

Palynomorphological study of the Bulgarian representatives of genus *Carduus* (Asteraceae)

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Absrtact. A detailed palynomorphological study of the Bulgarian species of genus *Carduus* L. (Asteraceae) is carried out for the first time by light and electron microscopy. New data have been obtained on the endemic species *C. armatus*, *C. candicans* subsp. *globifer*, *C. kerneri* subsp. *austro-orientalis*, *C. rhodopaeus*, and *C. thracicus*. The grouping of the species is traced out and their similarity is assessed by means of statistical processing and cluster analysis. The assumption of the species' belonging to one pollen type, the *Carduus crispus* type, has been confirmed. Within the framework of that type, two pollen groups have been distinguished: *Carduus nutans* and *Carduus crispus*. It has been established that the equatorial diameter, polar axis, height and width of the spikes are of the highest distinguishing value for the pollen grains.

Key words: Asteraceae, Bulgaria, *Carduus*, cluster analysis, LM, pollen morphology, SEM

Introduction

Morphology of the pollen is comparatively feebly influenced by environmental conditions, which makes it a reliable characteristic in the taxonomy of higher plants (Aytug 1959). Results of the palynomorphological studies are used successfully in the plant systematics to clarify the plant relationships and in tracing out the phylogeny and origin of different plant groups. The pollen of Asteraceae has been researched in numerous studies but only few of them could be defined as complete in terms of the broad geographical range of the researched species, or detailed studies of specific taxonomic groups. The first major studies of the morphology of pollen have been carried out by Wodehouse (1926, 1928, 1935), followed by Stix (1960), who studied 235 species of the family Asteraceae and distinguished 49 pollen types. The main taxonomic characteristics for distinguishing the types were the exine structure and the char-

acter of the spikes. Erdtman (1966), Faegri & Iversen (1975) and Moore & al. (1991) had provided comparatively scanty information about the morphology of pollen grains of that family, but left some practical instructions for their determining, while other researchers (Pragłowski & Nilsson in Erdtman & al. 1963) gave the basic keys for determination of the Asteraceae pollen. Important studies into the morphology of the pollen of Asteraceae were provided by Wagenitz (1955), Stix (1960), Bonnefille (1965), Skvarla & Larson (1965), Skvarla & Turner (1966), Valdes & al. (1987), Fægri & al. (1989), Moore & al. (1991), Osman (2006) Ghahreman & al. (2007) and Jafari & Ghanbarian (2007). For the Balkan countries, of interest are the studies into the pollen of genus *Achillea* from Turkey (Akyalcin & al. 2011), as well as of the subfamily Cichorieae (Wang & al. 2009).

Punt & Hoen (2009) presented one of the fullest researches into the pollen of subfamily Asteroideae

spread in Northwest Europe. In that research, the authors set genus *Carduus* apart as an independent pollen type, which included two pollen groups. The following seven species of the genus have been studied: *C. acanthoides* L., *C. crispus* L., *C. defloratus* L., *C. nigrescens* Villars, *C. nutans* L., *C. personata* L., and *C. tenuiflorus* Curtis.

Object of the present study is the pollen of genus *Carduus* L., represented by 14 species in the Bulgarian flora (Tutin & al. 1976; Delipavlov & Cheshmedzhiev 2003). Five of them are endemic: three Balkan and two Bulgarian endemics. The pollen of this genus' species distributed in Bulgaria has not been studied so far.

Material and methods

Plant material (anthodidiums) for the pollen analysis was collected in the period 2010–2013 from different floristic regions in Bulgaria. Herbarium samples of the species have been deposited at the Herbarium of the Agrarian University in Plovdiv (SOA). For one of the species (*Carduus thracicus*), material has been taken from a Herbarium sample stored in the herbarium (SOM) of IBER, BAS (Table 1).

The pollen has been processed according to the standard acetolysis method of Erdtman (1960), with subsequent inclusion of the material into glycerine gellatin preparations. The morphological descrip-

tions, measurements, comparisons, and analysis of the pollen grains have been made with a Magnum T light microscope, at magnification $\times 400$, with a digital photcamera Si 3000 and XLiCap software for image processing.

Calculations are based on the data from measurements of 30 pollen grains, registering for each species the minimum, average and maximum values. The following parameters have been measured out: apocolpium diameter (d), equatorial diameter (E), polar axis (P), height of spikes (h), width of spikes (w), number of spikes (n), nexine thickness (N) and sexine thickness (S). For statistical processing of the data, apocolpium index (AI) was also calculated, which expresses the correlation between apocolpium diameter and equatorial diameter (d/E).

The scanning electron microscopic studies have been carried out with a JEOL JSM 5510 microscope, at magnification of $\times 2000$ – $\times 10000$.

The software package Statistica v. 7.0 (STATSOFT INC., 2007) was used for the statistical data processing.

Results and discussion

Morphological specificities of the studied Bulgarian representatives of genus *Carduus* have shown that variations in the quantitative parameters of the analyzed characteristics do not permit differentiation

Table 1. Collection locality, altitude and voucher specimen of the studied *Carduus* species.

Species	Collection locality, floristic region in Bulgaria	Altitude (m)	Voucher specimen (SOA-Plovdiv)
<i>Carduus acanthoides</i> L.	Trigrad, Rhodopi Mountains (Central)	1100	059719
<i>Carduus acicularis</i> Betrol. [= <i>C. argentatus</i> L. subsp. <i>acicularis</i> (Bertol.) Meikle]	Tsarevo, Black Sea Coast (Southern)	30	059650
<i>Carduus armatus</i> Boiss. & Heldr. [= <i>C. tmoleus</i> Boiss. subsp. <i>armatus</i> (Boiss. & Heldr.) Franco]	Balgarka Nature park, Balkan Range (Central)	1415	059781
<i>C. candicans</i> Waldst. & Kit. subsp. <i>globifer</i> (Velen.) Kazmi	Rishki pass, Balkan Range (Eastearn)	320	059656
<i>Carduus carduelis</i> (L.) Gren. [= <i>C. alpestris</i> Willd.]	Yundola, Rila Mountains	1510	059779
<i>Carduus crispus</i> L.	Ivanski, Northeast Bulgaria	92	059725
<i>Carduus hamulosus</i> Ehrh.	Narechen, Rhodopes Mountains (middle)	588	059644
<i>Carduus kernerii</i> Simonkai ssp. <i>austro-orientalis</i> Franco [= <i>C. scardicus</i> (Griseb.) Wettst.]	Beglika, Rhodopi Mountains (Central)	1550	059651
<i>Carduus nutans</i> L.	Arkutino, Black sea Coast (southern)	30	059660
<i>Carduus personata</i> (L.) Jacq.	Balgarka Nature park, Balkan Range (Central)	815	059780
<i>Carduus pycnocephalus</i> L.	Tsarevo, Black Sea Coast (Central)	30	059649
<i>Carduus rhodopaeus</i> Velen. [<i>C. adpressus</i> C.A.Mey. subsp. <i>rhodopaeus</i> (Velen.) Kožuharov & N.Andreev]	Rozhen, Rhodopi Mountains (Central)	1431	059776
<i>Carduus thoermeri</i> Wienm. [= <i>C. nutans</i> subsp. <i>leiophyllus</i> (Petrovič) Stoj. & Stef.]	Novo selo, Thracian Lowland	217	059658
<i>Carduus thracicus</i> (Velen.) Hayek	Ivailovgrad, Rhodopi Mountains (Eastern)	–	153497 – Herbarium SOM – Sofia

of independent pollen types and that all species belonged to one pollen type, namely *Carduus crispus*, described by Punt & Hoen (2009). This is in conformity with the assumption about morphological changeability of the pollen of this genus expressed by other researchers (Beug 2004; Punt & Hoen 2009). Along with this, the differences between the various species permit their grouping into pollen groups in terms of the “pollen group” meaning suggested by Punt & Hoen (2009). Contrary to the pollen type, the pollen groups are differentiated on the basis of more changeable characteristics, often difficult for telling apart, which are not constant and are subject to variations.

***Carduus crispus* type**

Description: 3-zonocolporate pollen grains, oblate-spheroidal to spheroidal in shape; ectoaperture – a short to medium long colpus, comparatively narrow, covered with a membrane with almost smooth ornamentation; mesoaperture – a lalongate porus; endoaperture – a lalongate porus.

Nexine – dense, homogeneous; sexine – not represented; an indistinct cavea between the nexine and sexine. Tectum in all studied Bulgarian species of genus *Carduus* finely tectate-perforate. Perforations are fine, under 1 µm, well visible only under a scanning electron microscope. Pollen grains almost round in outline, only in some species could be described as rounded triangular, to nearly round.

The set of morphometric parameters and statistical data in this study give grounds for differentiation of two pollen groups. They differ from the groups distinguished by Punt & Hoen (2009), which are based on the specificities of mesoaperture; that is why the authors of this paper have not taken them under consideration. The present morphological study of the pollen grains of the species of genus *Carduus* made possible their grouping into the following pollen groups described below: *C. nutans* group and *C. crispus* group.

***Carduus nutans* group** (Plate I, Figs. 1-4, Plate IV)

The group includes the following species: *C. nutans* and *C. thoermeri*.

Description

1. Pollen class: 3-zonocolporate.
2. Form: Oblate spheroidal; P/E ratio = (0.79) 0.97 (1.16).

3. Outlines: polar view – circular; equatorial view – elliptical to almost circular.
4. Measurements: E = (40.85) 49.13 (58.50) µm; P = (37.00) 47.70 (60.90) µm; appocolpium index (AI) = (0.33) 0.38 (0.42), with small puncta between the echinae.
5. Ornamentation: Echinata, echinae evenly distributed on the surface of the entire pollen grain:
 - 5.1. Height of echinae (h) = (3.45) 4.67 (6.70) µm;
 - 5.2. Width of echinae (w) = (4.15) 5.65 (8.00) µm;
 - 5.3. Number (n): in polar view (13) 15 (20) echinae visible in the equatorial plane.
6. Apertures: ectoaperture – colpus, not very wide, with acute ends and without a distinct margin; colpus membrane smooth; mesoaperture – a lalongate porus; endoaperture – a lalongate porus, more or less indistinct.
7. Exine: two-layered; nexine (N) = (0.55) 0.78 (1.15) µm thick; sexine (S) = (2.05) 3.41 (5.65) µm thick; columellae small, circular and crowded.

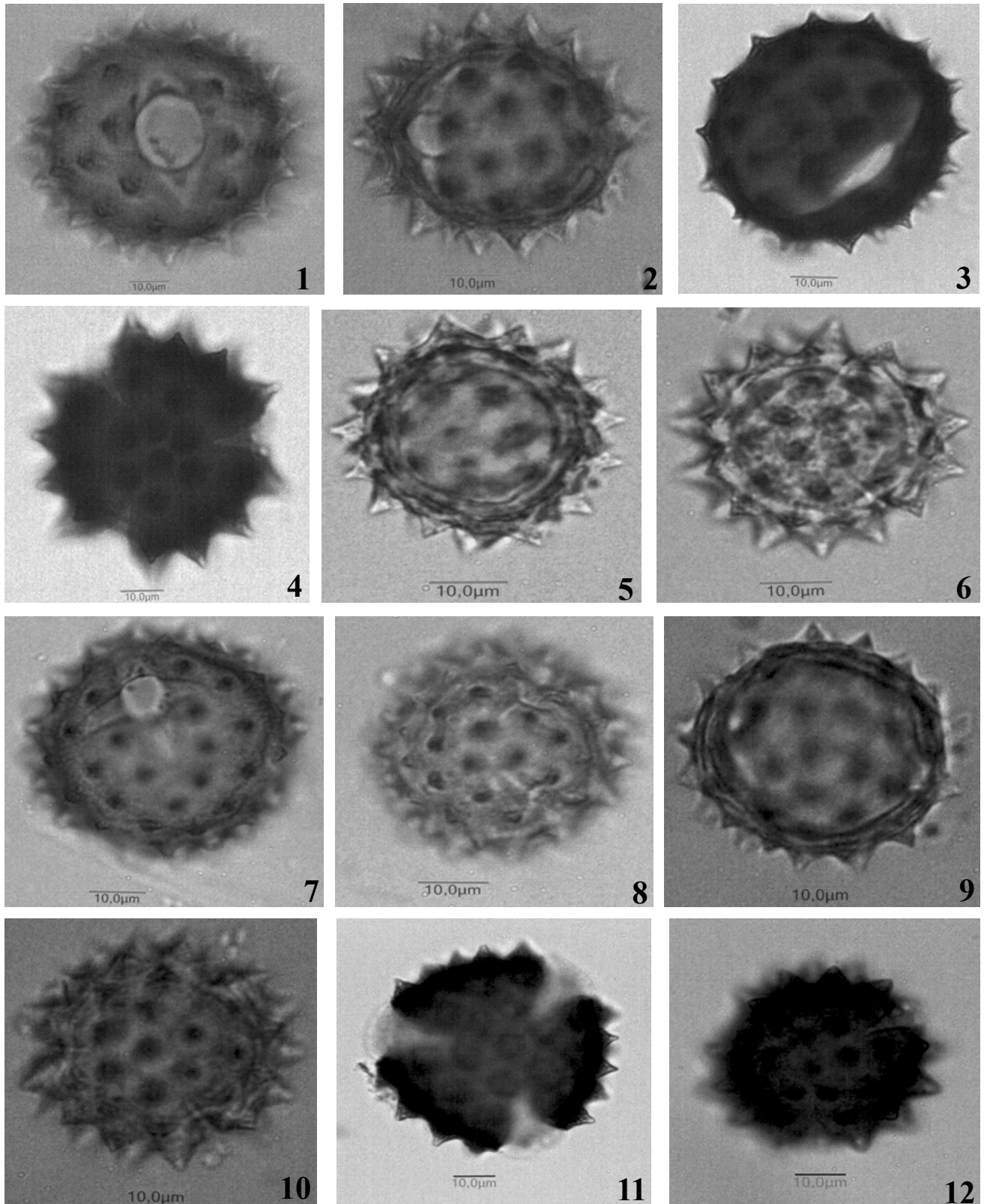
***Carduus crispus* group** (Plate I, Figs. 5-12; Plates II, III, V-VIII)

The group includes the following species: *C. crispus*, *C. pycnocephalus*, *C. personata*, *C. kernerii* subsp. *austror-orientalis*, *C. hamulosus*, *C. acanthoides*, *C. thracicus*, *C. carduelis*, *C. candicans* subsp. *globifer*, *C. rhodopaeus*, *C. armatus*, and *C. acicularis*.

Description

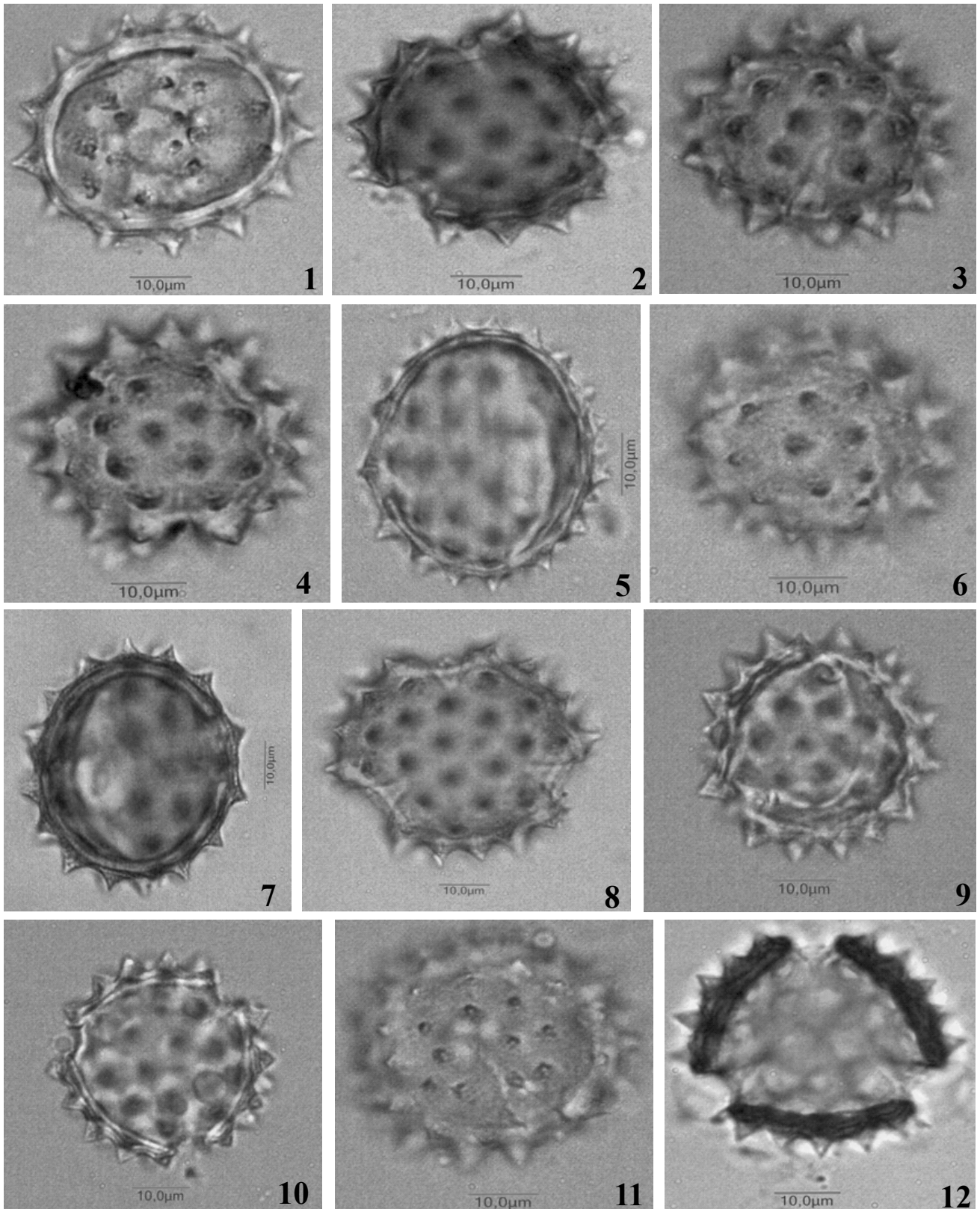
1. Pollen class: 3-zonocolporate.
2. Form: oblate spheroid; P/E ratio = (0.84) 0.96 (1.12).
3. Outlines: polar view – circular; equatorial view – elliptical to almost circular.
4. Measurements: E = (31.44) 36.99 (44.62) µm; P = (29.55) 35.72 (44.05) µm; appocolpium index (AI) = (0.29) 0.40 (0.48).
5. Ornamentation: echinata, echinae evenly distributed on the surface of the entire pollen grain:
 - 5.1. Height of echinae (h): (2.43) 3.38 (4.39) µm;
 - 5.2. Width of echinae (w): (3.11) 4.37 (5.68) µm;
 - 5.3. Number of echinae (n): in polar view (13) 14 (17) echinae visible in the equatorial plane.
6. Apertures: ectoaperture: colpus, comparatively narrow, with acuminate ends; mesoaperture: porus – elliptic, lalongate; endoaperture: lalongate, more or less indistinct.
7. Exine: two-layered; nexine: (0.40) 0.68 (0.99) µm; sexine: (2.03) 3.37 (4.88) µm.

Plate I.



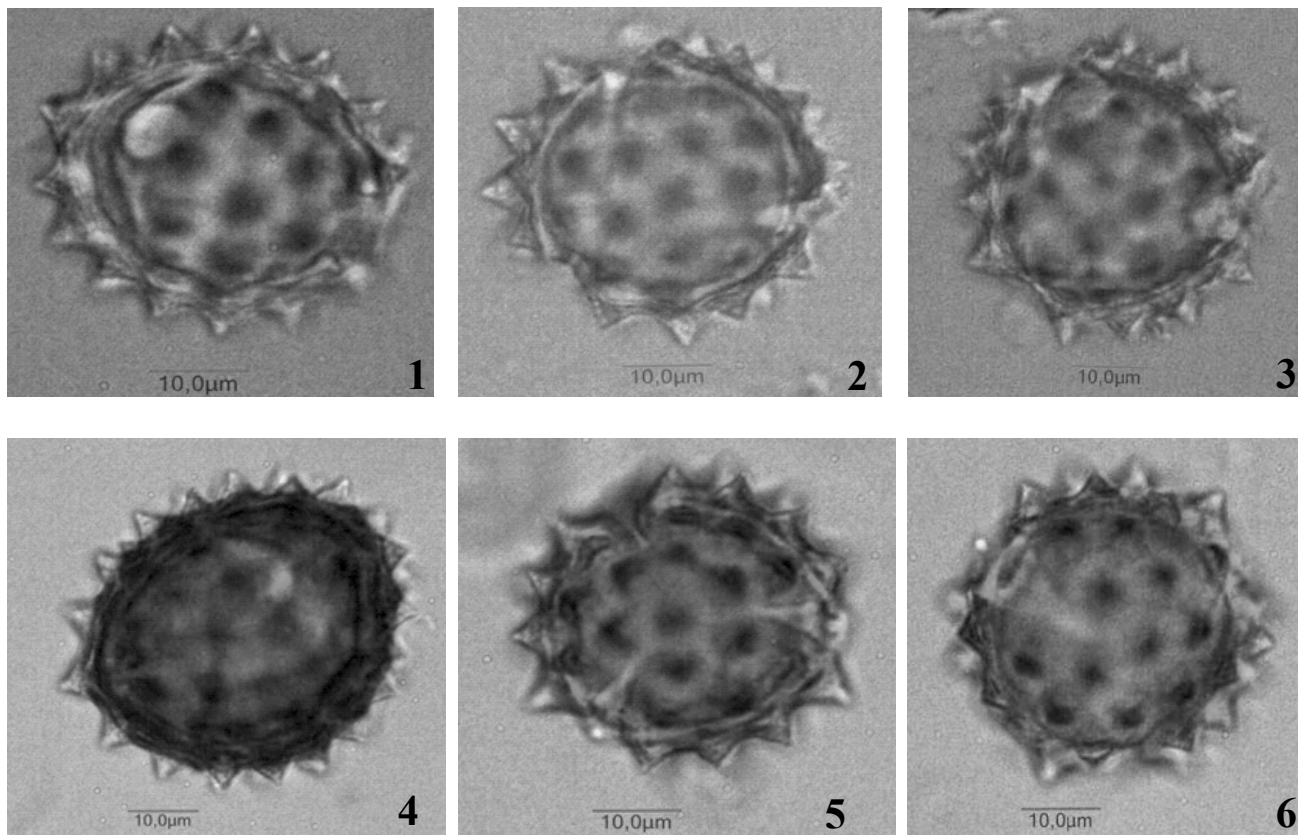
LM photographs of *Carduus* pollen: Figs. 1-4: *Carduus nutans* group: *C. nutans* (Figs. 1, 2. Equatorial view); *C. thoermeri* (Fig. 3. Equatorial view, Fig. 4. Polar view); Figs. 5-12: *Carduus crispus* group: *C. hamulosus* (Fig. 5. Equatorial view, 6. Polar view); *C. acicularis* (Fig. 7. Equatorial view, Fig. 8. Polar view); *C. armatus* (Fig. 9. Equatorial view, Fig. 10. Polar view); *C. rhodopaicus* (Figs. 11, 12. Polar view).

Plate II.



LM photographs of the pollen of *Carduus crispus* group: *C. candicans* ssp. *globifer* (Fig. 1. Equatorial view, Fig. 2. Polar view); *C. crispus* (Figs. 3, 4. Equatorial view); *C. carduelis* (Figs. 5, 6. Equatorial view); *C. thracicus* (Fig. 7. Equatorial view, Fig. 8. Polar view); *C. acanthoides* (Fig. 9. Equatorial view, Fig. 10. Polar view); *C. kernerii* ssp. *austro-orientalis* (Fig. 11. Equatorial view, Fig. 12. Polar view).

Plate III.



LM photographs of the pollen of *Carduus crispus* group: *C. personata* (Fig. 1. Equatorial view, Figs. 2, 3. Polar view); *C. pycnocephalus* (Fig. 4. Equatorial view, Figs. 5, 6. Polar view).

Cluster analysis

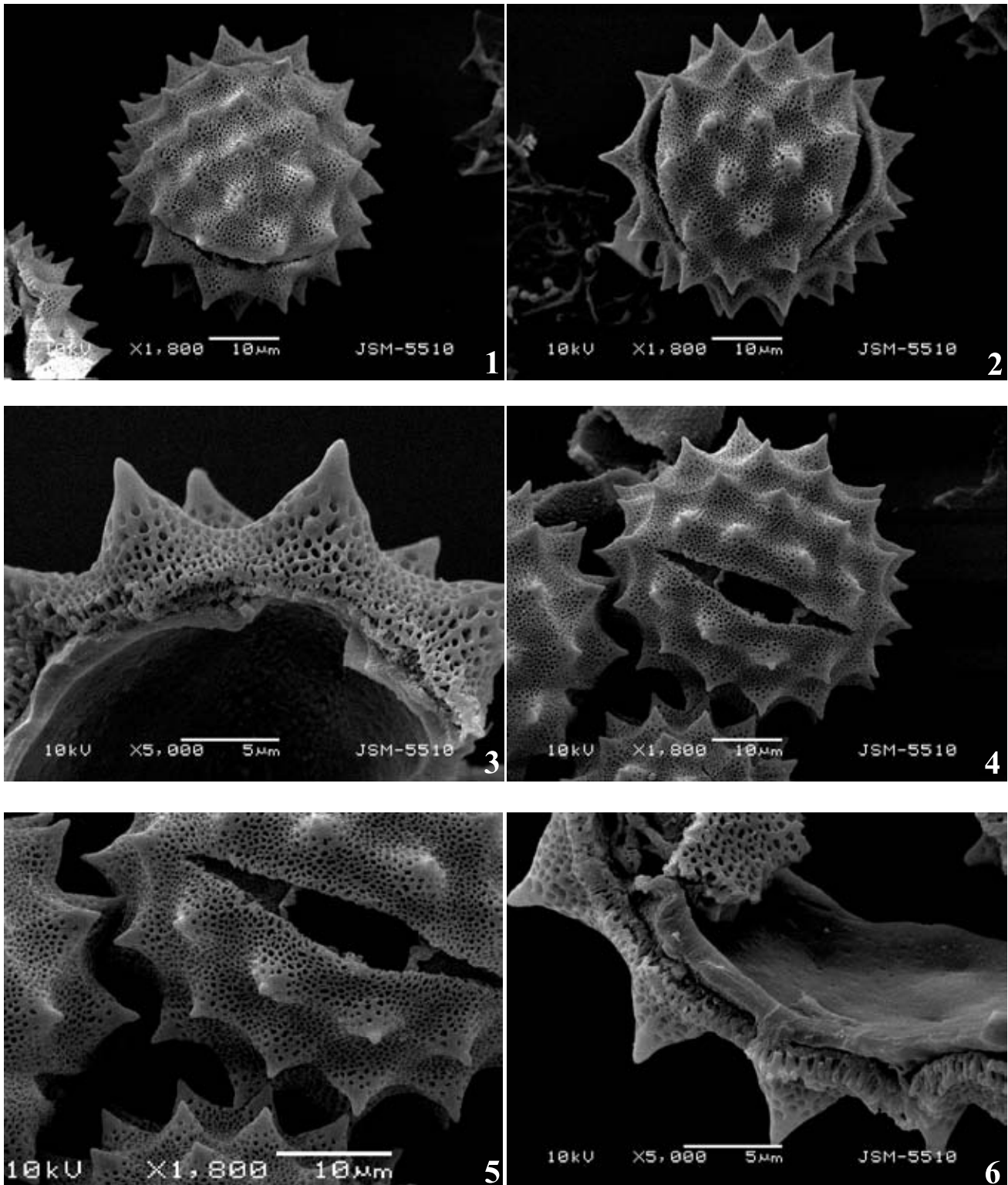
The obtained morphometric data about the pollen grains of genus *Carduus* were statistically processed by calculating their average values and standard error (Table 2). A cluster analysis was used to trace out the grouping the species in terms of their pollen grains (Fig. 1). The obtained dendrogram shows that the species of genus *Carduus* subdivide into two main groups in terms of the mean values of the studied parameters, with Euclidean distances of about 11.5%. The first group was presented by the species *C. nutans* and *C. thoermeri*. The second group comprised the remaining 12 species, including *C. acicularis* which differentiates into an independent subgroup relatively early. The remaining 11 species make a cluster with about 3% distance between the species. Within that cluster, a subgroup of the species *C. candidans*, *C. armatus* and *C. rhodopaeus* definitely stands out, with a subgroup of the remaining eight species. Two clusters were outlined within the framework of the second subgroup.

One of them comprises the species *C. crispus*, *C. carduelis*, *C. thracicus*, *C. acanthoides*, and *C. hamulosus*, while the second comprises the species *C. kernerii*, *C. personata* and *C. pycnocephalus*.

Conclusion

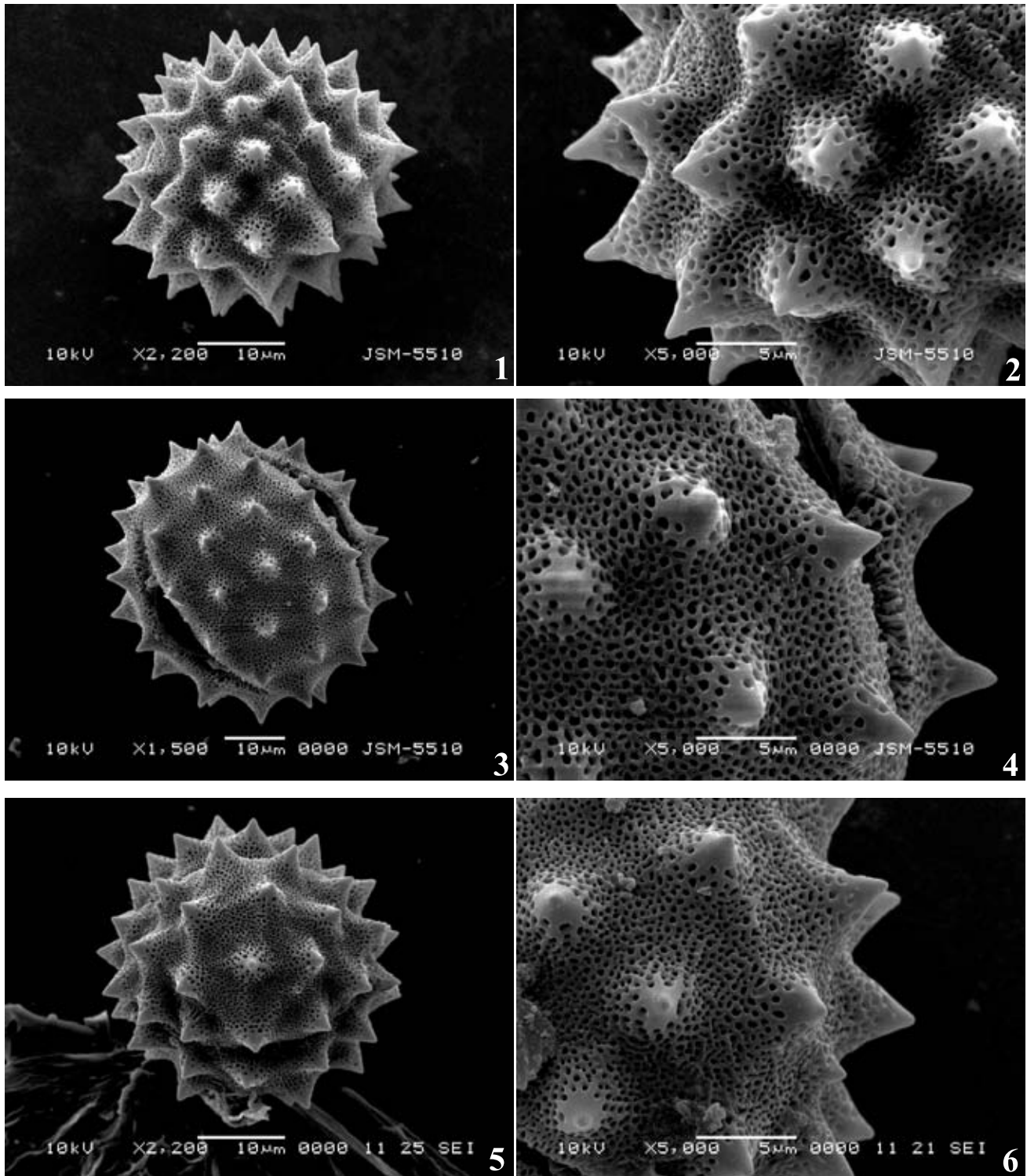
A palynomorphological study of the pollen of 14 species from genus *Carduus* distributed in the Bulgarian flora was made for the first time in the country. It provided data on the endemic species *C. armatus*, *C. candidans* subsp. *globifer*, *C. kernerii* subsp. *austro-orientalis*, *C. rhodopaeus*, and *C. thracicus* again for the first time and not only for Bulgaria. The biometric characteristics of pollen grains, statistical data processing and cluster analysis have confirmed the assumption of Beug (2004) and Punt & Hoen (2009) that the pollen of the species of genus *Carduus* is referred to one pollen type: the *C. crispus* type. Within the framework

Plate IV.



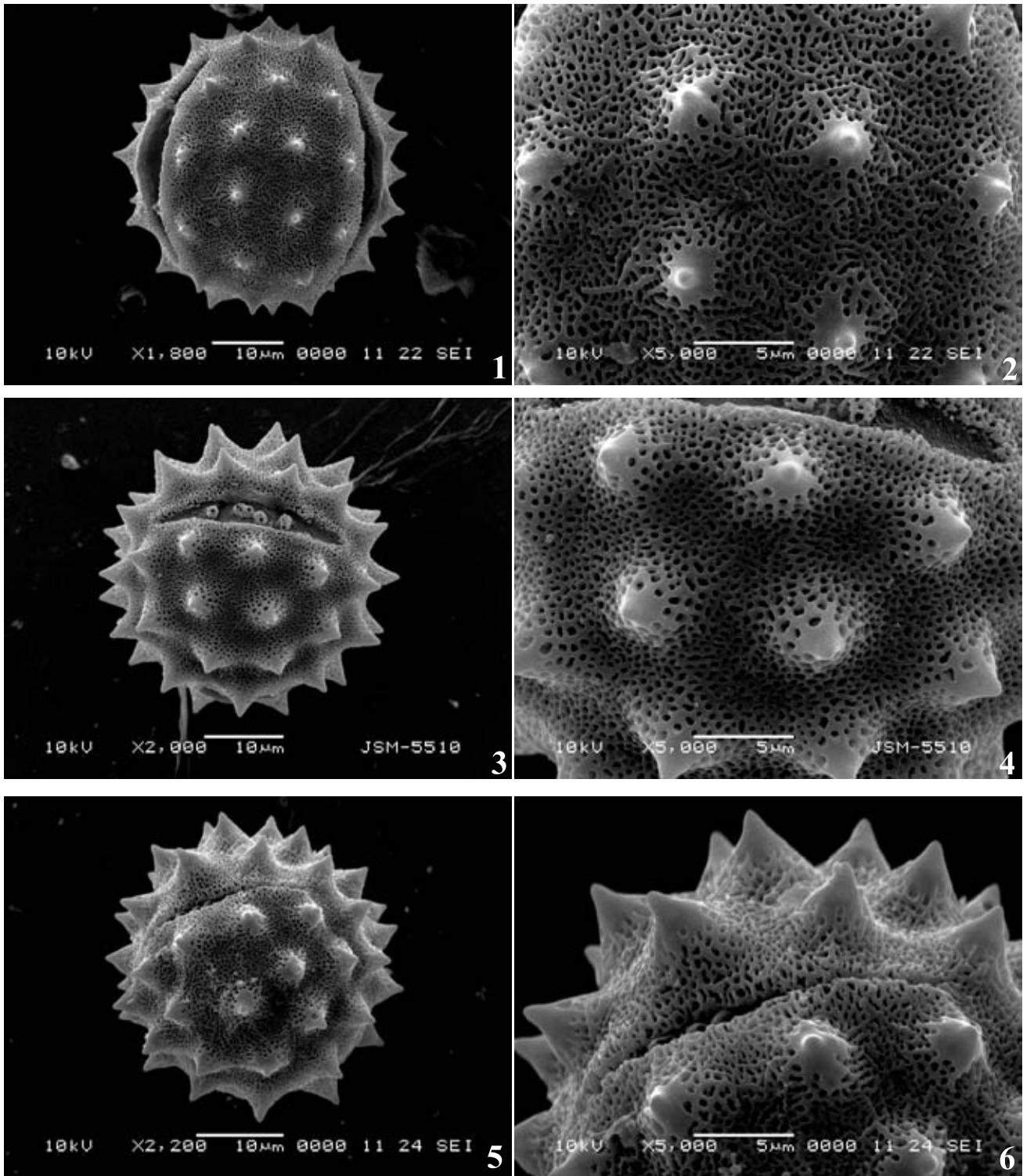
SEM photographs of the pollen of *Carduus nutans* group: *C. nutans* (Figs. 1-3); *C. thoermeri* (Figs. 4-6).

Plate V.



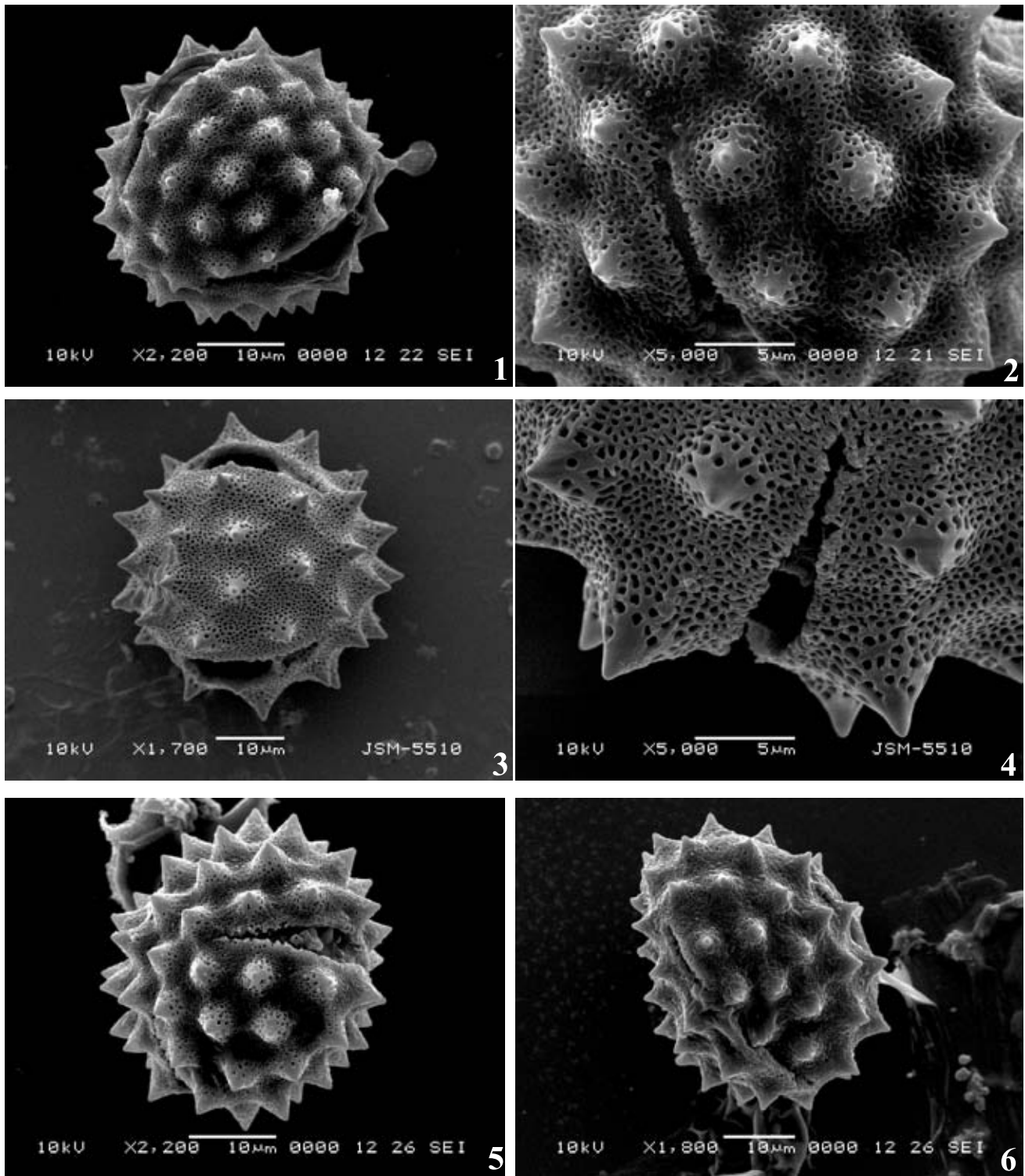
SEM photographs of the pollen of *Carduus crispus* group: *C. hamulosus* (Figs. 1-2); *C. acicularis* (Figs. 3-4); *C. armatus* (Figs. 5-6).

Plate VI.



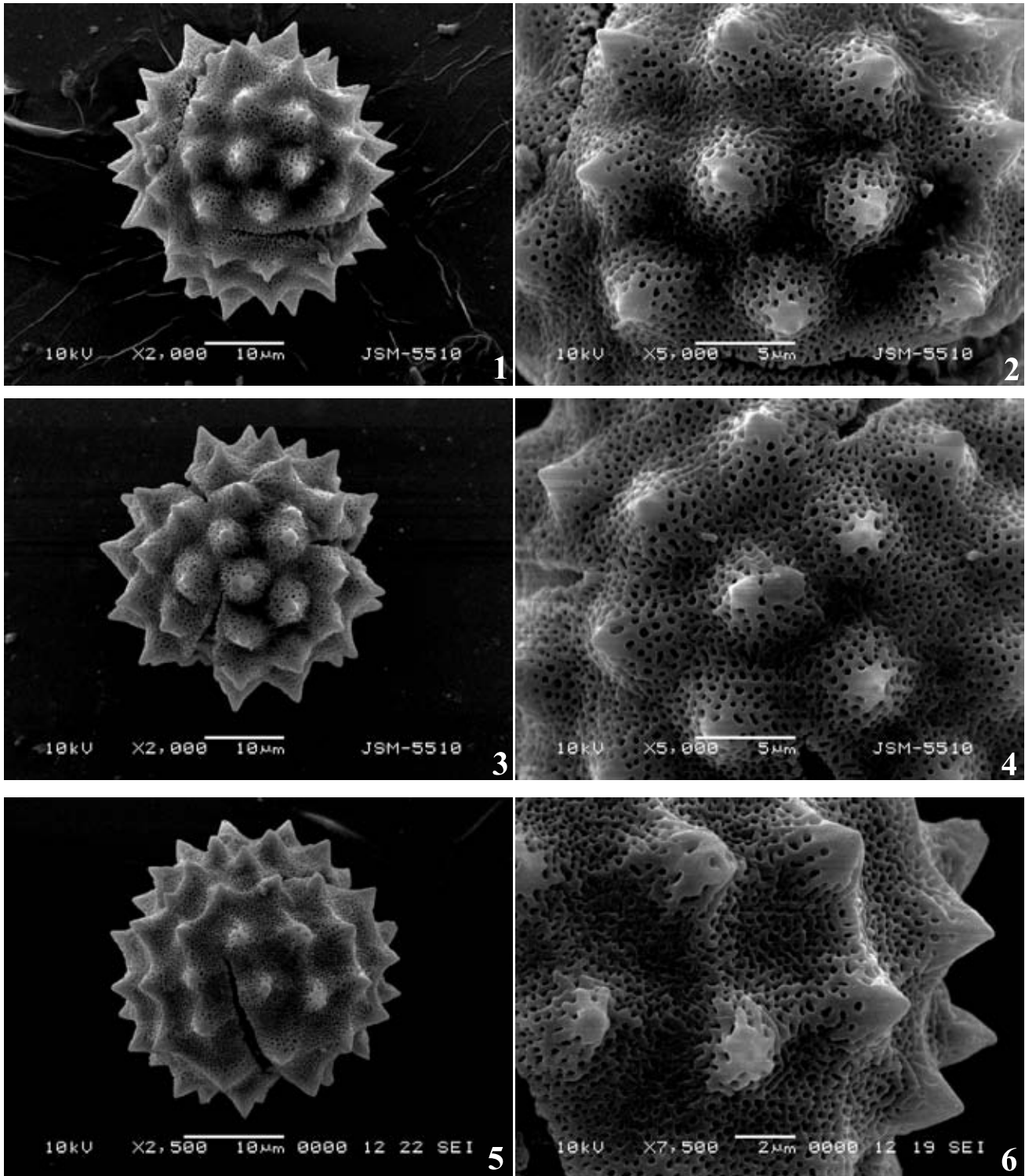
SEM photographs of the pollen of *Carduus crispus* group: *C. rhodopaeus* (Figs. 1-2); *C. candicans* ssp. *globifer* (Figs. 3-4); *C. crispus* (Figs. 5-6).

Plate VII.



SEM photographs of the pollen of *Carduus crispus* group: *C. carduelis* (Figs. 1-2); *C. thracicus* (Figs. 3-4); *C. acanthoides* (Figs. 5-6).

Plate VIII.



SEM photographs of the pollen of *Carduus crispus* group: *C. kernerii* ssp. *austro-orientalis* (Figs. 1-2); *C. personata* (Figs. 3-4); *C. pycnocephalus* (Figs. 5-6). Fig. 1. Grouping of the *Carduus* species by mean values of all measured pollen grains parameters (Cluster analysis).

of the *C. crispus* type, on the basis of a set of morphometric parameters and statistical data, two pollen groups have been distinguished (in the meaning of Punt & Hoen 2009): *Carduus nutans* group and *C. crispus* group.

The *C. nutans* group stands out with its large pollen grains, polar axis and equatorial diameter reaching 60.9 μm and average values of $P = 47.7 \mu\text{m}$ and $E = 49.1 \mu\text{m}$. The echinae are the largest, as compared to the other representatives, reaching 6.7 μm in height, and up to 8 μm in width. The average exine thickness is about 4.0–4.2 μm .

The *Carduus crispus* group is characteristic with pollen grains of average size, polar axis and equatori-

al diameter reaching 44 μm , and average values of $P = 30\text{--}37 \mu\text{m}$ and $E = 33\text{--}38 \mu\text{m}$. Echinae are average in size, about 3.3 μm high and about 4.4 μm wide. Exine thickness on the average reaches about 4–5 μm .

The present study has shown that the morphometric characteristics of equatorial diameter, polar axis and height and width of echinae have the highest taxonomic value for distinguishing the pollen grains.

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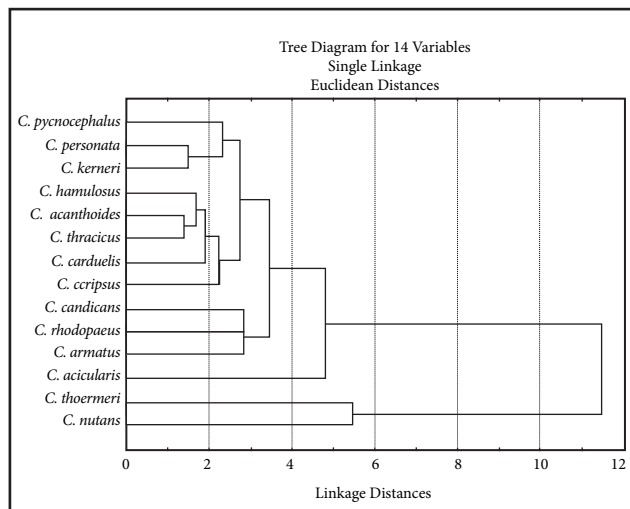


Fig. 1. Grouping of the *Carduus* species by mean values of all measured pollen grains parameters (Cluster analysis).

Table 2. Mean values of the measured parameters of *Carduus* pollen grains.

Parameter Species	d, μm M \pm SE	ED, μm M \pm SE	PO, μm M \pm SE	H, μm M \pm SE	W, μm M \pm SE	N M \pm SE	Ne, μm M \pm SE	Se, μm M \pm SE
<i>C. pycnocephalus</i>	16.44 \pm 0.73	38.73 \pm 0.69	35.96 \pm 0.83	3.19 \pm 0.12	4.60 \pm 0.13	15 \pm 0.38	0.66 \pm 0.04	3.43 \pm 0.13
<i>C. acicularis</i>	12.32 \pm 0.35	41.16 \pm 0.83	39.88 \pm 0.84	2.92 \pm 0.11	4.17 \pm 0.14	17 \pm 0.25	0.67 \pm 0.03	3.24 \pm 0.14
<i>C. thoermeri</i>	20.11 \pm 0.17	47.70 \pm 0.61	46.84 \pm 1.05	4.90 \pm 0.14	5.69 \pm 0.16	14 \pm 0.19	0.78 \pm 0.02	3.37 \pm 0.25
<i>C. nutans</i>	17.07 \pm 0.01	50.55 \pm 1.05	48.56 \pm 1.45	4.44 \pm 0.12	5.61 \pm 0.14	17 \pm 0.45	0.78 \pm 0.03	3.44 \pm 0.12
<i>C. personata</i>	14.93 \pm 0.29	36.65 \pm 0.52	36.55 \pm 0.99	3.22 \pm 0.07	4.11 \pm 0.09	15 \pm 0.33	0.69 \pm 0.04	3.01 \pm 0.12
<i>C. hamulosus</i>	13.41 \pm 0.26	34.12 \pm 0.45	32.55 \pm 0.42	3.59 \pm 0.09	4.72 \pm 0.12	15 \pm 0.22	0.69 \pm 0.03	4.74 \pm 0.15
<i>C. kernerii</i> subsp. <i>austro-orientalis</i>	15.66 \pm 0.07	36.70 \pm 0.50	35.33 \pm 0.66	3.50 \pm 0.07	4.25 \pm 0.10	15 \pm 0.23	0.69 \pm 0.02	3.33 \pm 0.13
<i>C. candicans</i> ssp. <i>globifer</i>	16.22 \pm 0.22	39.24 \pm 0.68	38.62 \pm 0.78	3.87 \pm 0.09	4.80 \pm 0.14	13 \pm 0.20	0.59 \pm 0.02	3.73 \pm 0.18
<i>C. carduelis</i>	13.65 \pm 0.11	32.76 \pm 0.59	31.75 \pm 0.52	2.93 \pm 0.09	3.83 \pm 0.11	15 \pm 0.24	0.67 \pm 0.02	2.77 \pm 0.08
<i>C. rhodopaeus</i>	18.41 \pm 0.43	40.39 \pm 0.63	37.99 \pm 0.84	4.10 \pm 0.13	5.28 \pm 0.19	12 \pm 0.20	0.85 \pm 0.02	3.72 \pm 0.14
<i>C. crispus</i>	13.54 \pm 0.30	35.87 \pm 0.33	34.23 \pm 0.43	3.29 \pm 0.07	4.20 \pm 0.09	14 \pm 0.22	0.86 \pm 0.21	3.35 \pm 0.09
<i>C. acanthoides</i>	13.75 \pm 0.22	34.71 \pm 0.35	32.62 \pm 0.37	3.50 \pm 0.08	4.28 \pm 0.09	15 \pm 0.18	0.71 \pm 0.02	3.25 \pm 0.10
<i>C. armatus</i>	15.49 \pm 0.14	39.37 \pm 0.63	40.86 \pm 0.79	3.19 \pm 0.08	4.09 \pm 0.09	14 \pm 0.19	0.64 \pm 0.02	3.02 \pm 0.10
<i>C. thracicus</i>	13.32 \pm 0.27	34.22 \pm 0.39	32.24 \pm 0.41	3.24 \pm 0.07	4.08 \pm 0.12	14 \pm 0.14	0.68 \pm 0.02	2.80 \pm 0.14

Abbreviations. d-Diameter; ED-equatorial diameter; PO-polar axis; H-height; W-width; N-number; Ne-nexine; Se-sexine; M-mean; SE-standard error.

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