski et al. 2014). The Paleogene hypothesis probably is not correct because the time of origin and early divergence of the whole genus *Taraxacum* is estimated to have taken place in the upper Middle Miocene (Tremetsberger et al. 2013) according to molecular clock analyses, or, the interpretation of *Taraxacum* fossil records, in the Upper Oligocene (Kirchheimer 1948).

As a remarkable Polish endemic, *Taraxacum pieninicum* attracted attention of botanists and conservationists. The most important studies were published by Małecka (1958, 1961, 1963) who studied *T. pieninicum* from the viewpoint of karyology and cytoembryology, and characterized it as a diploid species (2n = 2x = 16) with sexual reproduction, which, at that time, was the first record of a diploid sexual *Taraxacum* in Poland. Małecka (1961 and personal communication in 1984) used young inflorescences of cultivated plants in her research.

Kirschner & Štěpánek (1985: 124) described *T. erythrocarpum* as an agamospermous tetraploid species (2n = 32, see also Kirschner & Štěpánek 1992: 21) very close to *T. pieninicum*, but occurring in West Slovakia. At that time, only rather scanty herbarium material of *T. pieninicum* was available for comparison. The two species apparently are very close to each other, having very similar or almost identical features of achenes and outer phyllaries, and yellow stigmas. There is a difference in plant size and leaf shape, but the main features separating these taxa are (i) contrasting systems of reproduction (sexual, with sporophytic multiallelic self-incompatibility vs. agamospermous), (ii) different ploidy levels (diploid, with 2n = 16, vs. tetraploid, with 2n = 32 in *T. erythrocarpum*), and, as a consequence, (iii) presumably very different patterns of variation in populations (the Hardy-Weinberg multi-genotype equilibrium vs. uniclonal or oligoclonal structure found in the agamospermous taxon).

A new search of the Trzy Korony massif, including Mt Okrąglica, revealed two micropopulations of *T. pieninicum* in 1999 and 2000 (Zarzycki et al. 2001, Wróbel 2004). The rediscovered species was analysed in detail and included in Red Books (Zarzycki et al. 2001, Zarzycki & Szeląg 2006, Wróbel & Zarzycki 2008, Wróbel et al. 2014). The two micropopulations amounted to several dozen plants and seedling recruitment is considered to be very rare (Wróbel et al. 2014). The rediscovery of *T. pieninicum* was followed by an intensive research on its biology, and in particular the methods of

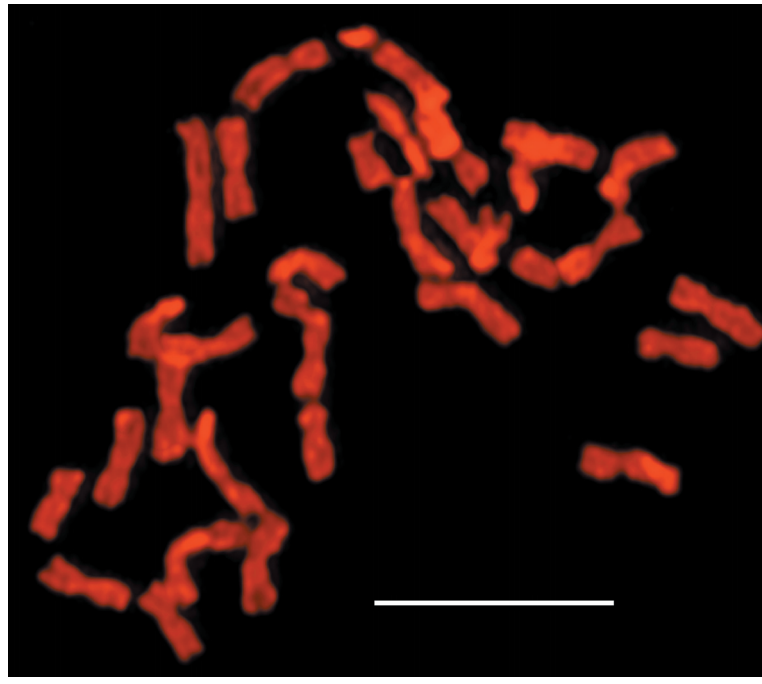

Fig. 1. – *Taraxacum pieninicum.* A metaphase chromosome plate with 2n = 32. Note the size asymmetry. Plant from source A (see Material and methods); scale bar = 5 μm. Photographed by M. Kamińska.

micropropagation and long-term storage, including the genetic stability of regenerants. (Trejgell et al. 2013, Kamińska et al. 2016, 2018, 2020a, b). Kula et al. (2013) studied the karyology of *T. pieninicum* again and confirmed its diploid status (see Discussion).

A large amount of experimental and biological data was accumulated during the exploration of *T. pieninicum*. However, our early observations also planted the seed of doubt about the diploidy and sexuality of *T. pieninicum*, and the separate position of *T. erythrocarpum*. The first, and substantial indication of a weakening of the traditionally accepted picture was the photographs of *T. pieninicum* published after its rediscovery, either directly from the original locality (Wróbel & Zarzycki 2008, Wróbel et al. 2014) or from cultivation (micropropagated regenerants, Kamińska et al. 2016: Fig. 1; Kamińska et al. 2020a: Fig. 6; A. Trejgell, unpublished). J. Kirschner and J. Štěpánek, based on 40 years of taxonomic experience working on *Taraxacum*, realized that the conspecific status of *T. pieninicum* and *T. erythrocarpum* was very probable, because the excellent photographs of *T. pieninicum* from the wild perfectly matched the features of wild *T. erythrocarpum*. Even the well-developed micropropagated plants in cultivation were extremely close to those of *T. erythrocarpum*.

Another indication was the very variable size of pollen grains of plants referred to as *T. pieninicum*, with a size range similar to other *Taraxacum* polyploids (Bednorz & Maciejewska-Rutkowska 2010). Judging from the picture and measurements of achenes, the plant material in the latter work almost surely comes from true *T. pieninicum* sampled near the type locality.

An additional factor to be mentioned is the complicated cultivation of what was originally called *T. erythrocarpum*; this species does not survive more than 2–3 years in cultivation and can be maintained in cultivation by growing it from fresh achenes. Thus, the cultivated *T. pieninicum* might have been replaced by other, more weedy taxa in botanical gardens, before it was sampled for the experimental study. As an example, pictures of plants under the name of *T. pieninicum* from the Lublin Botanical Garden, Poland (Anonymous 2020) undoubtedly belong to a species of *T.* sect. *Taraxacum* (*T. officinale* Wigg. s. lat.). In Kraków where *T. pieninicum* was cultivated, diploid sexuals of *T.* sect. *Taraxacum* (*T. linearisquameum* van Soest) occur quite frequently (J. Štěpánek, unpublished field observations) and in all likelihood, occurs in most of south-eastern Poland (there are records and specimens collected by B. Trávníček in the vicinity of Dukla, vouchers deposited at OL).

Because of the doubts about the status of *T. pieninicum* and the unclear relationship with *T. erythrocarpum*, we carried out a detailed experimental reassessment of *T. pieninicum* on the basis of new, verified plant material. In particular, we focus on genome size, ploidy level, chromosome number and mode of reproduction of *T. pieninicum*.

Material and methods

Plant material

For comparison with *T. pieninicum*, we used published data and chromosome numbers of *T. erythrocarpum* and the ample herbarium material of this species preserved at PRA. Duplicates of *T. erythrocarpum* from two localities, were issued in the exsiccate series of *Taraxaca exsiccata* (fasc. IV: 142; fasc. V: 151, see Kirschner & Štěpánek 1992, 1997), which are preserved in a number of institutions (PRA, M, S, BM, PRC etc.). The plant material examined in this study is deposited at PRA, if not otherwise stated. Most of our revision labels are numbered and refer to the specimens to which they are attached (as "no. det.", not necessarily to duplicates).

For *T. pieninicum*, great attention was devoted to reliable sources of plant material and a verified identification. For the verification, JK & JŠ are responsible. The material came from two independent sources:

(A) Samples used for micropropagation in the laboratory of A. Trejgell, Toruń, Poland. Achenes (source of material for micropropagation) were obtained from the collections of the seed bank of the Botanical Garden of the Polish Academy of Sciences, CBDC in Powsin, Warsaw. Their identification is based on an analysis of achenes (red-brown, large, about 5 mm long, with cylindrical cone usually 1.2–1.5 mm long), and on the examination of detailed photographic documentation of plants in cultivation (colour, margins and posture of outer phyllaries, and yellow stigmas). The samples consisted of a large collection of achenes and several fresh leaves for the flow cytometry analysis (FCM).

(B) Three samples of achenes from the two micropopulations of *T. pieninicum* in Pieniny National Park were collected by G. Vončina. Plants from the same site were photographed and the pictures inspected by JK & JŠ. Achenes in all the three samples have features corresponding to those of *T. pieninicum* (and not present in other Polish dandelions). A few of the achenes from each sample were grown in the Experimental Garden of the Institute of Botany, Czech Academy of Sciences, Průhonice, Czech Republic; plants

again correspond to the type material of *T. pieninicum*. Herbarium specimens of *T. pieninicum* from both micropopulations were deposited at PRA and most of the achenes obtained from cultivation will be returned to the authorities of the Pieniny National Park. The exact locality of samples (B):

1. Polska, Karpaty Zachodnie, Pieniny Centralne, Stromowce Niżne, Trzy Korony massif, Okrąglica peak, [so called] lower locality, in crevices in limestone rock, *Dendranthemo-Seslerietum variae* community, slope S, 960 m a.s.l. ATPOL: EG33 (two samples, cultivated as JK 7007 and JK 7008). Voucher specimen: PRA, no. det. 35799. This site is situated about 20–30 m from the probable locus classicus that was destroyed by a rock slide.

2. Polska, Karpaty Zachodnie, Pieniny Centralne, Stromowce Niżne, Trzy Korony massif, Okrąglica peak, [so called] upper locality, in crevices in limestone rock, *Dendranthemo-Seslerietum variae* community, slope E, 960 m a.s.l. ATPOL: EG33 (cultivated as JK 7009). Voucher specimen: PRA, no. det. 35801. This site is situated about 25 m from a locality of *T. pieninicum* mentioned in Zarzycki (1981, seen and communicated personally by B. Pawłowski in 1970 or 1971); the latter locality no longer exists.

Methods of cultivation

The cultivation methods follow those described in Kirschner & Štěpánek (1993) and Kirschner et al. (2020). For this study, it was important to cultivate all the plants side by side in a cultivation box to evaluate their general habit homogeneity or variability of half-siblings (if the cultivated plants, including the leaf rosettes, have almost identical size, leaf colour, leaf lobation and indentation, the outer phyllary and stigma colour, it is a good indicator of agamospermy).

Karyology

Counting chromosomes in meristematic cells of root tips of microplantlets from source A (counted by M. Kamińska) was made after micropropagation under *in vitro* conditions (seeds germinated on Murashige-Skoog [MS] medium, shoot proliferation onto MS medium supplemented with 1.1 µM benzylaminopurine and 0.14 µM naphthyl-1-acetic acid, and rooting in liquid 1/4 MS medium; Murashige & Skoog 1962, Trejgell et al. 2013). Roots were incubated for one hour with 0.8% colchicine, then root tips (2–3 mm) were isolated, macerated in 1N HCl at 70 °C for 45 min and rinsed in distilled water. Samples were stained with Schiff's reagent for 1 hour at room temperature in the dark, washed in distilled water and rinsed for 10 min in a solution of bleach (10% sodium pyrosulfate and 1N HCl). After rinsing in water meristematic cells of the root tips were squashed and frozen at –80°C. For dehydration a series of graded alcohols was used: 70%, 80% and 95%. Samples were mounted in DPX media (44581, Sigma). Microscopic observations and photography were performed with Fluoview fv 3000 Laser Scanning Microscope (Olympus).

Chromosome counts of plants from source B (counted by J. Kirschner) were made on root tips of germinating achenes, using the methods outlined in Štěpánek et al. (2011). Rootlets were pre-treated with a saturated water solution of p-dichlorobenzene for two hours and then stored in a mixture of ethanol and acetic acid (3:1). After maceration in a mixture of hydrochloric acid and ethanol (1:1), a squash was stained with lacto-

propionic orceine. Chromosome plates were examined using a Zeiss Axio Imager.Z2 microscope.

Genome size

Genome size was estimated using flow cytometry, generally following the methods used in Trávníček et al. (2013) and Prančl et al. (2018). The sample preparation followed the simplified two-step procedure described by Doležel et al. (2007). About 0.25 cm^2 of leaf tissue was chopped together with an appropriate volume of the internal standard using a sharp razor blade in a Petri dish containing 0.5 ml of ice-cold Otto I buffer (0.1M citric acid, 0.5% Tween 20). *Solanum pseudocapsicum* L. was selected as the reference standard, as its genome size is similar but does not overlap that of all the samples studied. The crude suspension was filtered through a 42-µm nylon mesh and incubated for about 5 min at room temperature. After incubation, isolated nuclei were stained with 1 ml of Otto II buffer (0.4 M $Na_2HPO_4 \cdot 12H_2O$) supplemented with 2-mercaptoethanol (2 µg/ml) and propidium iodide (PI) and RNase IIA (both 50 µg/ml), see Otto (1990). Samples were run on the flow cytometer after about five minutes of staining. The samples were analysed using a Partec CyFlow instrument equipped with a green diode-pumped solid-state laser (Cobolt Samba, 532 nm, 150 mW output power) and the fluorescence intensity of 5000 particles was recorded. Three plants from source A were analysed using propidium iodide and the analyses repeated three times on three different days in order to account for random measurement error; if the range of variation of the repeated measurements exceeded the 2% threshold, the outlying values were discarded and the sample reanalysed. Seven plants from sources B1 and B2 were analysed for comparison. Histograms were evaluated using FloMax software, ver. 2.4d (Partec GmbH). The genome size was expressed as the ratio of the mean fluorescences of the sample and the internal standard. Fresh leaves from three plants (each measured three times on three different days) were used. The genome size in absolute units was calculated based on the genome size of *Solanum pseudocapsicum* (2C = 2.59 pg, Temsch et al. 2010). Subsequently, the 1Cx-value was derived from the mean 2C value.

Flow cytometry seed screen

The flow cytometry seed screen (FCSS) was used as a reliable indicator of diplosporous apomixis, based on the ploidy level ratios of embryo and endosperm. The applicability of this method and modifications of techniques were published by Krahulcová & Rotreklová (2010), Dobeš et al. (2013) and Krahulcová & Suda (2006). In *Taraxacum*, the FCSS has been successfully used to disentangle complex relationships between diploids and triploids (and artificial hybrids) in *T.* sect. *Erythrosperma* (H. Lindb.) Dahlst. and *T.* sect. *Taraxacum* (Mártonfiová 2006, 2015, Mártonfiová et al. 2010). Direct flow cytometry on seeds is used to estimate the ploidy levels and ratios for embryos and endosperm (Doležel et al. 2007). Ten mature achenes (with pericarp) were chopped with an appropriate amount of an internal standard (*Solanum pseudocapsicum*, see the FCM methods above) in a Petri dish containing 0.5 ml of ice-cold Otto I buffer. The suspension was filtered through a nylon mesh (mesh size 42 µm) into 3.5 ml cuvettes and incubated for five minutes at room temperature. Isolated nuclei were stained using 1 ml of Otto II buffer supplemented with the AT-selective fluorochrome DAPI (4',6-diamidino-2-phenylindole) and

α-mercaptoethanol at final concentrations of 4 μg · ml^{-1} and 2 μl · ml^{-1}, respectively. After a five-minute incubation at room temperature, the relative fluorescence of 5000 particles was recorded using a CytoFLEX S cytometer (Beckman Coulter, Indianapolis, IN, USA) equipped with a near UV laser (375 nm, 60mW) as the source of excitation light. The resulting histograms were analysed using CytExpert 3.2.1.22 software (Beckman Coulter) and both fluorescence intensities of embryo and endosperm peaks were recorded.

Results

Chromosome number of Taraxacum pieninicum

Plants from both sources (A and B, see Material and Methods) proved to be tetraploid, with 2n = 32 (M. Kamińska, source A; J. Kirschner, plants of JK 7009, source B2, see Material). This chromosome number corresponds to that of *T. erythrocarpum* based on material from two remote localities (Mt Vršatec, Biele Karpaty Mts, and Ostrý kameň, Malé Karpaty Mts, see Kirschner & Štěpánek 1985, 1992). As regards the character of the karyotype, the sample is too small to make quantitative conclusions. The karyotype consists of metacentric and submetacentric chromosomes. What is obvious (Fig. 1) is the size asymmetry, with short and long chromosomes.

Genome size

The genome size of *T. pieninicum* was estimated using plants from source A. The mean 2C value was 3.385±0.021 pg. For comparison, seven plants from the B1 and B2 sources were analysed (plants cultivated, sampled in 2021); the mean 2C value was 3.363±0.014 pg, and therefore virtually identical with that of source A (a difference of 0.6% is below the instrument's accuracy threshold). In *Taraxacum*, this genome size corresponds to tetraploidy, and the $1C_x$ value (0.846 pg) is very close to the genome size of the related *T.* sect. *Erythrosperma* (H. Lindb.) Dahlst. (and the unrelated *T. linearisquameum* of *T.* sect. *Taraxacum*), see also Záveský et al. (2005).

Flow cytometry seed screen

The FCSS was carried out on achenes of sample B2. The embryo: endosperm ratio corresponds to 1: 2 (1: 1.991±0.007 in absolute figures), which is proof of diplosporous agamospermy, with a 4C embryo and 4C+4C endosperm in our tetraploid samples (Fig. 2).

Taxonomy and nomenclature

Based on our results, we conclude that the names *T. erythrocarpum* and *T. pieninicum* belong to the same species. The name *T. erythrocarpum* is a synonym of *T. pieninicum*. It represents an agamospermous tetraploid taxon occurring as a relict on limestone rocks in the Western Carpathians (the Pieniny, Poland; the Malé Karpaty, the Biele Karpaty, the Strážovské vrchy, the westernmost Veľká Fatra and probably also in the Javorníky Mts, all in Slovakia; see the map, Fig. 2, and Kliment 1999).

Among the European members of *T.* sect. *Erythrocarpa*, *T. pieninicum* (incl. *T. erythrocarpum*) is very distinct in having outer phyllaries arcuate-recurved, relatively narrow,

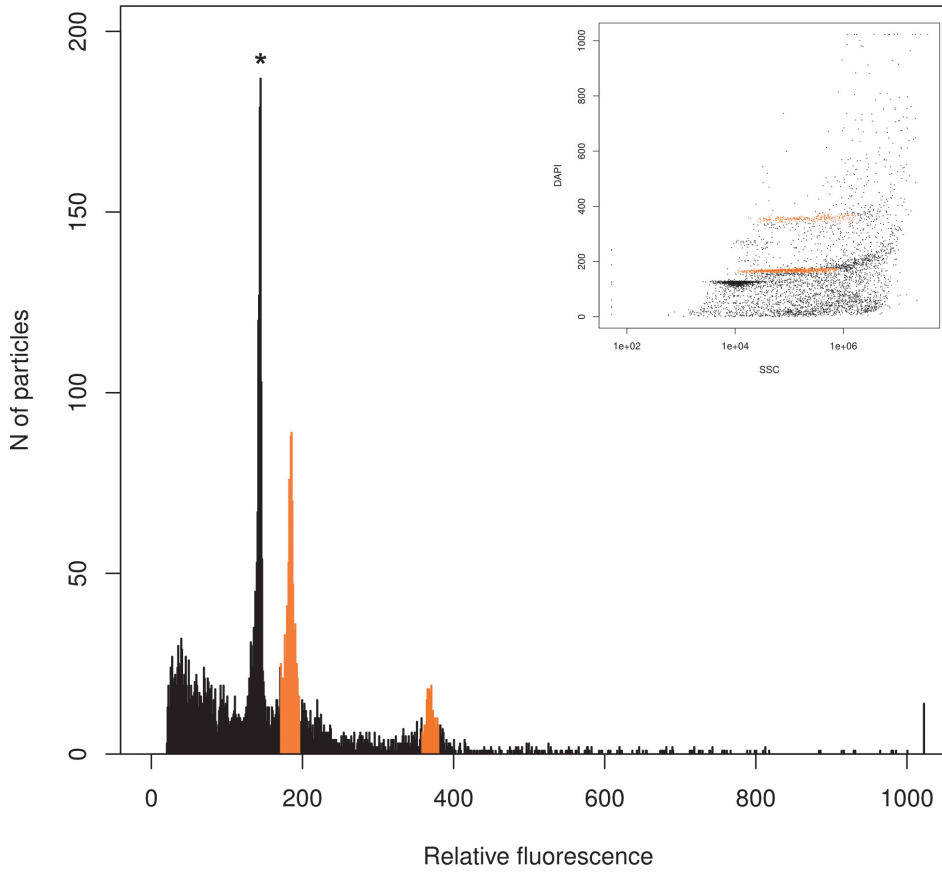

Fig. 2. – Flow cytometry output based on two achenes of *Taraxacum pieninicum*, with *Solanum pseudocapsicum* (indicated by the asterisk, 2C = 2.59 pg) as the standard. Fluorescence signal of nuclei attributed to achenes of *T. pieninicum* (embryo and endosperm) are highlighted in orange. The inserted figure is the dot plot of side-scatter (SSC) and fluorescence intensity (DAPI) of the nuclei in the histogram.

lanceolate to broadly lanceolate to narrowly triangular and pure yellow stigmas (the latter character is seldom found in other members of this section). The achene size, shape and colour of *T. pieninicum* is very close to the characters of core taxa of *T.* sect. *Erythrocarpa*. Achenes most frequently longer than 5.0–5.2 mm and the cone on average 1.4 mm long are diagnostic features when compared with sympatric members of *T.* sect. *Erythrosperma* Kirschner et Štěpánek.

Taraxacum pieninicum Pawłowski, Bull. Int. Acad. Polon. Sci. Lettr., Cl. Math. Natur., Sér. B, Sci. Natur. 1–2: 109 (1924)

≡ *Taraxacum hoppenaum* subsp. *pieninicum* (Pawłowski) Pawłowski, Ochrona Przyrody 11: 211 (1931); isonym: Domin, Preslia 13–15: 250 (1936)

Type: [Poland] Trzy Korony w Pieninach, skały wapienne tuż obok Okrągliny [the Pieniny Mts, Trzy Korony Group, calcareous rocks just next to Mt Okrąglica, the spelling

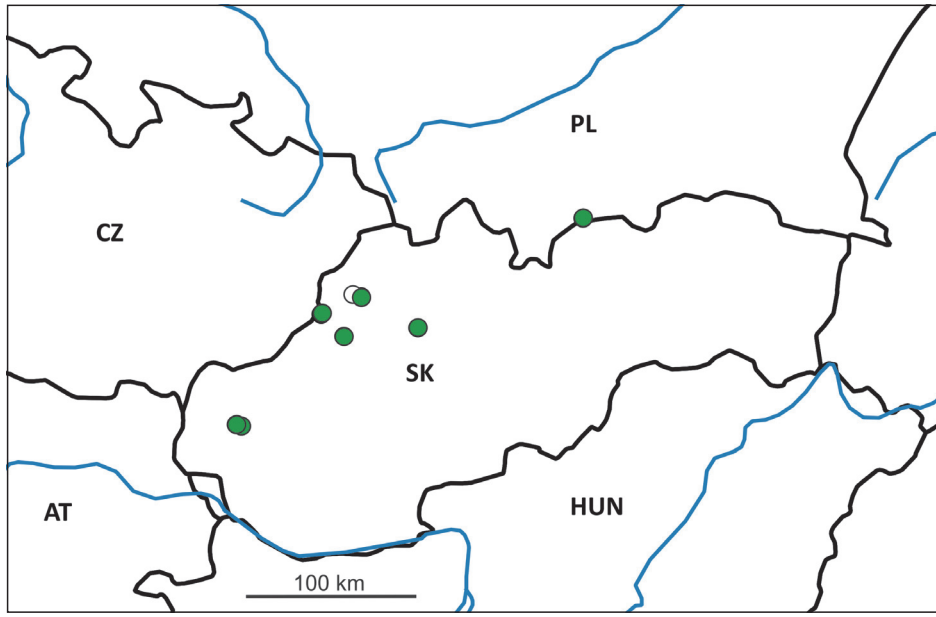

Fig. 3. – Map showing the distribution of *Taraxacum pieninicum*. The empty circle refers to a literature record (Kliment 1999).

of which is correct], 16 Jun 1922, *B. Pawłowski* (KRAM, no. det. 2444, lectotype, fide Kirschner & Štěpánek 1985: 127), in accordance with Tacik, personal communication in 1982; isolectotype: KRAM, no. det. 2443).

= *Taraxacum erythrocarpum* Kirschner et Štěpánek, Preslia 57: 122 (1985)

Type: Slovakia. Malé Karpaty, in rupibus calcareis in jugo ~0.5 km situ orientali a ruina arcis Ostrý kameň, inter pagos Buková et Smolenice [the Malé Karpaty Mts, calcareous rocks on a ridge ~0.5 km east of the ruin of Ostrý Kameň Castle], alt. ~660 m, 17 May 1984, *J. Kirschner* (PRA, no. det. 3701, holotype; isotypes: PRA, no. det. 3592, 3699, 3700 & 3702)

Illustrations: Pawłowski (1924: Fig. 2, Plate 3); Pawłowski (1931, Plate VIII); Kirschner & Štěpánek (1985: Fig. 6, as *T. erythrocarpum*), Fig. 7, Plate IX (1985); Procházka et al. (1999: 367, as *T. erythrocarpum*); Wróbel et al. (2014: 551); Wróbel & Zarzycki (2008: 406); Zarzycki et al. (2001: 392, photograph 126 and a line drawing).

Exsiccates: *Taraxaca Exs*., no. 142, 151.

A new, consolidated description

Plants small to medium-sized, sometimes robust, usually (8–) 10–15 cm tall. Petiole most often narrowly winged, pale green to light pinkish-brownish; plant base with tunic. Leaves greyish green, later greyish deep green, sparsely arachnoid to subglabrous, ± oblanceolate in outline, usually 6–13 × (1.5–) 2.0–2.5 (–3) cm; terminal segment usually broadly triangular, usually 1.5–3 × 1.5–3.5 cm, obtuse to subacute, with distal margin convex to subconvex, entire or with a symmetrical pair of shallow incisions, rarely with

a few minute teeth; lateral segments 3–4 (6), triangular to narrowly triangular, usually hamate-recurved, with distal margin convex, usually entire, sometimes with a few minute teeth, apex subobtuse to subacute, proximal margin straight to variously subconcave, entire or with sparse minute teeth; interlobes relatively broad (3–6 mm), short or relatively long (usually up to 7–8 mm), often with raised margins, usually with 1–2 short lobules and several little teeth; mid-vein usually pale greenish or slightly suffused pinkish-bronze proximally. Scapes irregularly arachnoid, usually suffused purplish at least proximally, ± overtopping leaves, distally very often with 1–2 bract-like rudimentary phyllaries. Involucre usually 10–11 mm wide at base at full anthesis. Outer phyllaries 16–19, regularly or slightly irregularly arcuate-patent to arcuate-recurved, narrowly to broadly lanceolate or narrowly triangular, (4.0–) 5.0–8.0 (–9.5) × (2.0–) 2.3–3.5 (–4.2) mm, initially pale green, at least proximally, most often suffused pink on both surfaces, distally often with an indistinct thin darker middle stripe (developed mostly abaxially), border narrow, ~0.2 mm wide, whitish, apex usually ± corniculate; inner phyllaries 10–14 mm long at anthesis, apex ± callose. Capitulum yellow, usually 2–3 cm wide, outer ligules flat, striped light greyish pink outside. Stigmas pure yellow. Anthers polliniferous, pollen grains irregular in size. Achenes deep brownish red, usually 4.5–5.7 × 1.0–1.2 mm, body ± densely shortly spinulose in upper 1/3 (sometimes spinulose to tuberculate throughout), subabruptly to subgradually narrowing into narrow, cylindrical cone (1.1–) 1.3–1.5 (–1.6) mm long; beak thin, usually 8.0–9.5 mm long, pappus ± white, usually 5.5–6.5 mm long (Figs 4–6).

Discussion

Sources of the conflicting data on the mode of reproduction and the ploidy level of Taraxacum pieninicum

It is a rather embarrassing task to analyse the origin of the conflict over the reproduction and ploidy in *T. pieninicum*. For 60 years, since the detailed and competent work of J. Małecka (1958, 1961, 1963) on *T. pieninicum*, the literature has been unanimous in presenting *T. pieninicum* as a sexual, diploid species, a taxon confined to a single locality in the Polish part of the Pieniny Mts. Now on the basis of an equally meticulous experimental and taxonomic evidence, it is proposed that *T. pieninicum* is an agamospermous (diplosporous) tetraploid, and, moreover, that it includes also populations in western Slovakia, previously described as a separate taxon, *T. erythrocarpum* (Kirschner & Štěpánek 1985).

As regards the relevance of our material, we analysed plants from repeated sampling of both extant micropopulations near the locus classicus of *T. pieninicum*. The sites we sampled are located less than 50 m from the two *T. pieninicum* localities known before 1980 (cf. Zarzycki 1981). The first sample (before 2006, Trejgell et al. 2013) was used to preserve *T. pieninicum* in a seed bank and for the production of regenerants and achenes (A. Trejgell), and for the FCM and chromosome counts in this study, the second sample (G. Vončina), from the current locality, was used for both types of analyses (FCM and FCSS) and again for chromosome counts. The voucher specimens are deposited at PRA.

Let us test first the hypothesis of dihaploidy or polysomaty in *T. pieninicum* (Kula et al. 2013). These authors report a chromosome count of 2n = 16 for *T. pieninicum* and note

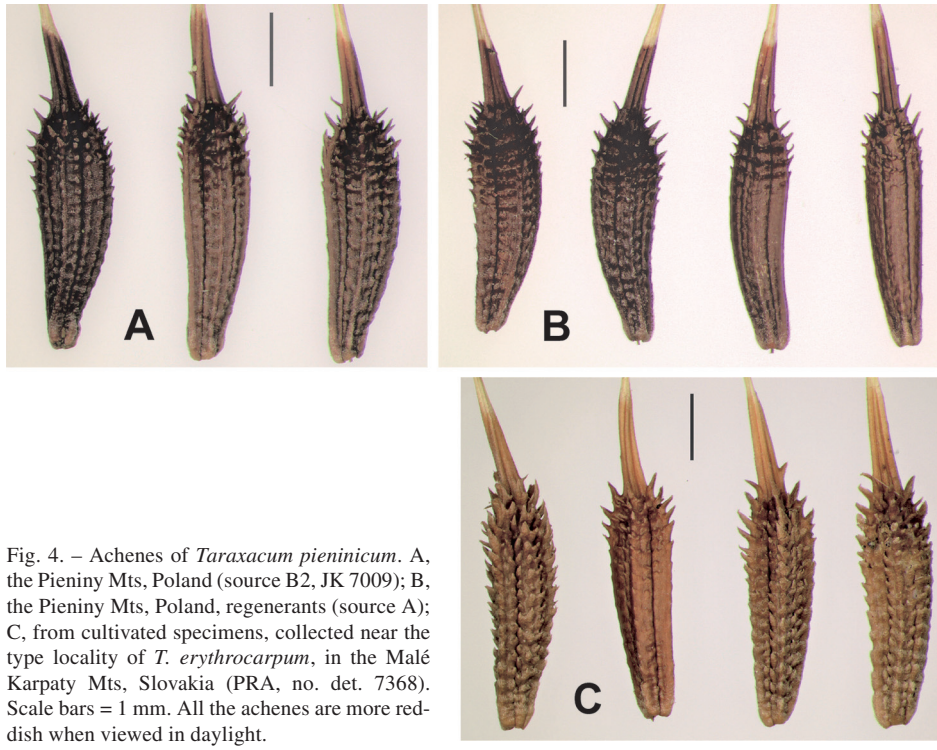

Fig. 4. – Achenes of *Taraxacum pieninicum*. A, the Pieniny Mts, Poland (source B2, JK 7009); B, the Pieniny Mts, Poland, regenerants (source A); C, from cultivated specimens, collected near the type locality of *T. erythrocarpum*, in the Malé Karpaty Mts, Slovakia (PRA, no. det. 7368). Scale bars = 1 mm. All the achenes are more reddish when viewed in daylight.

that "tetraploid metaphase plates were observed equally often in the studied material", then they attribute this observation to polysomaty of root-tip meristems or chromosomal instability of the specimens studied. The FCSS method is very suitable for detecting spontaneous dihaploidy in embryos; our analysis (see Fig. 2) effectively excludes dihaploidy. Polysomaty would manifest itself in the somatic tissues of *T. pieninicum*, and FCM is very suitable for its detection. Leaf tissues from both main sources were used in the FCM analyses; our results again effectively exclude polysomaty as a phenomenon occurring in *T. pieninicum*. Another argument against polysomaty is the numerous, exclusively tetraploid chromosome plates studied by us in the material of *T. erythrocarpum* and plants of *T. pieninicum* coming from sources A and B.

Taraxacum pieninicum (incl. *T. erythrocarpum*) appears to be a coherent agamospermous entity (cf. Kirschner et al. 2016), with a very limited variation, and invariably tetraploid at the several sites studied and in particular in all the current sources of material from the Polish part of its distribution. The possibility of coexistence of two ploidy levels within this species can be safely rejected, both because of the FCM, FCSS and karyological results and the populations of *T. pieninicum* are very small, with relict ecological and geographical distributions, and the repeatedly proven agamospermy in both parts of its distribution.

There is an important fact to be emphasized: there is not a single voucher specimen documenting the material used in the studies of J. Małecka or R. Czapik (Wróbel & Zarzycki 2008), or a description of the plants used in the karyological studies or picture

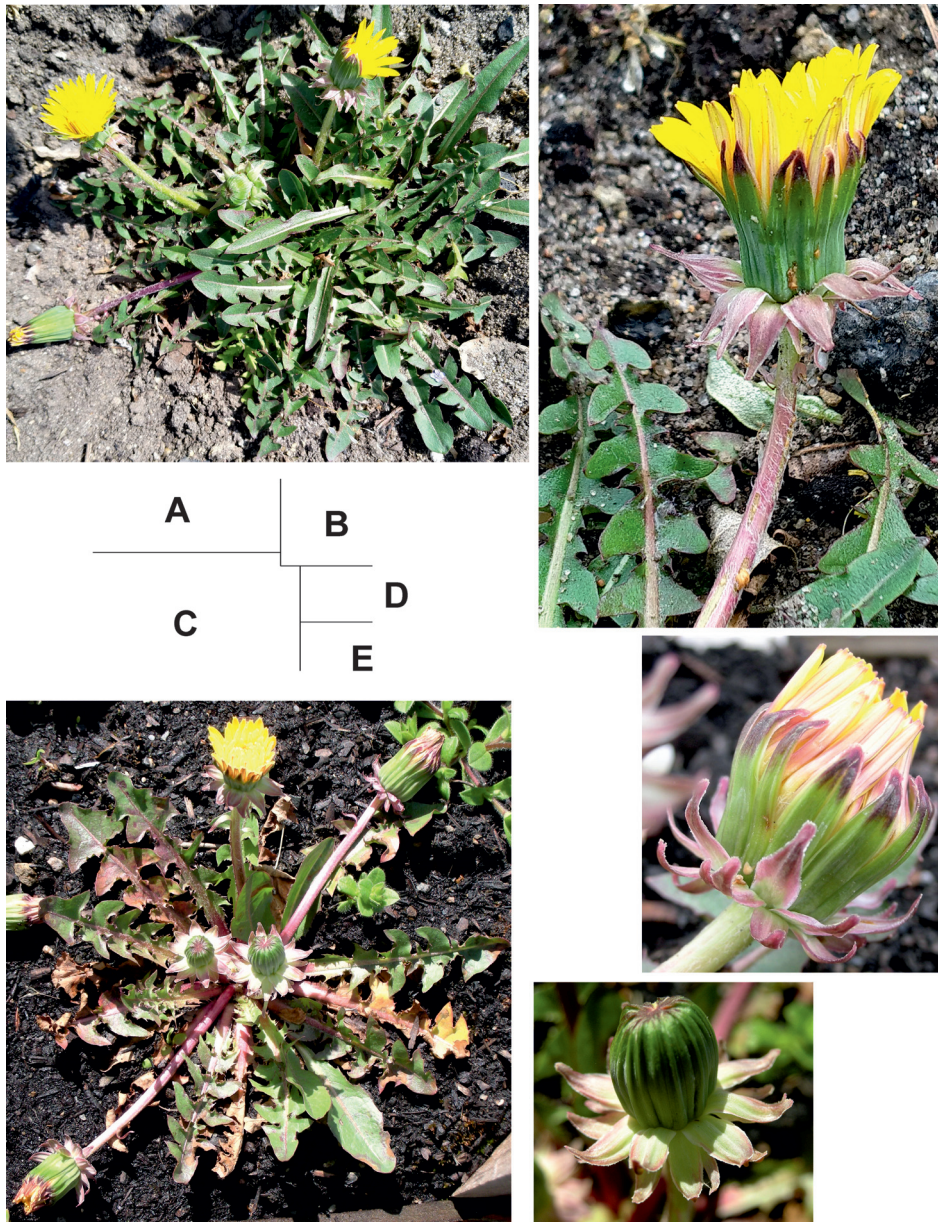

Fig. 5. – *Taraxacum pieninicum*. A, general habit of regenerants in cultivation at Toruń (note the wide range of leaf shapes in a single individual); B, detail of a capitulum (the same source). C, general habit of a plant cultivated as JK 7009 at Průhonice; D, E, details of involucres (the same source).

of the plant they studied. There is therefore no positive evidence in favour of the identity of the material previously used under the name of *T. pieninicum*.

Basic experimental studies of *T. pieninicum* were published by Małecka (1958 – the chromosome number, 1961 – cytoembryology, 1963 – chromosome number), the most

Fig. 6. – A representative specimen of *Taraxacum pieninicum* from Slovakia (PRC, no. 453645, distributed as *Taraxaca Exsiccata*, no. 142).

important paper is the cytoembryological one (Małecka 1961). All the cytoembryological analyses carried out about 60 years ago used young capitula from cultivated plants (Małecka, pers. comm. in 1984). We were shown remnants of the material originally used in the cytoembryological study, stored in fixative in brown glass flasks, which consisted

of numerous capitula (moreover, the number of young flower heads excludes field sampling). In her publications, Małecka either does not specify the details of the plant material (Małecka 1958) or mentions collecting flower heads in the four years, 1956–1959 (Małecka 1961), or reports the source of the root tips (from live plants from natural habitats) used in the chromosome counts for each of the nine species examined (Małecka 1963).

In conclusion, the validity of the experimental findings of Małecka (1961), particularly the diploidy and sexuality of her material, is beyond doubt. We therefore suppose that the cytoembryological studies were carried out on wrongly identified material.

Plants of *T. pieninicum* do not survive long in cultivation, usually not more than 2–3 years, unless transplanted. After that period, without being grown from seed again, they may be replaced by other dandelions in the garden. As an example, the pictures of plants under the name of *T. pieninicum* from the Lublin Botanical Garden, Poland (Anonymous 2020) undoubtedly belong to a species of *T.* sect. *Taraxacum* (*T. officinale* Wigg. s. lat.).

For the sake of completeness, we can add that the *in vitro* regenerants and plant material from one of the sources used in the present study, were screened to exclude the possibility of a risk of somaclonal variation. No changes or increased variation were observed in the regenerants (Kamińska et al. 2018, 2020b).

Sexuality in Taraxacum sect. Erythrocarpa

Taraxacum section *Erythrocarpa*, like a substantial number of other *Taraxacum* sections, includes a core sexual taxon. It is *T. pindicola* (Bald.) Hand.-Mazz., a species known from the mountains of north-western Greece and adjacent mountain ranges in Bulgaria, North Macedonia and Albania (J. Štěpánek & J. Kirschner, unpublished). The agamospermous taxa obviously related to *T. pindicola* and presumably derived from it, radiate from the diversity centre in northern Greece in various directions. This phenomenon is frequently called "geographical parthenogenesis" (Hörandl 2006), although this terminology is not accurate. Within this pattern, the peripheral northern populations are expected to be agamospermous. Another possible record of sexuality in *T.* sect. *Erythrocarpa* (cf. Siljak-Yakovlev et al. 2010) is discussed below and considered too uncertain to be accepted as a proven fact.

Genome size of Taraxacum pieninicum compared with other Taraxacum

The genome size of *T. pieninicum* is relatively small. Záveský et al. (2005) report several species with $1C_x$ values almost identical with that of *T. pieninicum*; it is noteworthy that species with a genome size close to that of *T. pieninicum* belong to derived European sections (*T.* sect. *Taraxacum*, *T.* sect. *Erythrosperma*, with $1C_x$ values 0.87–0.95 pg, cf. Záveský et al. 2005, Macháčková et al. 2018). However, in *Taraxacum*, the genome size alone is not a very reliable indicator because of enormous intrageneric variation. If we disregard old and possibly inaccurate genome size values, one of the smallest $1C_x$ values is reported for the diploid, relatively ancestral *T. stevenii* (Spreng.) DC. of *T.* sect. *Orientalia* Hand.-Mazz. ($1C_x$ = 0.85, T. Černý 2009, unpublished) while the largest values are reported for the derived, East Asiatic pentaploid *T. albidum* Dahlst. of *T.* sect. *Mongolica* (Dahlst.) Doll ($1C_x$ = 1.73, Záveský et al. 2005).

The only genome size reported for *T.* sect. *Erythrocarpa* is published under the name of *T. hoppeanum* Griseb. (Siljak-Yakovlev et al. 2010). It is difficult to speculate about the identity of the plant material on which this record was based. The locality (the Biokovo Mts, Croatia) is known as a site for the polyploid agamospermous *T. janchenii* Kirschner et Štěpánek, but the genome size, 2C = 2.07 pg ($1C_x$ = 1.04 pg), obviously is for a diploid taxon and it is quite possible that this record is not referable to *T.* sect. *Erythrocarpa*.

Possible origin of Taraxacum pieninicum

The unique morphology of *T. pieninicum* does not indicate any close relationships within *T.* sect. *Erythrocarpa*, but its inclusion in this section is beyond doubt and there is no contradictory evidence. The purely yellow stigma is a character not frequently found in this section and it points to the group of *T. janchenii*, widely distributed in the northern and central Balkan Peninsula, with similar taxa found in Velebit, Croatia and northern Greece. Achenes of both *T. pieninicum* and *T. janchenii* are similar, but the two taxa have very different outer phyllaries.

The distribution of *T. pieninicum* is characterized by a disjunction between the Pieniny and the limestone ranges in western Slovakia (~125–170 km). Ecologically, it is confined to refugia on limestone cliffs, ridges and ledges at medium altitudes, nowadays a scattered habitat occurring only in the outer fringes of the Western Carpathians (cf. Mráz et al. 2016). *Taraxacum pieninicum* is the northernmost representative of *T.* sect. *Erythrocarpa* in Europe, remarkably remote from the rest of this section. The closest localities of *T. janchenii* are in southern Romania, while in the west, there are representatives of *T.* sect. *Erythrocarpa* in the southern and south-western Alps.

The westernmost Carpathians in Slovakia, with a number of important isolated limestone localities, harbour a number of noteworthy relicts. We should mention *Poa crassipes* Domin (a taxonomically unclear but distinct form known from Vršatec in the Biele Karpaty Mts, Slovakia, and Kotouč hill near Štramberk, Moravia, intermediate between *Poa badensis* Willd. and *Poa alpina* L.). Another remarkable and geographically isolated relict is *Scabiosa lucida* subsp. *calcicola* Bloński (see also Kaplan et al. 2019). We refrain from drawing conclusions about two Balkan–South-Carpathian taxa with extremely remote sites in the western Slovak Carpathians, *Seseli rigidum* Waldst. et Kit. (Slavík 1968) and *Arabis procurrens* Waldst. et Kit. (Štěpánek et al. 2002) as their Slovak localities are suspected to be of secondary origin.

As an example of the western migration route, so called Noric migration element, i.e., migration from refugia in the western Balkan Peninsula and south-eastern Alps northwards, we can cite *Knautia drymeja* Heuffel. It is a woodland species of low altitudes and its north-easternmost sites are in the southern Malé Karpaty Mts, Slovakia (Štěpánek 1985).

Some of the relicts belong to species with wide distributions in central northern Mediterranean, or have close relatives among such species; the most noteworthy is *Pedicularis comosa* L., known from a single locality on Mt Vršatec in Slovakia. Another case is *Daphne arbuscula* Čelak., an endemic on limestone cliffs in the Muráňska planina in C. Slovakia, with relatives both in the eastern Alps and on the Balkan Peninsula (Kliment 1999). A similar situation is reported for *Hieracium* sect. *Cernua* Uechtr. (Szeląg 2006);

this section is primarily distributed in the southernmost Carpathians and reaches the high western Carpathians and the Krkonoše Mts in the north (included in this distribution is also an Alpine part).

The majority of species or species groups with relict limestone sites in the Western Carpathians are distributed, or have relatives, on the Balkan Peninsula and southernmost Carpathians. A strikingly similar distribution pattern to that of *T.* sect. *Erythrocarpa*, i.e., the Balkan–Western-Carpathian disjunction, is that of *Alchemilla obsoleta* Fröhner, a species described from the Tatra Mts (in Slovakia and Poland) and later found in Bulgaria (Kurtto et al. 2007, 2009), without known localities between these two remote areas. A similar case is *Alchemilla gorcensis* Pawł., with many localities in Greece, Bulgaria and other mountainous areas on the Balkan Peninsula and another distribution centre in the West Carpathians (this time, there are also two sites in the easternmost Carpathians). If we look for a similar pattern in *Taraxacum*, there is that of the narrow group of *T. alpestre* (Tausch) DC. and *T. nigricans* (Kit.) Rchb. (Štěpánek et al. 2011), with a diversity centre in the Tatra Mts and radiating to the Krkonoše Mts (the Giant Mountains) in the west, and another centre of species diversity in the southern Carpathians and adjacent mountains on the Balkan Peninsula. The group of *Pilosella alpicola* Steud. et Hochst. (Šingliarová & Mráz 2009), although confined to localities on acidic bedrock, well matches the pattern of the Balkan and South Carpathian–West Carpathian disjunction.

In conclusion, we consider it as probable that *T. pieninicum* originates from the Balkan diversity centre of *T.* sect. *Erythrocarpa* and is an element of the Balkan–Carpathian migration and diversification route.

Conservation issues

As a consequence of the new treatment of *T. pieninicum* (with *T. erythrocarpum* as a synonym), we are not now considering two locally endemic relics but a single, extremely morphologically isolated and remarkable endemic in the Western Carpathians. However, the conservation issues are similar, with the following exception: for agamospermous species the conservation issues do not include inbreeding and effective population size, decreasing frequency of pollinators, neighbourhood size or conservation of the entire range of variation of a species, all essential for sexual species. We should point out that the overall number of mature plants and regular recruitment at a locality remains an issue even in an agamospermous taxon. It is, nevertheless, possible to focus the activities on habitat conservation, or perhaps re-establishment of sibling seedlings at suitable sites.

In Poland, *T. pieninicum* is strictly protected by law (Anonymous 2014) and, despite its recent range extension, this status should be maintained. The conservation status, the CR category according to IUCN criteria, was adopted in the Polish Red List (Kaźmierczakowa et al. 2016), Polish Red Book (Wróbel et al. 2014) and Red Book of the Polish Carpathians (Wróbel & Zarzycki 2008). This status will also remain unchanged because it only concerns the status of species within Poland. In Slovakia, *T. pieninicum* (under the name of *T. erythrocarpum*) is also considered as critically endangered (CR, Procházka et al. 1999) but, due to the number of populations situated in protected areas, the Slovak Carpathians Red List (Turis et al. 2014) classifies it as endangered (EN). Nevertheless, all the relevant sources recognize *T. pieninicum* as a remarkable, but threatened species in Slovakia (see also Anonymous 2003, Eliáš et al. 2015).

The Pieniny population is the most threatened in the whole distribution of *T. pieninicum* (regularly monitored since its rediscovery in 1999 and 2000 by S. Wróbel, with two subpopulations fluctuating in the number of flowering scapes from 10 to 53 in the upper one, and from 0 to 25 in the lower one), but for most of the populations in Slovakia the conservation situation is almost equally critical. The richest population is that in the Gaderská dolina gorge (see Appendix 1), with ~ 100 individuals (Procházka et al. 1999). The negative factors include the overgrowing of suitable patches by *Sesleria caerulea* (L.) Ard. or by shrubby vegetation, and there is a threat of disturbance and trampling at some Slovak sites (Vršatec and Malé Karpaty sites).

It is important to summarize the pathways of the distribution of achenes collected after 1999 at Pieniny in order to preserve *T. pieninicum* ex situ. Already in 1999, two plants had been grown, which were transferred with seeds from the wild to the Department of Plant Cytology of the Jagiellonian University in Kraków. A successful attempt was also made to grow specimens in the gardens of the administration of the Pieniny National Park in Krościenko nad Dunajcem (Wróbel 2004). In 2007 and 2014, seeds were transferred to the Botanical Garden, Centre for Biological Diversity Conservation, Polish Academy of Sciences in Powsin, from where they were sent to Gołubieński Botanical Garden, Botanical Garden of the Adam Mickiewicz University in Poznań and Botanical Garden of the Maria Curie-Skłodowska University in Lublin (Ziarnek 2017).

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Shrnutí

Po více než 60 letech jsme znovu podrobili zkoumání význačný západokarpatský endemit, *Taraxacum pieninicum* Pawłowski, druh patřící do sekce *Erythrocarpa*. Na základě nového, taxonomicky bezpečně identifikovaného materiálu z locus classicus v Pieninách jsme znovu posoudili reprodukční způsob, chromozomový počet a ploidii. Důvodem pro to byly pochybnosti, zda předchozí četné zprávy o sexualitě a diploidní úrovni *T. pieninicum* nejsou založeny na nesprávně identifikovaném materiálu. Použili jsme průtokovou cytometrii, FCSS a počet chromosomů jako hlavní metody. Výsledkem bylo zjištění, že pieninská populace druhu *T. pieninicum* zahrnuje pouze tetraploidní rostliny (2n = 32) s apomiktickým (diplosporickým) rozmnožováním. Srovnání s tetraploidním diplosporickým druhem popsaným jako *T. erythrocarpum* Kirschner et Štěpánek z nejzápadnějších slovenských Karpat ukázalo, že slovenské populace též patří k *T. pieninicum*. *Taraxacum pieninicum* tak představuje pozoruhodný reliktní druh, endemit omezený na vápencová bradla nejsevernějších a nejzápadnějších Karpat. Představili jsme též hypotézu, že *T. pieninicum* má své předky na severu a v centru Balkánského poloostrova.

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Appendix 1. – Coordinates of the known localities of *Taraxacum pieninicum.*

Poland	
PeininyTrzy Korony, Okrąglica	49°24'49"N, 20°24'51"E
Slovakia	
Biele Karpaty, Vršatec	49°04'26"N, 18°09'41"E
Biele Karpaty, Babky, 650 m	49°04'45"N, 18°10'13"E
Veľká Fatra, Gaderská dolina	48°56'16"N, 18°56'20"E
Malé Karpaty, Ostrý Kameň	48°31'19"N, 17°22'17"E
Malé Karpaty, below Ostrý Kameň	48°31'20"N, 17°22'30"E
Malé Karpaty, Smolenice, Hlbočianský vodopád	48°30'40"N, 17°24'37"E
Strážovské vrchy, Malý Manín	49°08'39"N, 18°30'21"E
Strážovské vrchy, Manínska tiesňava, 350 m	49°08'23"N, 18°30'26"E
Strážovské vrchy, Vápeč	48°56'21"N, 18°19'34"E
Javorníky, Klapy (Kliment 1999)	49°09'41"N, 18°25'24"E