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Micromorphological, anatomical and cytogenetical studies in endemic *Crepis macropus* Boiss. & Heldr. (Asteraceae) from Turkey

Huseyin Inceer*, Nursen Aksu Kalmuk, Kemal Vehbi Imamoglu, Ozge Duman, Sema Hayirlioglu-Ayaz, Gokhan Arslan

Karadeniz Technical University, Faculty of Sciences, Department of Biology, 61080 Trabzon, Turkey

Abstract – In the present study, the micromorphological structure of achene, pappus and style using scanning electron microscope (SEM), stomatal characteristics, anatomy of stem and achene together with chromosome number and nuclear DNA content of the Turkish endemic *Crepis macropus* Boiss. & Heldr. are provided in order to expand knowledge of its taxonomy. The SEM studies in this species show that dense spiny cells are found on the achene surface, the pappus bristle has 3–5 spikes and the style possesses slender papillae. The stem structure is composed of epidermis, collenchyma, parenchymatous cortex and pith. The species has anomocytic stomata in both the upper and the lower surface of the leaves. The pericarp of the achene is mainly composed of several layers of sclerenchymatous cells. In this species, the chromosome number is 2n = 2x = 8, karyotype consists of two submetacentric and six subtelocentric chromosomes and nuclear DNA content (2C-value) is 12.96 pg. These data are presented here for the first time and their taxonomic values are discussed.

Keywords: anatomy, chromosome, *Crepis*, endemic, micromorphology, nuclear DNA content, Turkey.

Introduction

The genus *Crepis* L. belongs to the tribe Cichorieae of the Asteraceae family and comprises over 200 species (Bremer 1994). Its species are distributed throughout the northern hemisphere with single species occurring in South East Asia. Some species also occur in different regions of Africa, the Canary Islands and Madeira (Enke 2008). It is thought that the origin of the genus *Crepis* is in the Altai/Tien Shan region in the Central Asia (Babcock 1947a). The genus presently has its highest species diversity in the circum-Mediterranean area (Enke 2008). In the Turkish flora, the genus is represented by 42 taxa and eight taxa of them are endemic to Turkey (Ekim 2012).

Crepis is a problematic genus from a taxonomical point of view and it is notorious for its lack of discriminating characters (Enke 2008). Polymorphism is common in the genus and many taxonomic characters vary more within a species than between closely related species, and this often leads to unclear species-specific boundaries. Additionally, some species of *Crepis* are especially similar to *Hieracium* and *Lapsana* in their morphological characteristics and are also similar to many other Cichorieae in their habits. Therefore, they have been confused both taxonomically and nomenclaturally with each other and other Cichorieae genera.

The achenes of Cichorieae are in many cases indispensable for the identification of the genera and species and provide the systematically most valuable features on all taxonomic levels (Kilian et al. 2008). As studies in the Cichorieae have shown before (Pak and Kawano 1990, Pak 1993, Pak et al. 2001, Zhu et al. 2006, Sennikov and Illarionova 2008), achene anatomy is helpful in the generic delimitation and infrageneric classification of *Crepis* (Enke 2008, 2009, Jana and Mukherjee 2012). In general the achene surface features are also taxonomically valuable, mainly at species level, and more rarely concur with supraspecific delimitation (Kilian et al. 2008). The pappus has always been an important feature for the discrimination of groups on all taxonomic levels in the Cichorieae (Kilian et al. 2008). The style morphology has been an important morphological character in major clade delimitation of the Asteraceae (Bremer 1996). Some taxonomic value of micromorphology of the pappus and style has been reported in *Crepis* (Enke 2008, 2009). Stem and leaf anatomy are considered as diacritical character in some groups of the tribe Cichorieae (Carlquist 1967), but there are rare data on stem and leaf anatomy of *Crepis* in the literature (Metcalfe and Chalk 1979).

The relevance of cytological and cytogenetical information to knowledge of the taxonomy and evolution of *Crepis*

* Corresponding author, e-mail: inceer@ktu.edu.tr

was noted long ago. Karyotype and genome size similarities and differences, especially chromosome number have been used as criteria to infer species relationships in *Crepis* (Babcock 1947a, b, Siljak-Yakovlev and Cartier 1986, Siljak-Yakovlev and Wraber 1988, Kamari et al. 1991, Godelle et al. 1993, Dimitrova and Greilhuber 2000, 2001, Siljak-Yakovlev et al. 2010, Enke et al. 2011, 2015).

Most of the micromorphological, anatomical and cytogenetical studies conducted in *Crepis* have concentrated on common species, with some work having been interested in endemic species (Kamari et al. 1991, Kamari 1992, Enke 2009, Enke et al. 2011, Siljak-Yakovlev and Peruzzi 2012). To our knowledge, except the chromosome counting of *C. dioritica* (2n = 8, Davis et al. 1988) in Turkey, no micromorphological, anatomical and cytogenetic studies have been reported for Turkish endemic species of *Crepis*.

Crepis macropus is an endemic species that belongs to section *Berinia* (Babcock 1947a, b, Enke 2009). This endemic species is distributed in central, north and north-west Anatolia. It grows on chalky, dry, stony or rocky slopes, steppe and field sides at altitudes from 300 to 1600 m (Babcock 1947b, Lamond 1975). *Crepis macropus* exhibits closer similarities to its Balkan relatives, *C. turcica* and *C. albanica*, than to those of Anatolia, but it is more like *C. turcica* based on its habit, involucres, florets and achenes (Babcock 1947b). According to Babcock (1947a), *C. macropus* is an "intermediate" type with regard to its phylogenetic position in the genus *Crepis*, and there is strong positive correlation between this advanced type and adaptation to low altitude and arid environment, and it is relatively young species. Thus, *C. macropus* might be neo-endemic and according to the terminology Siljak-Yakovlev and Peruzzi (2012), it can be considered schizoendemic.

According to the relevant literature records, except for molecular analysis based on ITS and chloroplast *matK* sequences (Enke and Gemeinholzer 2008, Enke 2009), the micromorphological, anatomical and cytogenetical characteristics of *C. macropus* have not yet been studied. We aimed to give a detailed account of the micromorphological structure of achene, pappus and style, leaf epidermal characteristics, achene and stem anatomy, karyotype and nuclear DNA content for this Turkish endemic species.

Material and methods

Plant materials

Plant samples were collected from natural populations in 2013 and 2014 from Ankara (Beynam Forest, rocky slopes, 1320 m a.s.l., June, 11th, 2013, achene and pappus for micromorphological observations; leaf, stem and achene for anatomical observations; achene for cytogenetic analysis: Inceer 1009), Çankırı (near Çakmak Village, rocky slopes, 885 m a.s.l., June, 27th, 2014, leaf for cytogenetic analysis: Aksu 196) and Nevşehir (Ürgüp National Park, 1095 m a.s.l, July, 1st, 2013, capitulum for micromorphological observations of style: Inceer 1015) provinces. Vouchers are deposited in the herbarium at the Karadeniz Technical University, Department of the Biology (KTUB).

Scanning electron microscopy

Micromorphological structure of achene, pappus and style was analyzed on the scanning electron microscope (SEM). Dry and mature specimens were mounted directly on the stubs using double-sided adhesive tape, and then observed in Agilent FESEM 8500 scanning electron microscope. The micromorphological characterization was performed according to Enke (2008).

Anatomical studies

Anatomical observations were performed in the leaf, stem and achene. For this purpose, peripheral sections of upper (adaxial) and lower (abaxial) epidermis of the leaves were taken by hand using commercial razor blades from fixing materials in FAA (5% formaldehyde : 5% glacial acetic acid : 70% ethyl alcohol). Semi-permanent slides were mounted in glycerine. Stomatal index was assessed in both the upper and lower epidermis (Meidner and Mansfield 1968). Transverse sections from middle parts of the stem were taken by hand using commercial razor blades from fixing materials in formaldehyde-glacial acetic acid-ethyl alcohol and were stained with safranin and then mounted in entellan. Transverse sections of achene were carried out with the paraffin method (Algan 1981) and stained with hematoxylin and then mounted in entellan. Three slides obtained from three individuals were prepared and the anatomical characters were measured using an ocular micrometer under the light microscope. The photographs were taken using a Leica DM 4000 microscope and a Leica DFC 490 digital camera. Illustrations were drawn with a lucida camera attached to a light microscope.

Cytogenetic analyses

The root tip meristems obtained directly from germinated achenes were used for chromosome analysis. The root tips were pre-treated with 0.05% aqueous colchicine solution for 3–5 h at room temperature and then fixed in absolute ethanol-glacial acetic acid (3:1) for at least 24 h at 4 °C (Inceer and Hayirlioglu-Ayaz 2007). They were hydrolyzed in 1 N HCl at 60 °C for 12–15 minutes. Staining was carried out in 1% lacto-propionic orcein for 12–18 h at room temperature and squash preparations were made in 45% acetic acid.

The best metaphase plate of each individual was photographed with a Leica DM 4000 microscope with a Leica DFC 490 digital camera. Five well-spread metaphase plates of different five individuals were used for chromosome analysis and idiogram building. Karyotype analysis was performed according to Levan et al. (1964). Idiograms were prepared based on the average measurements of each chromosome pair. Chromosomes were classified on the basis of arm ratio in accordance with Stebbins (1971). The intrachromosomal asymmetry index (A_1) was calculated by use of the formula proposed by Romero Zarco (1986), and the interchromosomal asymmetry index (A_2) was measured as the ratio of chromosome length/mean chromosome length. In haploid idiograms, chromosomes were arranged according their length.

The young leaves were taken from three specimens in natural population for flow cytometric analysis. The leaves of *Lycopersicon esculentum* cv. 'Swanson' (2 pg/2C) potted and grown were used as an internal standard. Nuclear DNA content was assessed by flow cytometry as follows. Leaf fragments of the sample plant and the standard plant were chopped using a razor blade in 1 mL of woody plant buffer (Loureiro et al. 2007; 0.2 M Tris HCl, 4 mM $MgCl_2 \times 6H_2O$, 2 mM $Na_2EDTA \times 2H_2O$, 86 mM NaCl, 10 mM potassium metabisulphite, 1% PVP-10, 1% (v/v) Triton X-100, pH 7.5) supplemented with 50 µg mL^{-1} propidium iodide and 50 µg mL^{-1} DNAse-free RNAse, filtered through a 30 µm mesh and stored on ice, in the dark, until measurement. Three independent samples were extracted, filtered and measured on the same day. The measurements were made three consecutive days using BD Accuri™ C6. Usually 10,000 nuclei per sample were analyzed for nuclear DNA content.

Results

Micromorphology

In this species, all achene surfaces have prominent spiny cells (Figs. 1a, b). The pappus bristles are slender and made up of 3–5 cells in diameter. The pappus bristles have 3–5 spikes per 100 µm, diameter is 21–44 µm (Fig. 1c). The style and the style branches consist of papillae. The papillae are type A and more densely arranged in the style. The style diameter is 102.23 ± 2.91 µm, and the diameter of its arm is 119.43 ± 12.72 µm (Figs. 1d, e).

Anatomy

In the leaves of *C. macropus*, there is a single layered isodiametric epidermis with wavy walls on both the adaxial and the abaxial surface. Adaxial epidermal cells are more or less equal to the abaxial ones. Stomata are present on both surfaces of the leaf (amphistomatic) and anomocytic type (stomata without subsidiary cells). They lie more or less at the epidermis level. The guard cells are oval-shaped. The stomata on both the adaxial and abaxial sides are almost the same size. On the adaxial side, the size of stomata is 26.43 ± 0.65 × 37.66 ± 2.17 µm, on the abaxial side, 26.68 ± 0.80 × 37.74 ± 1.37 µm (Figs. 2b, c). However, the stomatal index on the abaxial side is 18.06, whereas it is 14.80 on the adaxial side.

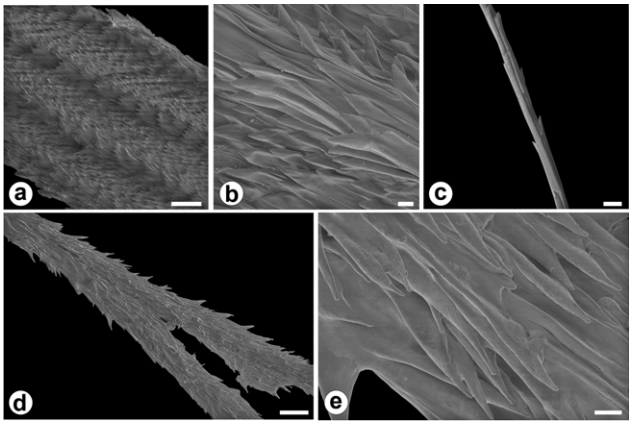

Fig. 1. Scanning electron microscope micrographs of *Crepis macropus*: a, b) achene (Inceer 1009), c) pappus (Inceer 1009), d) style (Inceer 1015), e) style arm (Inceer 1015), scale bars: a, d) = 100 µm; b, e) = 10 µm; c) = 40 µm.

The stem of *C. macropus* is more or less round in the transverse sections. In its stem anatomy, the epidermis consists of single layered, roundish-ovate, frequently arranged cells and is surrounded by a more or less thick cuticle layer. Cells dimensions are 15.86 ± 03.02 × 13.42 ± 01.29 µm. Collenchyma is located below the epidermis. It has 2–3 layers at the margins and its thickness is 183.20 ± 10.04 µm. Cortex is composed of 7–8 rows, parenchymatous and oval cells. Its thickness is 183.20 ± 10.04 µm. Vascular bundles are collateral, scattered in a circular order in the ground tissue. Phloem and xylem members are clear. The phloem thickness is 95.36 ± 07.81 µm, whereas the xylem thickness is 194.18 ± 15.29 µm. Width of the vascular bundle is 113.66 ± 14.89 µm. The cambium formation is distinguishable in the vascular bundles. The pith of the stem consists of large, round parenchymatic cells (Fig. 2a).

The achene of the species has a rounded outline with 12 ribs consisting of sclerenchymatous cell bundles in the transverse sections. The ribs are prominent without inter rib furrows. Pericarp consists of exocarp, mesocarp and endocarp. Exocarp is one layered, with a thick outer cell wall, but the cells are collapsed. Mesocarp only consists of sclerenchymatic bundles and cells. Endocarp is two layered and collapsed. The pericarp thickness is 91.09 ± 17.84 µm, and its width is 96.79 ± 19.74 µm. Testa is circular. Its thickness is 2.44 ± 0.14 µm. Endosperm consists of two layered cells. The thickness of the endosperm is 10.30 ± 0.47 µm. Embryo is formed by more or less oval or round, parenchymatic cells. The orientation of cotyledons is at right angles to the axis of achene (Fig. 2d).

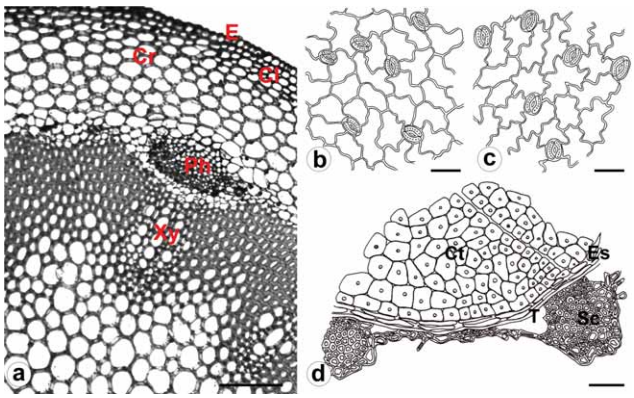

Fig. 2. Anatomical structure of *Crepis macropus* (Inceer 1009): a) stem, Cl – collenchyma, Cr – cortex, E – epidermis, Ph – phloem, Xy – xylem, scale bar = 100 µm, b) abaxial surface in the leaves, c) adaxial surface in the leaves, scale bars (b–c) = 50 µm, d) achene, Ct – cotyledons, Es – endosperm, Sc – sclerenchyma, T – testa, scale bar = 50 µm.

Cytogenetics

The chromosome number of *C. macropus* is 2n = 2x = 8. The two chromosome types in its karyotype are distin-

guished: one submetacentric and three subtelocentric chromosome pairs. The chromosome length ranges from 5.26 to 7.64 μm. The relative length ranges from 20.25 to 29.42. (Tab. 1, Figs. 3a, b). The classis of karyotype asymmetry is placed in 4A, the index of A_1 is 0.69 and the index of A_2 is 0.15.

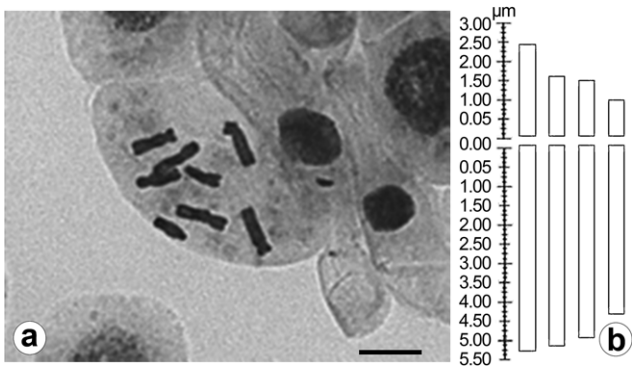

Fig. 3. Somatic metaphase of *Crepis macropus* (Inceer 1009): a) microphotograph of mitotic chromosome plate (2n = 8), b) haploid idiogram, scale bar = 10 μm.

The nuclear DNA content (2C-value) of this endemic species is 12.96 ± 0.11 pg. Its monoploid genome size (1C-value) is 6.48 pg (Fig. 4).

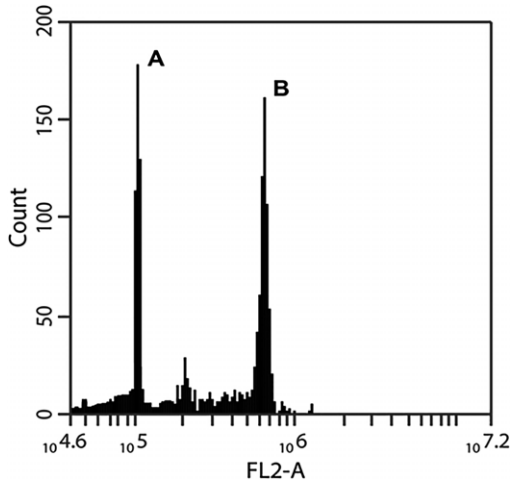

Fig. 4. Flow cytometry histograms: A) Peak of standard *Lycopersicon esculentum* cv. 'Swanson' (2C = 2.00 pg), B) Peak of *Crepis macropus*, Aksu 196 (2C = 12.96 pg).

Discussion

The results obtained from micromorphological, anatomical and cytogenetical studies on *C. macropus* are presented in Tab. 1 and Figs. 1–4. Our findings are in agreement with the previous results on the other species of the genus (Babcock 1947a, b, Metcalfe and Chalk 1979, Enke and Gemeinholzer 2008, Enke 2009, Enke et al. 2011, 2015, Yildirim et al. 2011). The additional morphological, anatomical and cytogenetical characters supporting systematic delimitation of the genus have been used because the molecular analyses by Enke (2009) could not support the current taxonomic sections (Babcock 1947b).

Enke (2008) pointed that the surface features of achene, pappus and style are taxonomically valuable, mainly at species level in *Crepis*. The present morphological analyses show that the achenes of *C. macropus* have prominent spiny cells on its surface. The pappus is comparatively homogenous in the species and it confirms the general description presented by Enke (2008). Additionally, Enke (2009) reported three papillae types as "type A, type B and type C" in style and style branches. The papillae type in the style and style branches of *C. macropus* is type A in this species. Similar results are reported by Enke (2008) for *C. leontodontoides* and *C. zacintha*.

Many anatomical characters of the leaves are useful for systematics, particularly the epidermal cells and stomata (Metcalfe and Chalk 1979, Dickison 2000, Araújo et al. 2010, Inceer and Ozcan 2011). Paradermal sections taken from the leaves show that *C. macropus* has isodiametric epidermal cells together with anomocytic stomata. The epidermal cell walls are also of the same structure, with anticlinal undulate walls on both the adaxial and abaxial surface. These characteristics agree with the results previously reported by Metcalfe and Chalk (1979) and Inceer and Ozcan (2011) for the family Asteraceae.

Metcalfe and Chalk (1979) reported that vascular bundles are taxonomically important and cambium can occur in the member of Asteraceae family. The stem anatomy of *C. macropus* is composed of epidermis, cortex, corticular vascular bundles with distinguishable cambium formation and parenchymatic pith. The present results are in agreement with previous data of Metcalfe and Chalk (1979).

The achene anatomy has proven so far to be of some relevance for classification of the *Crepis* species. Enke (2009) reported four different achene anatomy types, "type

Tab. 1. Morphometric data of karyotype of *Crepis macropus*. Data are arithmetic mean ± standard deviation, n = 5. S – short arm length, L – long arm length, T – total length, L/S – arm ratio, SAT – satellite, CI – centromeric index, RL – relative length, sm – submetacentric, st – subtelocentric.

Chromosome pairs	S (μm)	L (μm)	T (μm)	L/S	SAT (μm)	CI	RL	Chromosome type
1	5.28±0.48	2.36±0.25	7.64±0.53	2.24	–	30.89	29.42	sm
2	5.16±0.14	1.53±0.52	6.69±0.54	3.37	–	22.87	25.76	st
3	4.95±0.26	1.43±0.32	6.38±0.59	3.46	–	22.41	24.57	st
4	4.32±0.10	0.94±0.25	5.26±0.26	4.60	–	17.87	20.25	st

I, type II, type III and type IV" in *Crepis*. We observed that *C. macropus* possesses type IV in its achene anatomy. According to Enke (2009), in type IV, exocarp can be collapsed and the costae are very prominent with deep or no intercostal furrows.

Crepis is a model group for genetic studies explaining evolution and speciation in higher plants because it has low chromosome numbers. Therefore, many chromosomal studies on *Crepis* have been published so far (Babcock 1947a, b, Siljak-Yakovlev and Cartier 1982, Dimitrova and Greilhuber 2000, 2001). Different basic chromosome numbers x = 3, 4, 5 and 6, are present in the genus *Crepis* (Babcock 1947a, b, Kamari 1992, Dimitrova and Greilhuber 2000, 2001, Enke 2008) and karyotypes can be symmetrical or asymmetrical (Babcock 1947b). *Crepis macropus* is a diploid species with 2n = 2x = 8 chromosomes. This species has the same chromosome number with the members of the section *Berinia* such as *C. turcica*, *C. merxmuerlleri*, *C. sibthorpiana*, *C. sonchifolia* (Nazarova 1984, Kamari et al. 1991, Kamari 1992, Constantinidis et al. 2002, Enke 2008). There is a minor difference in the chromosome morphology between *C. macropus* and *C. turcica* (Kamari et al. 1991). The chromosome size values in *C. macropus* range from 5.26 to 7.64 μm, whereas the chromosome size values range from 4.7 to 6.7 μm in *C. turcica* (Kamari et al. 1991). The karyotypes in both species also consist of long chromosomes. *Crepis macropus* has an asymmetrical karyotype. It is widely accepted that the evolutionary trend is toward an increase in karyotype diversity (Stebbins 1971, Liu et al. 2006, Inceer et al. 2012). It was also reported that increasing karyotype asymmetry could occur via a shift of the centromere position from median to subterminal or terminal chromosome regions, or through accumulation of differences in relative size between individual chromosomes (Liu et al. 2006, Inceer et al. 2012).

Despite the impressive amount of genome size studies seen the last few years (Bennet and Leitch 2005, Enke et al. 2011, Garcia et al. 2013), taxonomic coverage remains very restricted, with C-values assessed for less than 40% of the *Crepis* species. The nuclear DNA content of *C. macropus* is presented with a histogram in Fig. 4, and its genome size (1C-value) is 6.48 pg. The genome size (1C-value) in *C. turcica* is 6.41 pg (Enke 2009), and thus there is slight difference in genome size between *C. macropus* and *C. turcica*. According to Leitch et al. (1998) and Soltis et al. (2003), genome sizes can be assigned to a series of distinct categories: very small (1C ≤ 1.4 pg), small (1.4 < 1C ≤ 3.5 pg), intermediate 3.5 < 1C < 14 pg), large (14 ≤ 1C < 35 pg) and very large (1C ≥ 35 pg). In these terms, the endemic *C. macropus* has an intermediate genome. Similar results are reported in other species of *Crepis* (Godelle et al. 1993, Siljak-Yakovlev et al. 2010, Enke et al. 2011). The similarities in the nuclear DNA content as well as karyotype and external morphology of *C. macropus* and its relative suggest that their genomes evolved from a common ancestral genome and underwent some structural differentiation of the genomes.

In conclusion, the present results obtained from micromorphological, anatomical and cytogenetical analyses will increase taxonomic information about *C. macropus* in the literature. Furthermore, features of achene, pappus, style, karyotype and nuclear DNA value can be used as taxonomic characters for this Turkish endemic species.

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