

*Comparative Anatomical Leaf Analyses of *Carduus nutans* and *Carduus thoermeri* from Bulgaria*

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Abstract: The present study provides a comparative anatomical leaf analysis of *Carduus nutans* L. and *C. thoermeri* Wienm., which belong to the Asteraceae family. Bifacial leaf structure, anomocytic and anisocytic types of stomatal apparatus and two types of trichomes (multicellular covering and glandular) were found on the leaves of both species. The quantitative anatomical characteristics varied considerably, however; basing on the statistical analyses, the most significant characteristics for distinguishing the two taxa are: the number of stomata in the abaxial epidermis, the number of capitated glandular trichomes and the number of multicellular covering trichomes in the upper (adaxial) and lower (abaxial) epidermis.

Key words: anatomical study, *Carduus nutans*, *Carduus thoermeri*, leaf, thistle.

Introduction

The Asteraceae (Compositae) family is the largest among the flowering plants, including about 1100 genera and 25000 species (HEYWOOD *et al.*, 1977), and according to STEVENS (2001) – about 1620 genera and over 23600 species. Genus *Carduus* (Thistle) belongs to this family and it is globally represented with about 90 species, naturally spread in Northern Europe, Asia and Africa (KAZMI, 1964; BOHM & STUESSY, 2001; KEIL, 2006; MABBERLEY,

2008). *Carduus nutans* L. and *Carduus thoermeri* Wienm. are widely distributed representatives of the genus in the Bulgarian flora (DELIPAVLOV & CHESHMEDZHIEV, 2003).

The basic anatomical characteristics of the representatives of the Asteraceae family were described by METCALFE & CHALK (1950, 1979, 1989), the description includes: types of trichomes, stomatal apparatus, type of mesophyll, and the sheath cells of the vascular bundles. In accordance with those findings, ZARINKAMAR (2007) confirmed the

anomocytic and anisocytic type of the stomatal apparatus of the species *C. thoermeri*, the stomata of the studied species found on the two leaf surfaces.

The taxonomic significance of the characteristics of the leaf epidermis in the representatives of the Asteraceae family was described by a number of authors (PALMER & GERBETH-JONES, 1986; JAYEOLA *et al.*, 2001; ADEDEJI & ILLOH, 2004; ADEDEJI, 2004). The secretory structures and their location also have an important diagnostic value in taxonomic studies (METCALFE & CHALK, 1950, 1979, 1989; FAHN, 1979).

The number, size, density and position of the stomata in the leaf epidermis are influenced by the environmental conditions and have an adaptive significance for plants (PARKHURST, 1978; ZARINKAMAR, 2006; NINOVA & DUSHKOVA, 1977, 1978a, 1978b; NINOVA *et al.*, 1984; CAIAZZA & QUINN, 1980; SALGARE & ACHARECAR, 1990; CASE, 1994). Changes in stomatal density might be a result of changing the amount of water in the environment (EDWARDS & MEIDNER, 1978), the light intensity (RESTALLACK, 2001; LU *et al.*, 1993), the temperature (CIHA & BRUN, 1978), the geographical location (RESTALLACK, 2001), the concentration of CO₂ in the atmosphere (BRISTOW & LOOL, 1968; ILKUN, 1978; WOODWARD, 1987; WOODWARD & BAZZAZ, 1988). VENDRAMINI *et al.* (2002) noted a thick leaf blade for *C. thoermeri* species.

There are a number of studies on the anatomical and morphological characteristics of the achenes of the representatives of tribe Cardueae (WAGENITZ *et al.*, 1982; PEREZ-GARCIA & DURAN, 1987; CHERNIK, 1984; HÄFFNER, 2000). OLIVIERI *et al.* (1983) investigated the type and location of the achenes in the head of the species *Carduus pycnocephalus* and *Carduus tenuiflorus*. Studying the morphology of the achenes of the species of the genus *Carduus*, KÖSTEKCI & ARABACI (2015) found that their characteristics could be used as diagnostic taxonomic traits for the representatives of the genus.

The aim of the present study was to differentiate the taxonomically significant anatomical features of *C. nutans* and *C. thoermeri* leaves, taking into account the scarce studies of those characteristics of the two species, belonging to the *C. nutans* group.

Material and Methods

The plant material (leaves) was collected in 2014-2015 from 4 floristic regions of Bulgaria (Table 1). The species were identified at the Department of Botany and Methods of Teaching Biology, Faculty of Biology, Plovdiv University "Paisii Hilendarski" according to DELIPAVLOV & CHESHMEDZHIEV (2003). The voucher materials was deposited in the Herbarium of Agricultural University - Plovdiv (SOA).

An anatomical analysis of the leaf epidermis and leaf lamina of the studied species was made following classical methods (METCALFE & CHALK, 1950). For this purpose, the leaves were fixed in 70% ethanol, followed by making histological preparations of epidermis and cross section of the lamina. The following qualitative and quantitative anatomical characteristics were investigated: type and number of trichomes, type and number of stomata in the adaxial and abaxial epidermis, lamina thickness, cuticle thickness and palisade tissue thickness. Quantitative data about the characteristics number of trichomes and number of stomata were based on the observations of 50 visible adaxial and abaxial epidermis areas for each species. The results obtained for the anatomical features lamina thickness, adaxial and abaxial cuticle thickness, as well as thickness of the palisade tissue, were based on measurements of 10 leaf blades for each species from each site. Light microscopic images were taken with a Magnum T Trinocular microscope, equipped with a photo-documentation system Si5000 (zoom x100 up to x400) at the Department of Botany and Methods of Teaching Biology, Faculty of Biology, Plovdiv University "Paisii Hilendarski". The electron microscopic

analysis was done with a Scanning electron microscope JEOL JSM-5510 (Faculty of Chemistry and Pharmacy, Sofia University "St. Kliment Ohridski" (zoom x50 to x10000). Statistical data processing was performed with the IBM SPSS, ver. 20.

Results and Discussion

The leaf epidermis of the studied species is represented by 4 types of cells: basic epidermal cells, stomatal guard cells, subsidiary cells and hair cells. The stomata are disorderly scattered in both epidermis-amphistoma leaves.

Table 1. List of studied taxa and localities of their collection.

Species	Locality (latitude/longitude and altitude)	Name of locality, Floristic region, year of sampling	Voucher specimen
	41°:40'N/24°:44'E/1431 m a.s.l.	Rozhen, Rhodopes Mts (<i>Central</i>), 2015	062053
<i>Carduus nutans</i> L.	42°:19'N/27°:43'E/30 m a.s.l.	Arkutino, Black Sea Coast(<i>South</i>), 2015	062047
	41°:76'N/25°:35'E/451 m a.s.l.	Tchernootchene, Rhodopes Mts (<i>East</i>) 2015	062049
<i>Carduus thoermeri</i> Wienm. [syn. <i>C.</i> <i>nutans</i> subsp. <i>lejophyllus</i> (Petrovič) Stoj. &Stef.]	42°:12'N/25°:21'E/212 m a.s.l.	Chirpan, Thracian Plain, 2015	060240
	41°:95'N/24°:86'E/367 m a.s.l.	Bachkovo Rhodopes Mts (<i>Central</i>), 2014	062040
	41°:51'N/24°:49'E/663 m a.s.l.	Laki Rhodopes Mts (<i>Central</i>), 2015	062051

METCALFE & CHALK (1979, 1989) indicated the same type of leaves in the Asteraceae family. The type of trichomes (syn. hairs) is the same in both species – multicellular covering trichomes and sparsely located capitate glandular ones, on both leaf surfaces. Covering trichomes are uniseriate, consisting of 4 to 6 cells (Fig. 1). Glandular trichomes have one secretory cell – the head of the trichome, which is above the epidermal surface, on a short one-celled stalk (Fig. 2).

In our study, we did not find a flagellum type of trichomes, as FREIRE *et al.* (2005) reported in another species of the genus *Carduus* (*C. acanthoides*). As a result of the study, a mixed type of stomatal apparatus was found for both species – anisocytic (predominant) and anomocytic types (Fig. 3).

The cross section of the lamina showed a dorsiventral structure (Fig. 4), which is typical of the Asteraceae family according to METCALFE & CHALK (1950, 1979, 1989): presence of adaxial and abaxial leaf epidermis, covered with a cuticle; a mesophyll (differentiated into palisade and spongy parenchyma tissues). The palisade tissue is located under the adaxial epidermis and it is

represented by 2 to 3 layers of tightly packed cells. The spongy parenchyma is located on the lower leaf surface.

The veins were clearly outlined with vascular bundles in them (Fig. 5). The vascular bundles are closed collaterally, covered with sclerenchyma bow. Only in the midrib they may be opened (Fig. 5). METCALFE & CHALK (1950, 1979, 1989) announced that the vascular bundles of the Asteraceae are covered by a parenchyma sheath structured by large cells. Their statement was also confirmed in our study, where the vascular bundles are covered with parenchyma sheath.

The comparative statistical analysis of the data from the three localities about *C. nutans* (Fishers' criterion) distinguishes the characteristics with significant differences within the species (Table 2).

The statistical analysis of the quantitative anatomical features of *C. nutans* leaves from the three studied localities showed that there are intraspecific differences in the following features: number of stomata per unit area, number of glandular trichomes and number of covering trichomes in the adaxial and abaxial epidermis (Table 2). The number of stomata in

the adaxial epidermis between Arkutino and Tchernoochene localities, as well as between Tchernoochene and Rozhen was significantly different ($p \leq 0.001$). As far as their number in the abaxial epidermis is concerned, there were

differences between all the studied localities: Arkutino and Rozhen ($p \leq 0.001$), Tchernoochene and Rozhen ($p \leq 0.001$), Arkutino and Tchernoochene ($p \leq 0.05$), respectively.

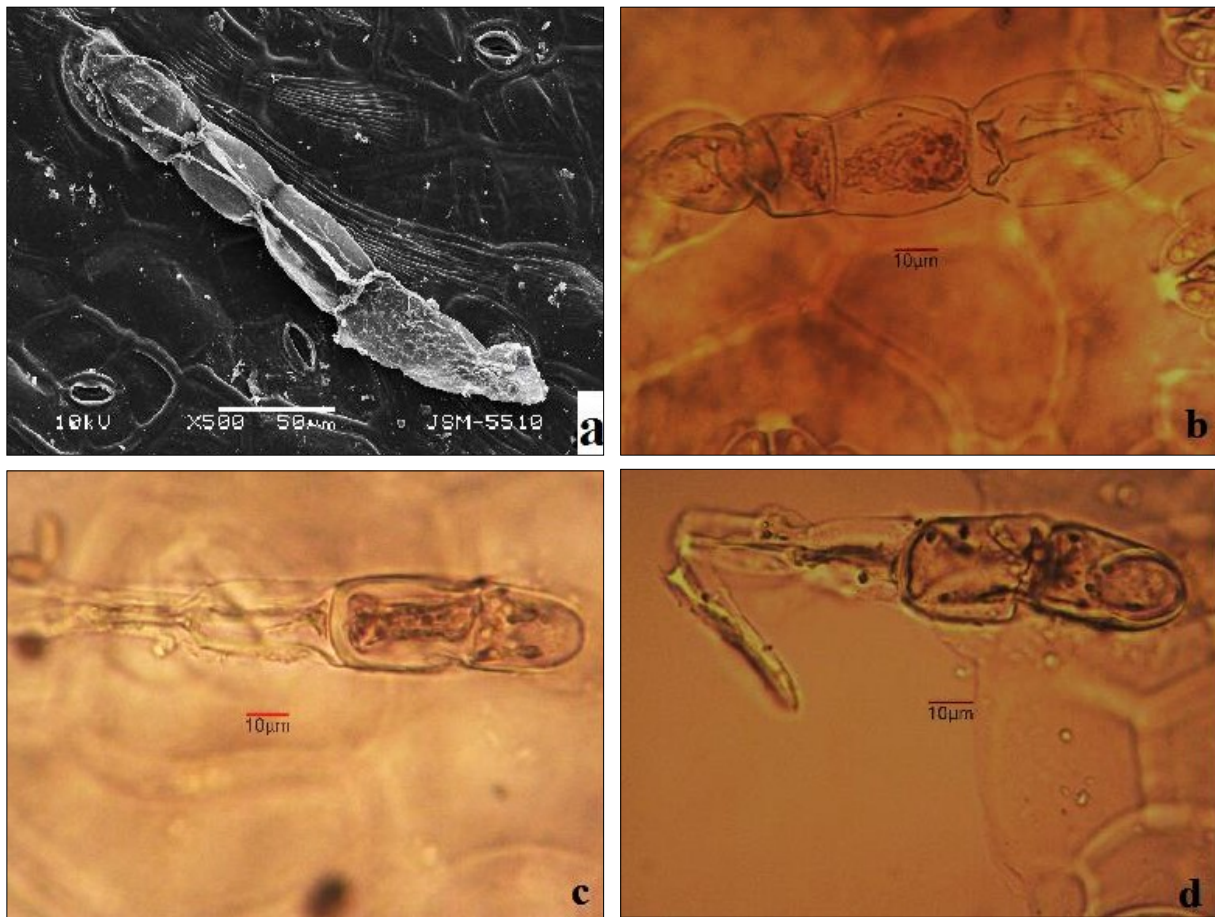


Fig. 1. Scanning electron microscopy (a) and light microscopy (b, c, d) of *Carduus nutans* (a, b) and *Carduus thoermeri* (c, d) trichomes.

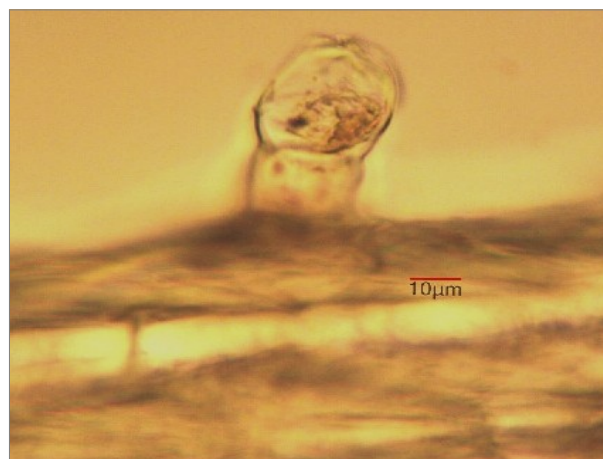


Fig. 2. Glandular trichome of leaf epidermis.

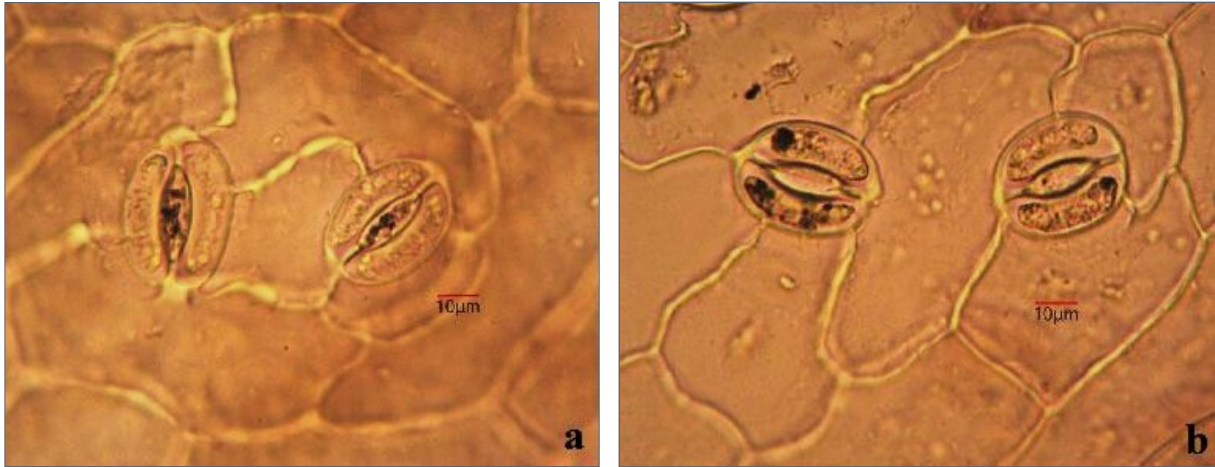


Fig. 3. Anomocytic and anisocytic stomatal type of *Carduus nutans* (a) and *Carduus thoermeri* (b).

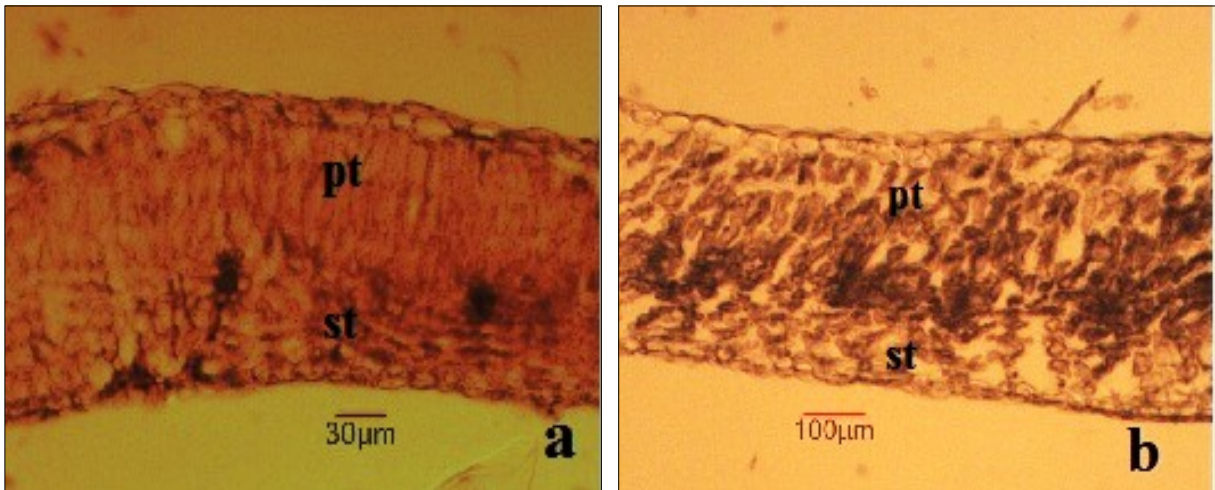


Fig. 4. Cross section of *Carduus nutans* (a) and *Carduus thoermeri* (b) leaf lamina.
Legend: pt - Palisade tissue; st - Spongy tissue.

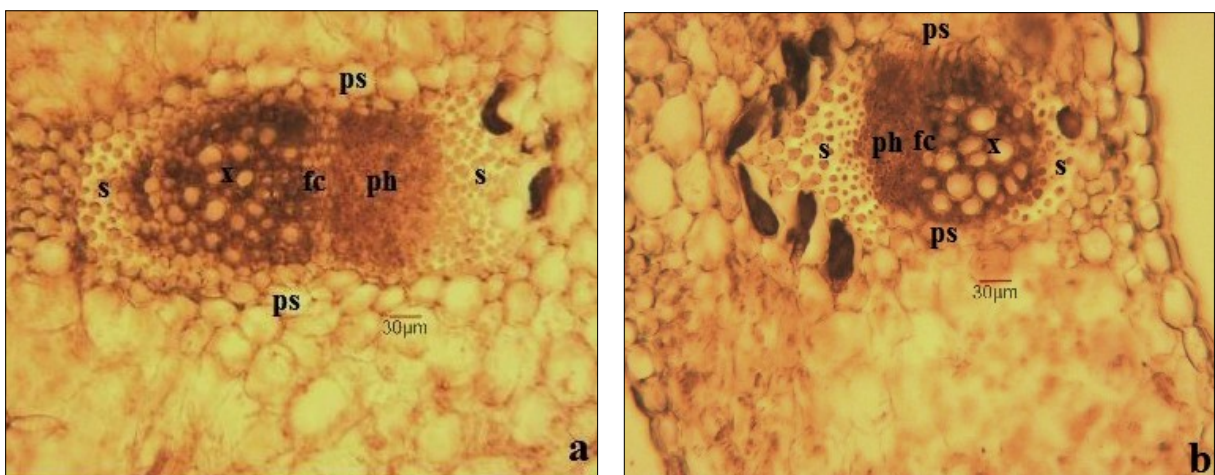


Fig. 5. Middle rib with opened collateral vascular bundles at *Carduus nutans* (a) and *Carduus thoermeri* (b). Legend: x - Xylem; ph - Phloem; fc - Fascicular cambium; s - Sclerenchyma bow; ps - Parenchyma sheath.

The amount of the two types of trichomes varied greatly between the different localities. Referring to the number of capitated glandular trichomes in the adaxial epidermis, there were differences between the studied specimens from Arkutino and Tchernoochene ($p \leq 0.001$) and those from Tchernoochene and Rozhen ($p \leq 0.05$). In the abaxial epidermis statistically significant difference ($p \leq 0.05$) was found only between Arkutino and Rozhen. The difference in the amount of the covering trichomes in the adaxial epidermis was statistically significant between Arkutino and Tchernoochene localities ($p \leq 0.001$), whereas in the abaxial epidermis differences were established between Arkutino and Rozhen and Tchernoochene and Rozhen ($p \leq 0.001$).

The results of the statistical processing of the measured quantitative indicators for the three localities of *C. thoermeri* are presented in Table 3.

The results of the statistical processing of the measured quantitative indicators for the three localities of *C. thoermeri* show that, similar to *C. nutans*, there was a great intraspecific variation of the quantitative anatomical characteristics (Table 3). Statistically significant differences between the localities were established in terms of the following characteristics: the number of stomata per unit area of the adaxial and abaxial epidermis, the number of multicellular covering trichomes in the adaxial and abaxial epidermis, the leaf blade thickness and the thickness of the palisade tissue. The difference in the number of stomata in the adaxial epidermis was statistically significant only between Bachkovo and Chirpan ($p \leq 0.01$), while significant differences in their number in the abaxial epidermis were found between Laki and Bachkovo ($p \leq 0.01$) and between Bachkovo and Chirpan ($p \leq 0.001$). The number of multicellular covering trichomes in the adaxial leaf surface varied greatly between the different localities and was statistically significant, as follows: Laki and Chirpan ($p \leq 0.001$), Laki and Bachkovo ($p \leq 0.01$), Bachkovo and Chirpan ($p \leq 0.05$), respectively. In the abaxial epidermis, the difference in the number of those trichomes was not found only among the specimens collected from Laki and

Bachkovo, while between Laki and Chirpan and Bachkovo and Chirpan the differences were significant ($p \leq 0.001$). The lamina thickness was statistically different between Laki and Bachkovolocalities ($p \leq 0.05$). In terms of the thickness of the palisade tissue there were differences between Laki and Bachkovo ($p \leq 0.001$) and Bachkovo and Chirpan ($p \leq 0.05$).

Both species were collected from the Central Rhodopes Mts., which necessitated the comparison of the mean values of the anatomical indicators in the frames of the floristic region (Students' T-Test). The results obtained are presented in Table 4.

In the floristic region of the Central Rhodopes Mts., the largest statistically significant difference was found in the characteristics: the number of capitated glandular trichomes in the abaxial epidermis and the number of multicellular covering trichomes in the adaxial epidermis ($p \leq 0.001$), followed by the number of multicellular covering trichomes in the abaxial epidermis ($p \leq 0.01$) and the number of capitated glandular trichomes in adaxial epidermis ($p \leq 0.05$). The aggregated mean values of the quantitative anatomical characteristics in the two studied species were compared applying the Student's T-test (Table 5).

The statistical processing of the data obtained for *C. nutans* and *C. thoermeri* showed a difference in the number of stomata in the abaxial epidermis, the number of capitated glandular trichomes in both epidermis, the number of multicellular covering trichomes in the abaxial epidermis ($p \leq 0.001$) and the number of multicellular covering trichomes in the adaxial leaf surface ($p \leq 0.05$). The established differences in the above-mentioned quantitative indicators supplement the characteristic of the two taxa and support the results of the molecular genetic analysis by [DENEV et al. \(2017\)](#) to distinguish them at the subspecies level.

Conclusions

The comparative anatomical leaf analysis of *Carduus nutans* L. and *Carduus thoermeri* Wienm was carried out for the first time in Bulgaria.

Table 2. Comparison of the mean values of anatomical features by localities of *Carduus nutans*. Legend: X_1 , X_2 , X_3 - Mean value; SE - Standard Error of Mean value; F -Fishers' criterion; p - Accuracy indicator; $p \leq 0.05$ - *; $p \leq 0.01$ - **; $p \leq 0.001$ - ***

Feature	1. Arkutino $X_1 \pm SE$	2. Tchernotchene $X_2 \pm SE$	3. Rozhen $X_3 \pm SE$	F	p	Multiple comparisons (Scheffe)		
						$X_1 - X_2$	$X_1 - X_3$	$X_2 - X_3$
Stomata number (Adaxial epidermis)	51.06±2.40	78.20±2.28	54.28±2.69	36.32	0.000	-27.14***	-3.22	23.92***
Stomata number (Abaxial epidermis)	114.54±3.80	129.26±3.83	92.00±2.94	28.00	0.000	-14.72*	22.54***	37.26***
Glandular hair number (Adaxial epidermis)	0.30±0.09	0.93±0.13	0.51±0.12	7.82	0.001	-0.63***	-0.21	0.42*
Glandular hair number (Abaxial epidermis)	0.27±0.08	0.60±0.11	0.69±0.13	4.23	0.016	-0.33	-0.42*	-0.09
Non glandular hair number (Adaxial epidermis)	2.40±0.15	1.41±0.20	1.86±0.21	6.92	0.001	0.99***	0.54	-0.45
Non glandular hair number (Abaxial epidermis)	1.14±0.16	1.11±0.15	2.19±0.21	12.22	0.000	0.03	-1.05***	-1.08***
Leaf lamina (Width) μm	385.10±19.27	352.70±24.37	404.10±18.06	1.57	0.227	32.40	-19.00	-51.40
Upper cuticle (Width) μm	6.20±0.25	6.60±0.43	6.40±0.54	0.22	0.801	-0.40	-0.20	0.20
Lower cuticle (Width) μm	6.80±0.25	6.10±0.38	6.30±0.30	1.32	0.284	0.70	0.50	-0.20
Palisade tissue (Width) μm	190.60±3.85	177.90±10.47	166.80±9.44	1.99	0.156	12.70	23.80	11.10

Table 3. Comparison of the mean values of anatomical features by localities of *Carduus thoermeri*. Legend: X_1 , X_2 , X_3 - Mean value; SE - Standard Error of Mean value; F -Fishers' criterion; p - Accuracy indicator; $p \leq 0.05$ - *; $p \leq 0.01$ - **; $p \leq 0.001$ - ***

Feature	1. Laki $X_1 \pm SE$	2. Bachkovo $X_2 \pm SE$	3. Chirpan $X_3 \pm SE$	F	p	Multiple comparisons (Scheffe)		
						$X_1 - X_2$	$X_1 - X_3$	$X_2 - X_3$
Stomata number (Adaxial epidermis)	56.58±2.20	63.02±2.35	51.98±2.26	5.96	0.003	-6.44	4.60	11.04**
Stomata number (Abaxial epidermis)	103.04±3.43	87.40±3.01	107.64±3.44	10.33	0.000	15.64**	-4.60	-20.24***
Glandular hair number (Adaxial epidermis)	0.30±0.10	0.09±0.05	0.12±0.06	2.55	0.081	0.21	0.18	-0.03
Glandular hair number (Abaxial epidermis)	0.06±0.04	0.12±0.06	0.03±0.03	1.04	0.36	-0.06	0.03	0.09
Non glandular hair number (Adaxial epidermis)	3.12±0.18	2.28±0.21	1.50±0.18	18.46	0.000	0.84**	1.62***	0.78*
Non glandular hair number (Abaxial epidermis)	3.33±0.17	2.70±0.23	1.50±0.18	21.96	0.000	0.63	1.83***	1.20***
Leaf lamina (Width) μm	364.40±24.82	431.50±17.95	396.00±9.47	3.29	0.053	-67.10*	-31.60	35.50
Upper cuticle (Width) μm	5.60±0.27	6.10±0.18	5.80±0.42	0.69	0.512	-0.50	-0.20	0.30
Lower cuticle (Width) μm	5.50±0.31	6.70±0.42	6.30±0.50	2.16	0.135	-1.20	-0.80	0.40
Palisade tissue (Width) μm	147.70±5.06	197.80±4.28	162.40±10.66	12.63	0.000	-50.10***	-14.70	35.40*

Table 4. Comparison of the mean values of the anatomical indicators of *Carduus nutans* and *Carduus thoermeri* from the floristic region of the Rhodopes Mts (Central). Legend: X – Mean value of *C. nutans*; Y – Mean value of *C. thoermeri*; SE – Standard Error of Mean value; T – Students’ T-Test; p – Accuracy indicator; p≤0.05 – *; p≤0.01 – **; p≤0.001 – ***

Feature	<i>C. nutans</i> x±SE	<i>C. thoermeri</i> y±SE	T	p	(x-y)±SE
Stomata number (Adaxial epidermis)	54.28±2.69	59.80±1.63	-1.85	0.067	-5.52±2.99
Stomata number (Abaxial epidermis)	92.00±2.94	95.22±2.40	-0.81	0.420	-3.22±3.98
Glandular hair number (Adaxial epidermis)	0.51±0.12	0.20±0.06	2.42	0.018	0.32±0.13*
Glandular hair number (Abaxial epidermis)	0.69±0.13	0.09±0.04	4.45	0.000	0.60±0.13***
Non glandular hair number (Adaxial epidermis)	1.86±0.21	2.70±0.14	-3.38	0.001	-0.84±0.25***
Non glandular hair number (Abaxial epidermis)	2.19±0.21	3.02±0.15	-3.23	0.002	-0.83±0.26**
Lamina (Width) µm	404.10±18.06	397.95±16.78	0.23	0.822	6.15±27.03
Upper cuticle (Width)µm	6.40±0.54	5.85±0.17	0.98	0.353	0.55±0.57
Lower cuticle (Width)µm	6.30±0.30	6.10±0.29	0.43	0.669	0.20±0.46
Palisade tissue (Width)µm	166.80±9.44	172.75±6.59	-0.52	0.608	-5.95±11.46

Table 5. Comparison of the mean values of anatomical characteristics in *Carduus nutans* and *Carduus thoermeri*. Legend: X – Mean value of *C. nutans*; Y – Mean value of *C. thoermeri*; SE – Standard Error of Mean value; T – Students’ T-Test; p – Accuracy indicator; p≤0.05 – *; p≤0.01 – **; p≤0.001 – ***

Feature	<i>C. nutans</i> x±SE	<i>C. thoermeri</i> y±SE	T	p	(x-y)±SE
Stomata number (Adaxial epidermis)	61.18±1.72	57.19±1.35	1.82	0.070	3.99±2.19
Stomata number (Abaxial epidermis)	111.93±2.39	99.36±2.02	4.02	0.000	12.57±3.13***
Glandular hair number (Adaxial epidermis)	0.58±0.07	0.17±0.04	5.08	0.000	0.41±0.08***
Glandular hair number (Abaxial epidermis)	0.52±0.06	0.07±0.03	5.08	0.000	0.41±0.08***
Non glandular hair number (Adaxial epidermis)	1.89±0.11	2.30±0.12	-2.48	0.014	-0.41±0.17*
Non glandular hair number (Abaxial epidermis)	1.48±0.11	2.51±0.13	-6.08	0.000	-1.03±0.17***
Lamina (Width) µm	380.63±12.21	397.30±11.50	-0.99	0.325	-16.67±16.78
Upper cuticle (Width) µm	6.40±0.24	5.83±0.17	1.93	0.059	0.57±0.29
Lower cuticle (Width) µm	6.40±0.18	6.17±0.25	0.75	0.45	0.23±0.31
Palisade tissue (Width) µm	178.43±5.04	169.30±5.62	1.21	0.23	9.13±7.54

There were no differences found between the two species, regarding the following qualitative anatomical characteristics – leaf lamina structure (bifacial structure), stomatal type (anisocytic and anomocytic) and hair type (capitated glandular hairs and multicellular uniseriate covering hairs), while intraspecific variations

in the quantitative leaf characteristics were found in the different localities, probably, related to ecological adaptation. Statistically significant differences between the two taxa were observed in the following characteristics: the number of stomata in the abaxial epidermis, the number of glandular hairs and the number of covering hairs in the adaxial and abaxial epidermis.

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