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Pollen morphology of Polish species from the genus *Rubus* L. (Rosaceae) and its systematic importance

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Abstract

The genus *Rubus* L. (Rosaceae) not been investigated satisfactorily in terms of palynology. This genus is taxonomically very difficult due to the large number of species and problems with their delimitation, as well as very different distribution areas of particular species. The aim of this study was to investigate pollen morphology and for the first time the ranges of intrageneric and interspecific variability of *Rubus* species, as well as verify the taxonomic usefulness of these traits in distinguishing studied taxa from this genus. The selected species of the genus Rubus were analysed for 11 quantitative pollen characteristics and the following qualitative ones: exine ornamentation, pollen outline and shape, as well as bridge structure. Analyses were conducted on a total of 1740 pollen grains, which represent 58 blackberry species belonging to a majority of subgenera and all the sections and series found in Poland. The most important characters included exine ornamentation (exine ornamentation type, width and direction of grooves and striae, number and diameter of perforations) and length of the polar axis (P). The arrangement of the examined species on the dendrogram does not corroborate division of the genus *Rubus* into subgenera, sections and series currently adopted in taxonomy. This fact is not surprising because the taxonomy of the genus was not based on pollen characters. Pollen features should be treated in taxonomy as auxiliary, because they fail to differentiate several (10) individual species, while the other ones create groups with similar pollen traits.

Introduction

Rubus L. is a large and diverse genus in the Rosaceae family with a worldwide distribution, including hundreds or even thousand of published species names and infrageneric taxa [1, 2]. Depending on which classification you follow, historic or modern, the number of *Rubus* species may vary from 429 to 750 or up to 1000 worldwide [3–9].

The genus *Rubus* L. belongs to the tribe *Rubeae* Dumort., subfamily *Rosoideae*, family Rosaceae Juss. [10, 11]. The studied genus belongs to the clades Superrosids, Rosids and the order

Rosales [12]. The genus *Rubus* was traditionally divided into 12 subgenera [13, 14]. The current classification recognises 13 subgenera, with the largest subgenus *Rubus* in turn divided into 12 sections [10]. However, this classification is clearly arbitrary, as many of the subgenera have been shown to be poly- or paraphyletic [15]. Most of the European blackberries belong to the typical subgenus—*Rubus*. Other subgenera were also distinguished from it: *Chamaerubus*, *Cylactis*, *Anoplobatus* and *Idaeobatus*, which were represented by individual species [9, 16].

According to Weber [9], about 250 to 300 species of blackberries are found in Central and North-Western Europe. In turn, Stace [17] described approx. 300 species from the British Isles alone. In Poland, the occurrence of 108 species from the genus *Rubus* has been confirmed so far [18]. Since the publication of the genus *Rubus* monograph written by the Polish batologist, prof. Jerzy Zieliński [16], five new blackberry species have been described in Poland and 10 new species for the Polish flora have been recorded [18]. Although blackberries have been a group of plants widespread throughout Europe, their phytogeographic, ecological and genetic diagnosis is still incomplete.

The genus *Rubus* is a highly complex one, particularly the subgenus *Rubus*, with polyploidy hybridisation and apparently frequent facultative apomixis, thus leading to great variation in the subgenus and making species classification one of the grand challenges of systematic botany [9, 16, 19]. Apomixis is characteristic almost exclusively to the subgenus *Rubus*, embracing most of the European blackberry species. Apomixis in blackberries gives rise to grains that are mature and of typical structure, as well as much smaller and not fully developed pollen. Facultative apomicts produce fewer undeveloped grains (several per cent) than obligate ones, in which they constitute from 10 to 25% [20].

Because pollen grains have a unique biological characteristics, contain a large amount of genetic information, and exhibit strong genetic conservation, they can be used for species identification [21–23]. Due to considerable difficulties in recognising particular blackberry species, pollen grains of most blackberry species have not been described in the palynological literature so far. To date only a few authors have studied pollen morphology of European taxa from this critical genus, and they are mostly older works, in which only several selected species (from 3 to 18) or the most important pollen grain features (pollen shape and exine ornamentation) were described. As a result, pollen grains of only 48 European blackberry species have been described [18, 24–33]. Among the 108 Polish blackberries species, pollen of just 15 species has been characterised so far, of which six are endemic species [31, 33, 34].

The most important characteristics of blackberry pollen grains include exine ornamentation (ornamentation type, width and orientation of striae and grooves), lenght of colpori, type of the bridge (clamped vs. stretched), costae colpi and the number and size of perforations [24, 25, 27, 28, 30, 31, 33–48]. According to Tomlik-Wyremblewska [31, 46], pollen size and shape prove to be poor criteria in species identification.

Despite relatively numerous publications, our knowledge concerning blackberry pollen morphology is far from complete, because the available descriptions are usually brief and sometimes limited to mean dimensions. Moreover, researchers typically analyse individual, most important pollen grain characters (such as pollen size and exine ornamentation); alternatively, only some selected species were characterized. Therefore, the aim of the presented study was to perform a comprehensive analysis of relationships among the species within the taxonomically challenging genus *Rubus* L., based on pollen features of 58 species, representing four subgenera, all three sections and 23 series found in Poland. Many of the studied blackberry species are distributed throughout Europe. Another aim of this study was to discuss the taxonomic significance of pollen morphology with reference to the current classification of this genus according to Zieliński [16]. In addition, the intrageneric and interspecific variability of pollen grains in the *Rubus* species under investigation has not yet been comprehensively analysed.

Materials and methods

Pollen morphology

The collected plant material was stored in the herbarium of the Faculty of Forest Botany of the Poznań University of Life Sciences (PZNF), which did not require any permits to conduct research.

The study was conducted on 58 Polish and European *Rubus* species, which represent four out of five subgenera, all three sections and all 23 series of blackberries found in Poland, including all six Polish endemic species (*R. capitulatus*, *R. chaerophylloides*, *R. ostroviensis*, *R. posnaniensis*, *R. seebergensis* and *R. spribillei*). A list of the species analysed with their affiliation to particular taxa is shown in Table 1.

In this paper, the taxonomic classification of the studied taxa from the genus *Rubus* was adopted from Zieliński [16], with further modifications [18]. The verification of the taxa was made by Prof. Jerzy Zieliński (Institute of Dendrology, Polish Academy of Sciences in Kórnik), a batologist—taxonomist specialising in the genus *Rubus*.

Several, randomly selected inflorescences (flowers) were collected from 58 natural blackberry localities in Poland (Table 2).

Pollen grains were acetolysed according to the method of Erdtman [49]. The inflorescences collected from the herbarium were placed in tubes and then centrifuged with glacial acetic acid. Grains were mixed with the acetolysis solution, which consisted of nine parts acetic anhydrite and one part concentrated sulphuric acid. The mixture was then heated to boiling and kept in the water bath for 2–3 min. Samples were centrifuged in the acetolysis mixture, washed with acetic acid and centrifuged again. The pollen grain samples were then mixed with 96% alcohol and centrifuged 4 times, with processed grains subsequently divided into two groups. One half of the processed sample was immersed in an alcohol-based solution of glycerin for LM, while the other was placed in 96% ethyl alcohol in preparation for scanning electron microscopy (SEM). The SEM observations were made using a Zeiss Evo 40 and the LM measurements of acetolysed pollen grain were taken using a Biolar 2308 microscope at a magnification of 640x. Pollen grains were immersed in glycerin jelly and measured using an ocular eyepiece with a scale. Measurements taken from 30 mature, randomly selected, properly developed pollen grains were made by using the light microscopy (LM), with 1740 pollen grains measured in total. Measurement results were then converted into micrometres by multiplying each measurement by two.

The pollen grains were analysed for 11 quantitative characters: length of the polar axis (P) and equatorial diameter (E), length of the ectoaperture (Le), thickness of the exine along the polar axis and equatorial diameter (Exp, Exe), distance between apices of two ectocolpi (d) and P/E, Le/P, Exp/P, Exe/E, d/E (apocolpium index P.A.I) ratios. The pollen shape classes (P/E ratio) were adopted according to the classification proposed by Erdtman [50]: oblate-spheroidal (0.89–0.99), spheroidal (1.00), prolate-spheroidal (1.01–1.14), subprolate (1.15–1.33), prolate (1.34–2.00) and perprolate (>2.01). In addition, the following qualitative characters were also determined: outline, shape, operculum structure and exine ornamentation.

Exine ornamentation types (I-VI) were identified based on the classification proposed by Ueda [47]. The types and subtypes of the striate exine ornamentation were characterised by the height and width of grooves, width of striae and the number and diameter of perforations.

Descriptive palynological terminology followed Punt et al. [51] and Halbritter et al. [52].

Statistical analysis

The normality of the distributions for the studied traits (P, E, Le, d, Exp, Exe, P/E, Le/P, d/E, Exp/P and Exe/E) was tested using Shapiro-Wilk's normality test [53]. Multivariate analysis of

Table 1. The taxonomic classification of the *Rubus* species studied.

No	Species	Subgenus	Section	Subsection	Series
1	*R. saxatilis*	*Cylactis*	-	-	*Saxatiles*
2	*R. xanthocarpus*				*Xanthocarpi*
3	*R. odoratus*	*Anoplobatus*	-	-	-
4	*R. idaeus*	*Idaeobatus*	-	-	-
5	*R. nessensis*	*Rubus*	*Rubus*	*Rubus*	*Nessenses*
6	*R. scisus*				
7	*R. constrictus*				*Rubus*
8	*R. plicatus*				
9	*R. opacus*				
10	*R. divaricatus*				
11	*R. canadensis*				*Canadenses*
12	*R. allegheniensis*				*Alleghenieses*
13	*R. bifrons*			*Hiemales*	*Discolores*
14	*R. montanus*				
15	*R. grabowskii*				
16	*R. henrici-egonis*				
17	*R. parthenocissus*				
18	*R. perrobustus*				*Rhamnifolii*
19	*R. marssonianus*				
20	*R. gracilis*				
21	*R. wimmerianus*				*Sylvatici*
22	*R. angustipaniculatus*				
23	*R. circipanicus*				
24	*R. macrophyllus*				
25	*R. sprengelii*				*Sprengeliani*
26	*R. chlorothyrsos*				
27	*R. pyramidalis*				*Vestiti*
28	*R. micans*				*Micantes*
29	*R. glivicensis*				
30	*R. chaerophylloides*				
31	*R. acanthodes*				
32	*R. clusii*				
33	*R. radula*				*Radulae*
34	*R. posnaniensis*				*Pallidi*
35	*R. pfuhlianus*				
36	*R. koehleri*				*Hystrix*
37	*R. bavaricus*				
38	*R. schleicheri*				
39	*R. apricus*				
40	*R. ostroviensis*				*Glandulosi*
41	*R. siemianicensis*				
42	*R. pedemontanus*				
43	*R. hercynicus*				
44	*R. orthostachys*		*Corylifolii*	*Sepincoli*	*Subrectigeni*
45	*R. lamprocaulos*				
46	*R. czarnunensis*				*Sepincoli*
47	*R. hevellicus*				*Subthyrsoidei*
48	*R. gothicus*				
49	*R. camptostachys*				*Subsylvatici*
50	*R. mollis*				*Subcanescentes*
51	*R. fasciculatus*				
52	*R. fabrimontanus*				*Subradulae*
53	*R. capitulatus*				*Hystricopes*
54	*R. dollnensis*				
55	*R. seebergensis*				
56	*R. spribillei*				
57	*R. corylifolius*				-
58	*R. caesius*		*Caesii*		-

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Table 2. List of localities of the *Rubus* species studied.

No	Species	Localities	Geographical coordinates	Collector, herbarium
1	*R. acanthodes*	Poland, Dolnośląskie, Nowe Łąki near Pielgrzymka	51°07′06,1"N, 15° 46′37,5"E	Boratyńska, Dolatowska, Tomlik, Zieliński; KOR
2	*R. allegheniensis*	Poland, Zachodniopomorskie, Łukęcin near Świnoujście	54°02′34,9"N, 14° 52′23,8"E	Boratyńska, Dolatowska, Zieliński; KOR
3	*R. angustipaniculatus*	Poland, Mazowieckie, Zakrzew near Radom	50°26′27,3"N, 21° 00′02,4"E	Maliński, Zieliński; POZNF
4	*R. apricus*	Poland, Wielkopolskie, Bachorzew near Jarocin	51°59′39,9"N, 17° 33′49,9"E	Maliński, Zieliński; POZNF
5	*R. bavaricus*	Poland, Wielkopolskie, Robczysko near Leszno	51°48′41,4"N, 16° 45′38,6"E	Danielewicz, Maliński; POZNF
6	*R. bifrons*	Poland, Podkarpackie, Łukowe near Sanok	49°25′20,1"N, 22° 14′14,1"E	Oklejewicz; KOR
7	*R. caesius*	Poland, Lubuskie, Osiecznica near Krosno Odrzańskie	52°04′45,0"N, 15° 03′11,0"E	Maliński, Zieliński; POZNF
8	*R. camptostachys*	Poland, Wielkopolskie, Raków near Kępno	51°11′16,8"N, 18° 05′54,1"E	Zieliński; KOR
9	*R. canadensis*	Poland, Dolnośląskie, Bialskie Mts. near Stronie Śląskie	50°14′59,9"N, 16° 57′45,7"E	Kosiński; KOR
10	*R. capitulatus*	Poland, Wielkopolskie, Psienie-Ostrów near Pleszew	51°57′48,2"N, 17° 45′51,5"E	Danielewicz, Maliński; POZNF
11	*R. chaerophylloides*	Poland, Wielkopolskie, Laskowo near Chodzież	53°01′19,2"N, 17° 05′45,4"E	Maliński, Zieliński; POZNF
12	*R. chlorothyrsos*	Poland, Pomorskie, Bargędzino near Łeba	54°43′53,4"N, 17° 43′19,3"E	Boratyńska, Dolatowska, Zieliński; KOR
13	*R. circipanicus*	Poland, Zachodniopomorskie, Jarosławiec near Ustka	54°32′21,3"N, 16° 32′31,6"E	Zieliński; KOR
14	*R. clusii*	Poland, Małopolskie, Dobronków near Tarnów	49°59′28,2"N, 21° 20′37,5"E	Maliński, Zieliński; POZNF
15	*R. constrictus*	Poland, Małopolskie, Lipinki near Gorlice	49°40′20,4"N, 21° 17′31,6"E	Oklejewicz; KOR
16	*R. corylifolius*	Poland, Lubuskie, Różanówka near Bytom Odrzański	51°46′05,4"N, 15° 52′29,5"E	Maliński, Zieliński; POZNF
17	*R. czarnunensis*	Poland, Pomorskie, Drzewicz, Bory Tucholskie National Park	53°51′07,3"N, 17° 34′08,4"E	Tomlik, KOR
18	*R. divaricatus*	Poland, Lubuskie, Bielawy near Bytom Odrzański	51°46′21,3"N, 15° 55′09,6"E	Maliński, Zieliński; POZNF
19	*R. dollnensis*	Poland, Dolnośląskie, Młynowiec near Stronie Śląskie	50°16′36,1"N, 16° 54′04,8"E	Kosiński, Tomaszewski, Zieliński; KOR
20	*R. fabrimontanus*	Poland, Lubuskie, Tarnów Jezierny Nowa Sól	51°51′45,1"N, 15° 59′07,7"E	Maliński, Zieliński; POZNF
21	*R. fasciculatus*	Poland, Podkarpackie, Gruszowa near Przemyśl	49°40′57,4"N, 22° 40′47,2"E	Maliński, Zieliński; POZNF
22	*R. glivicensis*	Poland, Małopolskie, Maga near Tarnów	50°00′09,8"N, 21° 20′24,7"E	Maliński, Zieliński; POZNF
23	*R. gothicus*	Poland, Wielkopolskie, Pakówka near Bojanowo	51°40′20,7"N, 16° 46′07,9"E	Maliński, Zieliński; POZNF
24	*R. grabowskii*	Poland, Lubuskie, Tarnów Jezierny Nowa Sól	51°51′45,1"N, 15° 59′07,7"E	Maliński, Zieliński; POZNF
25	*R. gracilis*	Poland, Podkarpackie, Pod Lasem, near Rzeszów	49°53′42,5"N, 21° 35′52,1"E	Maliński, Zieliński; POZNF
26	*R. henrici-egonis*	Poland, Opolskie, Barnice near Głubczyce	50°03′02,5"N, 17° 47′38,5"E	Kosiński, Tomaszewski, Zieliński; KOR
27	*R. hercynicus*	Poland, Dolnośląskie, Stare Bogaczowice near Wałbrzych	50°50′53,7"N, 16° 11′37,4"E	Boratyńśki, Zieliński; KOR

(*Continued*)

Table 2. (Continued)

No	Species	Localities	Geographical coordinates	Collector, herbarium
28	*R. hevellicus*	Poland, Wielkopolskie, Tarce near Jarocin	52°00′02,4"N, 17° 35′26,1"E	Maliński, Zieliński; POZNF
29	*R. idaeus*	Poland, Kujawsko-Pomorskie, Brodnica near Bydgoszcz	53°15′29,2"N, 19° 23′57,9"E	Tomlik; KOR
30	*R. koehleri*	Poland, Dolnośląskie, Mirsk near Świeradów-Zdrój	50°58′19,9"N, 15° 23′08,9"E	Boratyński; KOR
31	*R. lamprocaulos*	Poland, Dolnośląskie, Serby near Głogów	51°41′04,1"N, 16° 06′42,9"E	Maliński, Zieliński; POZNF
32	*R. macrophyllus*	Poland, Dolnoslążkie, Przywsie near Rawicz	51°34′37,1"N, 16° 52′36,1"E	Maliński, Zieliński; POZNF
33	*R. marssonianus*	Poland, Pomorskie, near Kartuzy	54°20′03,2"N, 18° 11′50,5"E	Boratyński; KOR
34	*R. micans*	Poland, Opolskie, Wieszczyna near Prudnik	50°19′18,2"N, 17° 34′48,4"E	Kosiński, Tomaszewski, Zieliński; KOR
35	*R. mollis*	Poland, Dolnoslążkie, Lądek-Zdrój, Trzykrzyska Mt.	50°20′54,6"N, 16° 52′39,9"E	Kosiński, Tomaszewski, Zieliński; KOR
36	*R. montanus*	Poland, Dolnośląskie, Kowary near Kostrzyca	50°47′37,5"N, 15° 50′01,8"E	Zieliński; KOR
37	*R. nessensis*	Poland, Dolnośląskie, Karczmisko near Kłodzko	50°17′56,7"N, 16° 49′32,8"E	Kosiński; KOR
38	*R. odoratus*	Poland, Lubelskie, Niedrzwica Duża near Lublin	51°06′51,3"N, 22° 23′16,2"E	illegible name; KOR
39	*R. opacus*	Poland, Wielkopolskie, Starkowo near Leszno	51°58′37,7"N, 16° 18′35,7"E	Zieliński; KOR
40	*R. orthostachys*	Poland, Wielkopolskie, Ostatni Grosz near Krotoszyn	50°39′54,4"N, 17° 21′18,9"E	Maliński, Zieliński; POZNF
41	*R. ostroviensis*	Poland, Wielkopolskie, Wielkopolski National Park near Poznań	52°16′26,5"N, 16° 46′50,1"E	Zieliński, Maliński; POZNF
42	*R. parthenocissus*	Poland, Podkarpackie, Koniusza near Przemyśl	49°40′57,4"N, 22° 40′47,2"E	Maliński, Zieliński; POZNF
43	*R. pedemontanus*	Poland, Dolnośląskie, Nowy Kościół near Złotoryja	51°04′20,1"N, 15° 52′05,3"E	Boratyńśki, Zieliński; KOR
44	*R. perrobustus*	Poland, Podkarpackie, Dudyńce near Sanok	49°39′04,9"N, 22° 04′31,9"E	Oklejewicz; KOR
45	*R. pfuhlianus*	Poland, Wielkopolskie, Mieczewo near Kórnik	52°14′20,8"N, 17° 00′27,8"E	Zieliński; KOR
46	*R. plicatus*	Poland, Lubuskie, Różanówka near Bytom Odrzański	51°46′05,4"N, 15° 52′29,5"E	Maliński, Zieliński; POZNF
47	*R. posnaniensis*	Poland, Opolskie, Szybowice near Prudnik	50°21′09,5"N, 17° 29′11,9"E	Kosiński, Tomaszewski, Zieliński; KOR
48	*R. pyramidalis*	Poalnd, Wielkopolskie, Chruszczyny near Ostrów Wielkopolski	51°38′41,4"N, 17° 35′42,6"E	Maliński, Zieliński; POZNF
49	*R. radula*	Poland, Podkarpackie, Hermanowa near Rzeszów	49°56′07,4"N, 22° 00′40,4"E	Maliński, Zieliński; POZNF
50	*R. saxatilis*	Sweden, Abisko Östra	68°20′56,3"N, 18° 49′43,7"E	illegible name; KOR
51	*R. schleicheri*	Poland, Wielkopolskie, Kościan	52°05′10,7"N, 16° 38′41,9"E	Maliński, Zieliński; POZNF
52	*R. scisus*	Poland, Śląskie, Rudniki near Częstochowa	50°52′33,6"N, 19° 14′28,5"E	Zieliński; KOR
53	*R. seebergensis*	Poland, Wielkopolskie, Wielkopolski National Park near Poznań	52°16′26,5"N, 16° 46′50,1"E	Danielewicz; POZNF

(*Continued*)

Table 2. (Continued)

No	Species	Localities	Geographical coordinates	Collector, herbarium
54	*R. siemianicensis*	Poland, Wielkopolskie, Psienie-Ostrów near Pleszew	51°57′48,2"N, 17° 45′51,5"E	Danielewicz, Maliński; POZNF
55	*R. sprengelii*	Poland, Wielkopolskie, Borownica near Zduny	51°38′20,8"N, 17° 24′23,3"E	Maliński, Zieliński; POZNF
56	*R. spribillei*	Poland, Wielkopolskie, Gądki near Kórnik	52°18′45,4"N, 17° 02′47,8"E	Zieliński; POZNF
57	*R. wimmerianus*	Poland, Podkarpackie, Gniewczyna Łańcucka near Przeworsk	50°06′19,5"N, 22° 29′43,7"E	Oklejewicz, Zatorski; POZNF
58	*R. xanthocarpus*	Poland, Świętokrzyskie, Miedzianka near Kielce	50°50′22,5"N, 20° 22′03,3"E	Maciejczak, Bróż, Zieliński; KOR

KOR—Herbarium of the Institute of Dendrology, Polish Academy of Sciences, Kórnik, Poland, PZNF—Herbarium of the Department of Forest Botany, Poznań University of Life Sciences.

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variance (MANOVA) was performed on the basis of the following model using the MANOVA procedure in GenStat (18th edition): $\mathbf{Y} = \mathbf{XT}+\mathbf{E}$, where: $\mathbf{Y}$ is the $(n \times p)$-dimensional matrix of observations, n is the number of all observations, p is the number of traits (in this study $p = 11$), $\mathbf{X}$ is the $(n \times k)$-dimensional matrix of design, k is the number of species (in this study $k = 58$), $\mathbf{T}$ is the $(k \times p)$-dimensional matrix of unknown effects and $\mathbf{E}$—is the $(n \times p)$-dimensional matrix of residuals. Next, one-way analyses of variance (ANOVA) were carried out to determine the effects of species on the variability of examined traits, for each trait independently, on the basis of the following model: $y_{ij} = \mu+\tau_i+\varepsilon_{ij}$, where: y_{ij} is the jth observation of the ith species, μ is the grand mean, τ_i is the effect of the ith species and ε_{ij} is an error observation. The arithmetical means and standard deviations of traits were calculated. Moreover, Fisher's least significant differences (LSDs) were also estimated at the significance level $\alpha = 0.001$. The relationships between observed traits were assessed on the basis of Pearson's correlation. Results were also analysed using multivariate methods. The canonical variate analysis was applied in order to present multitrait assessment of similarity for the tested species in a lower number of dimensions with the least possible loss of information [54]. This makes it possible to illustrate variation in species in terms of all the observed traits in the graphic form. The Mahalanobis distance was suggested as a measure of "polytrait" species similarity [55], which significance was verified by means of critical value D_α called "the least significant distance" [56]. Mahalanobis distances were calculated for species. The differences between the analysed species were verified by cluster analysis using the nearest neighbour method and Euclidean distances [57]. All the analyses were conducted using the GenStat (18th edition) statistical software package [58].

Results

General morphological description of pollen

A description of pollen grain morphology of the *Rubus* species studied is given below and illustrated with several SEM photographs (Figs 1–3). The morphological observations for the other quantitative characters of pollen grains are summarised in Table 3.

Pollen grains of the *Rubus* species studied were tricolporate, isopolar monads (Fig 1A–1H). According to the pollen size classification by Erdtman [50], analysed pollen grains were medium (25.1–50 μm; 56.7%) or small (10–25 μm; 43.3%). The analysed pollen had a small range of average values for trait P, ranging from 20.57 to 30.20 μm. Therefore, most of the

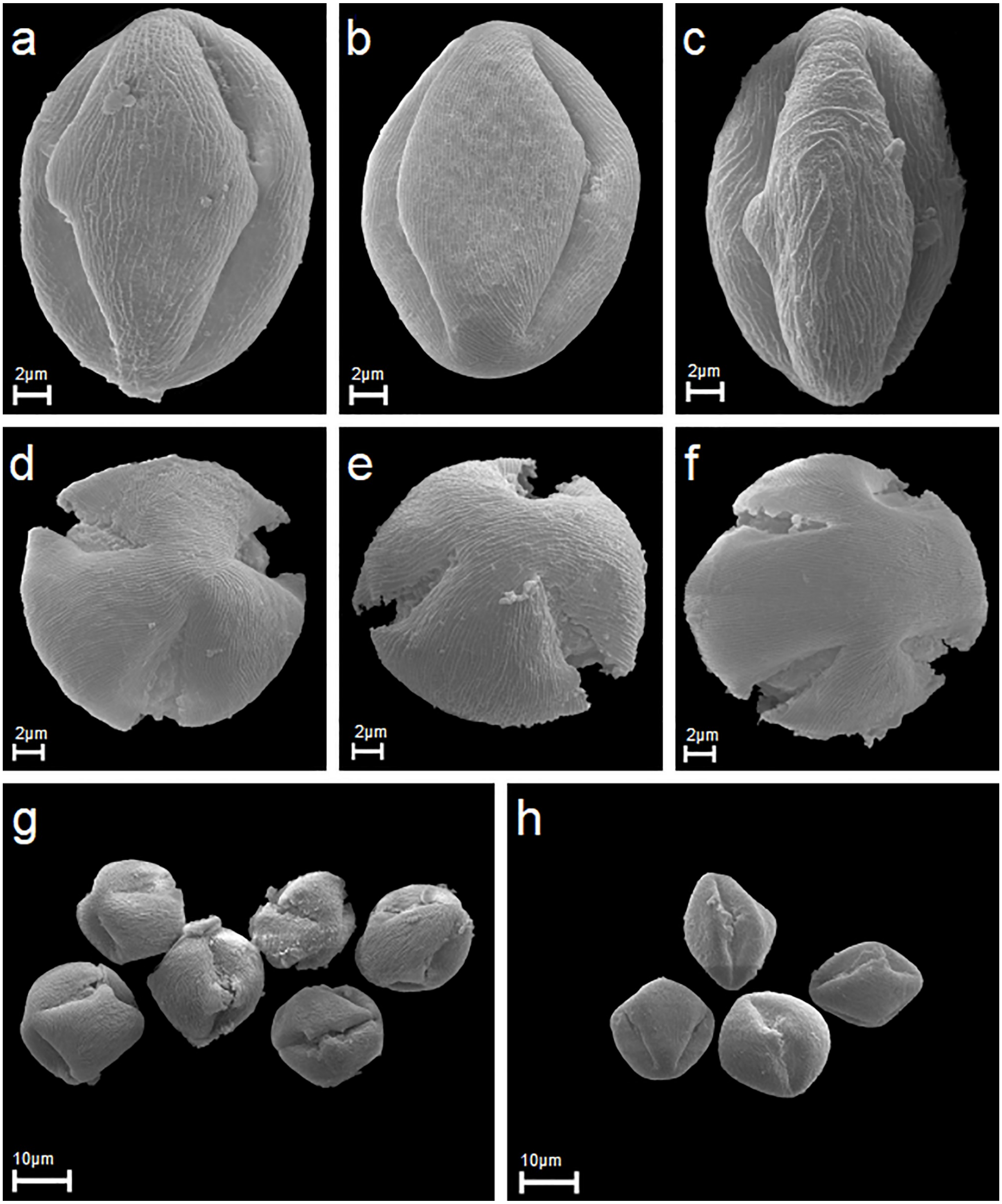

Fig 1. Equatorial and polar views, apertures and exine ornamentation in scanning electron microscope (SEM). (A-C) *R. chlorothyrsos*, *R. pedemontanus*, *R. mollispollen* grains in equatorial views, two colpori and exine ornamentation. (D-F) *R. fabrimontanus*, *R. pfuhlianus*, *R. lamprocaulos* pollen in polar views, three colpori and exine ornamentation. (G-H) *R. angustipaniculatus*, *R. hevellicus* six and four pollen grains in equatorial and polar views.

https://doi.org/10.1371/journal.pone.0221607.g001

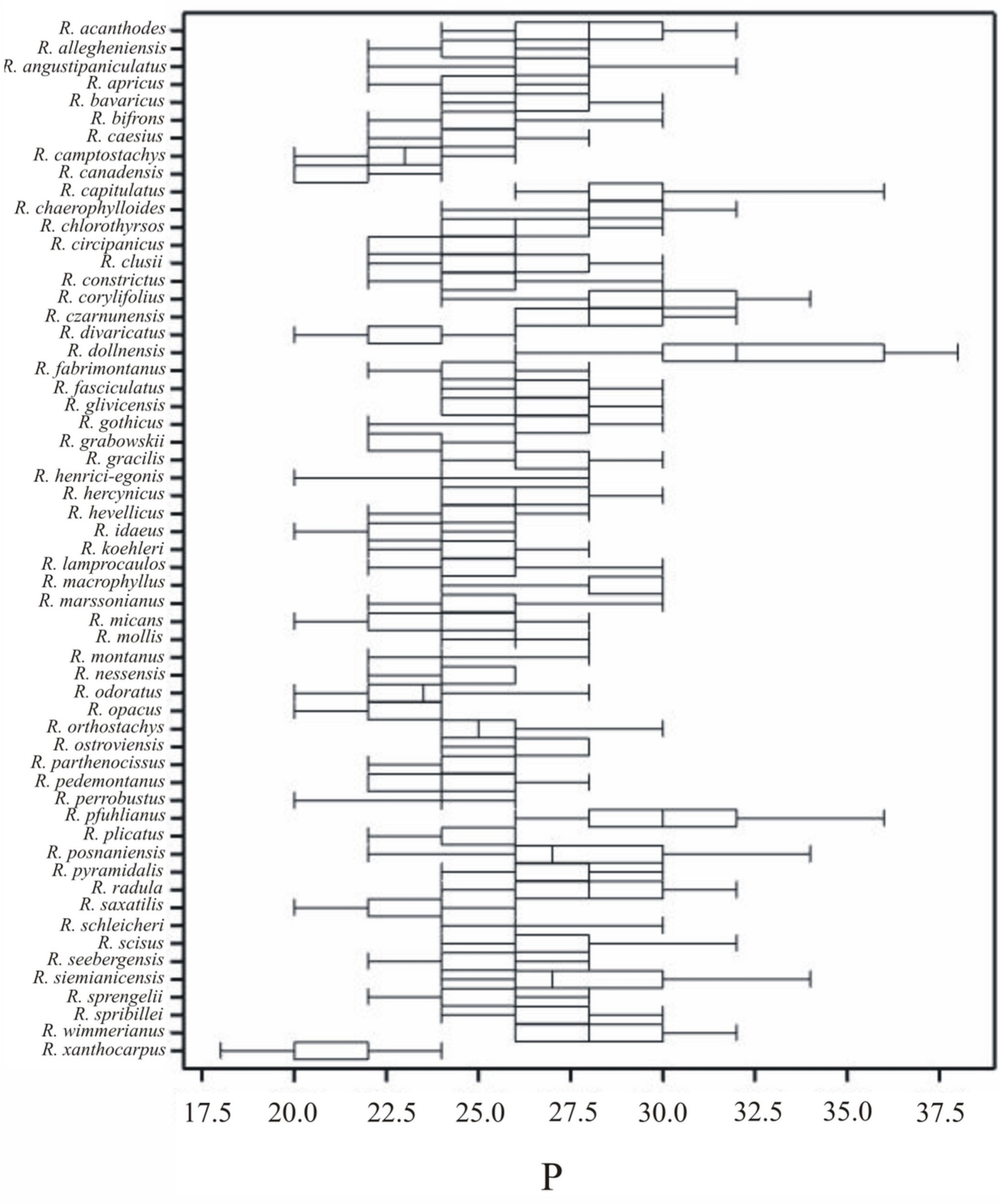

Fig 2. Box-and-whisker diagram of P values for 58 studied *Rubus* species. The mean length of the equatorial diameter (E) was 21.66 (14–32) μm. The shortest mean equatorial diameter was recorded in pollen of *R. canadensis* (18.47 μm), while the longest was found in *R. czarnunensis* (26.87 μm; Table 3).

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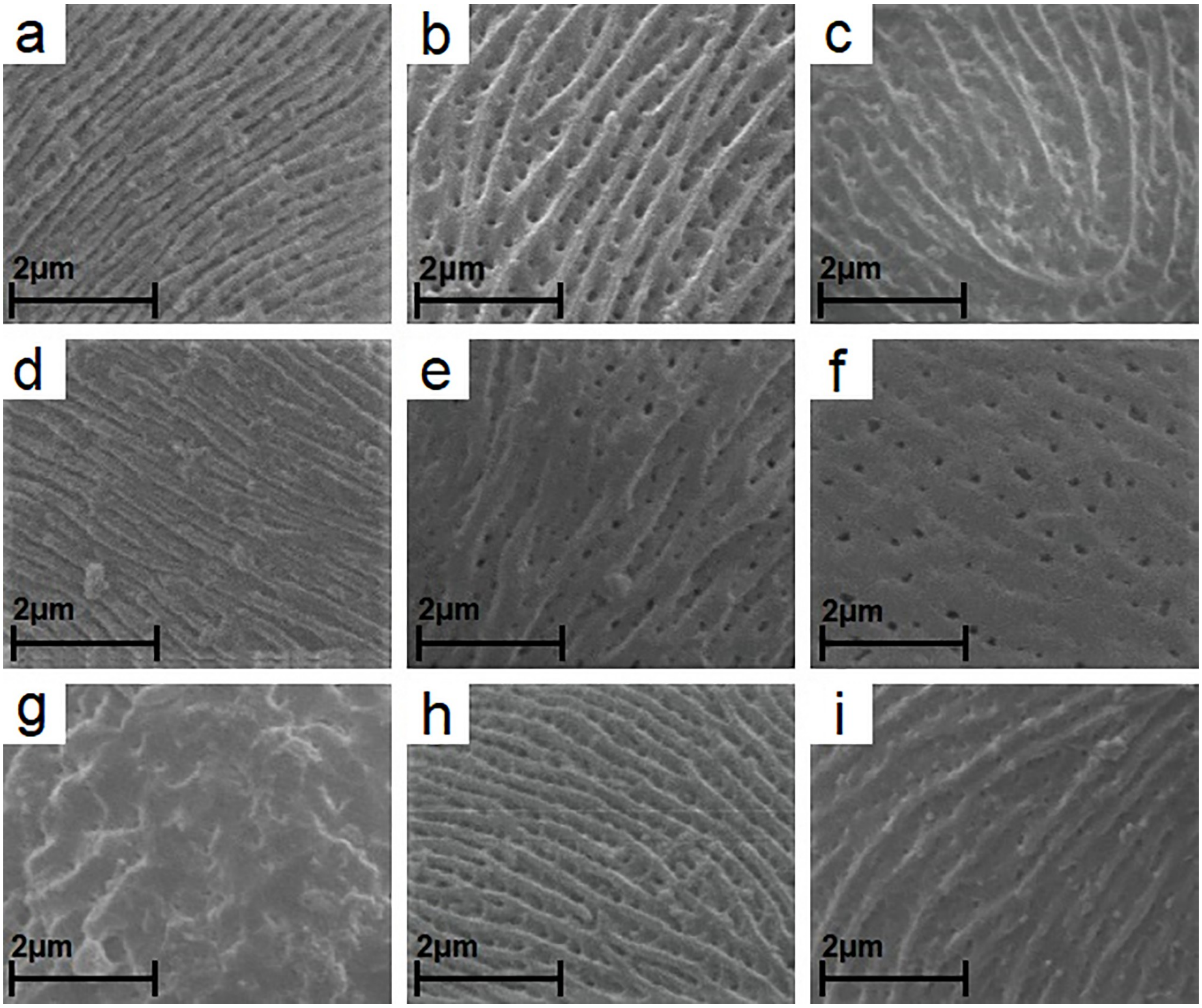

Fig 3. The participation of studied species in types and subtypes of striate exine ornamentation (according to Ueda [47]). (A) *R. lamprocaulos* (subtype—IA). (B) *R. angustipaniculatus* (IIA). (C) *R. orthostachys* (IIB). (D) *R. canadensis* (IIIA). (E) *R. montanus* (IIIB). (F) *R. saxatilis* (V). (G) *R. odoratus* (striate-verrucate ornamentation). (H) *R. plicatus* (IA/IIA), (I) *R. apricus* (IIA/IIB).

https://doi.org/10.1371/journal.pone.0221607.g003

pollen grains belong to the upper limit of small pollen or to the lower medium-sized pollen range.

The average length of the polar axis (P) was 25.72 (18–38) μm (Fig 2, Table 3). The smallest mean P was found for pollen of *R. xanthocarpus* (20.57 μm), while the largest—for *R. dollnensis* (32.27 μm) (Fig 2, Table 3). In the *R. xanthocarpus* sample all measured pollen grains were small at a narrow range of polar axis length (18–24 μm). On the other hand, the longest pollen grains were found in *R. dollnensis* (26–38 μm).

The outline in the polar view was mostly circular with obtuse apices, more rarely elliptic, whereas in the equatorial view the outline was mostly elliptic, rarely circular (Fig 1).

The mean P/E ratio was 1.19, ranging from 0.85 in *R. pedemontanus* to 1.71 in *R. saxatilis* (Table 3). On average the P/E ratio values were always above 1 and they ranged from 1.05 in *R. pedemontanus* to 1.32 in *R. chaerophylloides*. Pollen grains of the species examined were most frequently subprolate (57.3% - 997 pollen grains) or prolate-spheroidal (24.3% - 422), rarely

Table 3. Mean values and standard deviations (s.d.) for individual species and observed traits.

Species	P		E		Le		d		Exp		Exe		P/E		Le/P		d/E		Exp/P		Exe/E	
	Mean	s.d.	Mean	s.d.	Mean	s.d.	Mean	s.d.	Mean	s.d.	Mean	s.d.	Mean	s.d.	Mean	s.d.	Mean	s.d.	Mean	s.d.	Mean	s.d.
R. acanthodes	27.47	2.097	23.27	2.196	22.8	2.325	4.267	1.363	1.4	0.332	1.45	0.442	1.185	0.084	0.829	0.041	0.183	0.057	0.051	0.013	0.063	0.020
R. allegheniensis	24.47	1.717	21.2	1.448	20.8	1.627	4.267	1.363	1.967	0.434	1.883	0.215	1.158	0.095	0.851	0.058	0.201	0.062	0.081	0.021	0.089	0.011
R. angustipaniculatus	26.8	2.203	22.53	1.961	22	1.965	4.867	1.252	1.85	0.233	1.933	0.173	1.195	0.106	0.821	0.038	0.216	0.053	0.069	0.009	0.086	0.010
R. apricus	25.2	1.627	20.6	2.581	20.2	1.215	4.533	1.737	1.85	0.268	1.883	0.215	1.237	0.132	0.803	0.045	0.216	0.067	0.074	0.012	0.093	0.018
R. bavaricus	26.53	1.889	20.73	1.530	22.47	1.871	4.067	1.437	1.967	0.127	1.967	0.127	1.283	0.089	0.846	0.015	0.195	0.062	0.074	0.007	0.095	0.010
R. bifrons	25.6	1.694	20.93	1.946	21.47	1.570	3.667	1.061	1.817	0.308	1.767	0.365	1.23	0.106	0.839	0.040	0.174	0.047	0.071	0.012	0.085	0.020
R. caesius	25.6	1.694	23.27	1.112	21.2	1.710	4.8	0.997	1.85	0.233	1.85	0.233	1.102	0.074	0.828	0.031	0.207	0.045	0.073	0.010	0.080	0.011
R. camptostachys	22.67	1.845	19.4	1.070	18.07	1.780	4.533	1.279	2	0.000	1.783	0.364	1.172	0.117	0.797	0.040	0.232	0.061	0.089	0.007	0.092	0.019
R. canadensis	21.27	1.230	18.47	1.456	18.13	1.570	2.6	0.855	1.083	0.437	1.1	0.462	1.157	0.096	0.853	0.054	0.140	0.042	0.051	0.021	0.059	0.025
R. capitulatus	29.67	2.468	26.13	2.623	24.27	2.559	5.667	1.749	1.26	0.302	1.1	0.227	1.143	0.115	0.818	0.058	0.217	0.065	0.043	0.010	0.042	0.009
R. chaerophylloides	28.4	1.850	21.73	2.333	24	2.464	4.133	1.570	1.633	0.370	1.633	0.370	1.321	0.162	0.844	0.046	0.190	0.070	0.058	0.014	0.076	0.018
R. chlorothyrsos	26.2	1.769	22.33	1.749	21.13	1.717	4.733	1.437	1.883	0.252	1.9	0.242	1.177	0.081	0.807	0.040	0.210	0.055	0.072	0.010	0.085	0.012
R. circipanicus	23.93	1.530	19.8	1.424	19.67	1.583	3.867	1.042	1.733	0.286	1.833	0.240	1.213	0.097	0.823	0.054	0.195	0.052	0.073	0.013	0.093	0.014
R. clusii	26.47	2.389	20.27	1.799	20.67	1.845	5.333	1.322	1.833	0.330	1.817	0.334	1.319	0.194	0.789	0.111	0.264	0.062	0.070	0.015	0.090	0.018
R. constrictus	25.47	1.961	22.2	1.690	21.4	1.754	4.733	1.701	1.917	0.190	1.95	0.153	1.15	0.086	0.842	0.055	0.213	0.074	0.076	0.009	0.088	0.010
R. corylifolius	29.73	2.815	25.8	1.690	25.27	2.852	5.133	1.008	1.7	0.282	1.733	0.286	1.154	0.096	0.849	0.031	0.199	0.038	0.058	0.011	0.067	0.012
R. czarnunensis	28.53	2.097	26.87	2.330	23.2	2.497	7.333	1.688	2	0.000	2	0.000	1.068	0.106	0.812	0.045	0.274	0.063	0.070	0.005	0.075	0.007
R. divaricatus	22.87	1.634	19.67	1.295	19.2	1.126	3.167	0.950	1.883	0.215	1.867	0.225	1.165	0.084	0.842	0.049	0.160	0.044	0.083	0.012	0.095	0.014
R. dollnensis	32.27	3.629	25.27	1.617	26.8	3.736	6.067	1.617	2	0.000	2	0.000	1.279	0.133	0.829	0.035	0.240	0.064	0.063	0.007	0.079	0.005
R. fabrimontanus	25.67	1.749	22.87	1.717	21.13	1.456	4.933	1.258	1.933	0.173	1.9	0.275	1.127	0.094	0.825	0.046	0.217	0.057	0.076	0.008	0.084	0.014
R. fasciculatus	27.2	1.937	23.27	1.929	23	1.875	3.667	1.398	1.733	0.314	1.683	0.359	1.174	0.104	0.845	0.021	0.157	0.056	0.064	0.013	0.073	0.017
R. glivicensis	26.07	2.067	21.53	1.634	21.47	1.655	4.933	1.230	1.717	0.284	1.733	0.286	1.214	0.100	0.826	0.062	0.228	0.050	0.066	0.012	0.081	0.015
R. gothicus	26.4	1.773	23.4	1.905	22.07	1.780	3.933	1.337	1.95	0.201	1.917	0.231	1.133	0.087	0.836	0.038	0.167	0.051	0.074	0.008	0.082	0.011
R. grabowskii	23.53	1.137	19.93	1.437	19.67	1.061	3.9	1.125	1.667	0.401	1.7	0.385	1.186	0.092	0.837	0.050	0.196	0.053	0.071	0.018	0.085	0.019
R. gracilis	26.87	1.925	21.97	2.236	22.4	1.923	5.6	1.276	1.85	0.375	1.767	0.410	1.231	0.102	0.834	0.042	0.254	0.050	0.069	0.015	0.080	0.018
R. henrici-egonis	24.13	1.814	19.4	1.404	19.87	1.479	3.7	1.022	1.8	0.282	1.8	0.282	1.247	0.089	0.825	0.050	0.190	0.050	0.075	0.011	0.093	0.017
R. hercynicus	26.2	1.919	20.27	1.639	22.07	1.929	4.067	1.112	1.933	0.173	1.933	0.173	1.297	0.103	0.842	0.021	0.200	0.052	0.074	0.009	0.096	0.012
R. hevellicus	24.47	1.634	21.13	1.358	20.53	1.570	3.467	1.042	1.817	0.308	1.817	0.308	1.16	0.082	0.839	0.017	0.164	0.048	0.075	0.014	0.086	0.014
R. idaeus	22.6	1.673	20.37	1.497	18.53	1.655	4.2	0.925	1.817	0.359	1.733	0.430	1.114	0.095	0.822	0.071	0.207	0.049	0.081	0.017	0.085	0.022
R. koehleri	25.47	1.570	22.13	1.570	21.53	1.456	3.733	1.015	1.933	0.217	1.933	0.217	1.155	0.089	0.845	0.015	0.169	0.046	0.076	0.009	0.088	0.011
R. lamprocaulos	24.67	1.768	21.47	1.655	20.67	1.768	3.6	1.329	1.833	0.330	1.817	0.334	1.152	0.084	0.837	0.011	0.167	0.058	0.075	0.014	0.085	0.016
R. macrophyllus	28.13	1.655	23.33	1.516	22.47	2.209	4.6	1.673	1.867	0.225	1.833	0.240	1.21	0.103	0.798	0.056	0.199	0.074	0.066	0.008	0.079	0.011
R. marssonianus	25.47	2.403	22.3	1.985	20.73	1.617	4.5	1.167	1.55	0.422	1.533	0.370	1.147	0.113	0.817	0.051	0.202	0.051	0.061	0.018	0.069	0.018
R. micans	24.33	2.294	20.2	1.215	20.4	1.773	4.267	1.363	1.85	0.268	1.9	0.242	1.206	0.109	0.840	0.039	0.212	0.069	0.077	0.014	0.094	0.014
R. mollis	26	1.287	21.47	1.655	21.87	1.279	4.133	1.167	1.899	0.205	1.9	0.203	1.217	0.099	0.841	0.019	0.193	0.054	0.073	0.009	0.089	0.011
R. montanus	24.27	1.363	20	1.287	19.93	0.980	4.067	0.868	1.933	0.173	1.867	0.225	1.217	0.083	0.823	0.054	0.204	0.044	0.080	0.009	0.094	0.013
R. nessensis	24.27	1.363	20.03	1.450	19.33	1.422	3.967	0.964	1.967	0.127	1.933	0.254	1.216	0.099	0.797	0.049	0.199	0.051	0.081	0.007	0.097	0.014
R. odoratus	23.4	2.387	19.37	1.450	18.53	2.285	5.633	1.033	1.65	0.494	1.617	0.583	1.211	0.113	0.791	0.041	0.291	0.053	0.071	0.021	0.084	0.030
R. opacus	22.4	1.221	19.27	1.780	18.2	1.518	3.233	0.898	1.75	0.254	1.783	0.252	1.172	0.124	0.812	0.049	0.168	0.045	0.078	0.012	0.093	0.017

(*Continued*)

Table 3. (Continued)

Species	P		E		Le		d		Exp		Exe		P/E		Le/P		d/E		Exp/P		Exe/E	
	Mean	s.d.	Mean	s.d.	Mean	s.d.	Mean	s.d.	Mean	s.d.	Mean	s.d.	Mean	s.d.	Mean	s.d.	Mean	s.d.	Mean	s.d.	Mean	s.d.
R. orthostachys	25.53	1.871	21.07	1.946	20.53	1.737	4.8	1.448	1.933	0.217	1.917	0.190	1.219	0.109	0.804	0.036	0.227	0.062	0.076	0.011	0.092	0.011
R. ostroviensis	26.33	1.493	22.67	1.688	22.13	1.655	4.4	0.968	1.667	0.303	1.75	0.254	1.167	0.091	0.841	0.048	0.194	0.040	0.063	0.011	0.078	0.013
R. parthenocissus	24.47	1.252	20.47	1.358	20.33	1.061	3.333	0.959	1.917	0.231	1.933	0.217	1.199	0.077	0.832	0.032	0.163	0.046	0.079	0.010	0.095	0.012
R. pedemontanus	24.27	1.946	23.2	1.710	19.93	2.132	5	1.259	1.983	0.091	1.95	0.201	1.051	0.103	0.822	0.072	0.216	0.053	0.082	0.007	0.085	0.011
R. perrobustus	23.97	1.299	20.53	1.889	19.73	1.461	3.633	0.615	1.783	0.387	1.867	0.346	1.173	0.088	0.824	0.048	0.178	0.032	0.075	0.017	0.091	0.018
R. pfuhlianus	30.2	2.592	22.33	1.583	25.73	2.504	4.733	1.337	1.783	0.252	1.767	0.254	1.357	0.135	0.852	0.031	0.211	0.053	0.060	0.012	0.080	0.014
R. plicatus	24.4	1.102	21.4	1.831	20	1.050	3.867	1.570	1.767	0.430	1.833	0.379	1.146	0.088	0.820	0.030	0.179	0.063	0.072	0.017	0.086	0.018
R. posnaniensis	27.4	2.737	21.33	1.093	22.87	2.389	6	1.819	1.767	0.286	1.783	0.252	1.285	0.113	0.836	0.051	0.280	0.079	0.065	0.013	0.084	0.013
R. pyramidalis	27.4	1.831	23.6	1.694	22.47	2.209	4.8	1.243	1.717	0.252	1.733	0.254	1.164	0.076	0.819	0.047	0.203	0.049	0.063	0.009	0.074	0.012
R. radula	27.4	2.298	23.6	2.127	23	2.449	5.133	1.634	1.783	0.284	1.783	0.252	1.165	0.091	0.839	0.045	0.218	0.072	0.065	0.011	0.076	0.013
R. saxatilis	22.27	1.461	18.67	1.605	18.2	1.606	4	1.462	1.817	0.278	1.817	0.334	1.201	0.131	0.817	0.051	0.212	0.069	0.082	0.013	0.098	0.022
R. schleicheri	26.2	1.424	21.87	1.961	21.27	1.617	5.133	1.456	1.7	0.249	1.717	0.252	1.205	0.096	0.812	0.042	0.235	0.062	0.065	0.009	0.079	0.014
R. scisus	27	2.393	22.93	1.799	21.8	2.369	5.667	1.398	1.867	0.320	1.883	0.252	1.18	0.099	0.808	0.058	0.248	0.061	0.069	0.012	0.083	0.013
R. seebergensis	25.27	1.856	22.87	2.330	21.07	1.639	5	1.554	1.75	0.341	1.75	0.341	1.112	0.101	0.834	0.019	0.216	0.057	0.070	0.015	0.078	0.018
R. siemianicensis	27.4	2.527	21.6	1.773	22.73	2.545	4.867	1.548	1.767	0.286	1.75	0.341	1.275	0.136	0.830	0.045	0.225	0.070	0.065	0.013	0.081	0.016
R. sprengelii	25.07	1.639	21.13	2.013	20.53	1.479	4.267	1.258	1.833	0.240	1.867	0.225	1.192	0.097	0.820	0.043	0.201	0.053	0.073	0.009	0.089	0.013
R. spribillei	27.67	1.668	22.07	1.999	22.8	1.789	3.467	1.074	1.44	0.338	1.2	0.288	1.261	0.103	0.825	0.054	0.156	0.045	0.052	0.013	0.055	0.015
R. wimmerianus	28.2	1.789	23.33	2.354	22.57	2.192	4.5	1.196	1.983	0.091	1.817	0.382	1.215	0.088	0.800	0.053	0.192	0.047	0.071	0.005	0.079	0.018
R. xanthocarpus	20.57	1.431	17.6	1.545	16.23	1.305	3.867	1.074	1.75	0.388	1.8	0.337	1.175	0.110	0.791	0.055	0.219	0.054	0.085	0.019	0.103	0.021
$LSD_{0.001}$	1.63		1.5		1.61		1.1		0.244		0.251		0.089		0.040		0.048		0.011		0.013	

P—the length of polar axis, E—the length of equatorial axis, Le—the length of ectocolpi, d—the distance between the apices of two ectocolpi, Exp—the thickness of exine along polar axis, Exe—the thickness of exine along equatorial axis

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prolate (8.9% - 155) or spheroidal (8.6% - 150) and very rarely oblate-spheroidal (0.7% - 12) and perprolate (0.2% - 4). The highest number of subprolate pollen grains was recorded in *R. henriciegonis* and *R. montanus* (each at 80%, - 24 grains), of prolate-spheroidal pollen–in *R. idaeus* (53.3% - 16 grains) and of prolate grains—in *R. chaerophylloides* (50% - 15).

The exine was two-layered, with the ectexine and endexine of about the same thickness. Mean exine thickness was 1.79 (0.5–4.0) μm; on average Exp—1.79 μm and Exe—1.78 μm. The exine was the thinnest in *R. canadensis* (Exp—0.8 μm; Exe—1.1 μm), while it was the thickest in *R. czarnuensis* and *R. dollensis* (Exp and Exe—2.0 μm; Table 3). The relative thickness of the exine (Exp/P ratio) averaged 0.07 (0.02–0.18) and (Exe/E ratio) 0.08 (0.02–0.14). The above results were similar, indicating a more or less equal exine thickness along the entire pollen grain (Table 3).

In all the studied species, exine ornamentation was striate-perforate and very rarely striate, with the exception of *R. odoratus*, which had a striate-verrucate ornamentation with small perforations (Fig 3). Exine ornamentation elements were highly variable (Fig 3). Striae and grooves usually ran parallel to colpori and the polar axis, but frequently they also formed fingerprint-like twists. Striae were straight or forked and of varying length, width and height.

The investigated pollen of the individual *Rubus* species was classified according to the striate exine ornamentation classification proposed by Ueda [47] into four types (I-III and V) and five subtypes (I A, II A,B and III A,B). The cited author distinguished six types (I-VI) and six subtypes (I-III, each A and B). In our study types IV, VI and subtype IB were not found (Fig 3, Table 4). The greatest number of species (18) belonged to the IIA subtype, which was characterised by fairly distinct striae, narrow grooves and frequently by prominent, numerous perforations. Subtypes IA, IIA/IIB, IIB and IIIA were represented by a relatively large number of species (8, 11, 8 and 9 species, respectively), while types IA/IIA, IIIB and V—by only one species. Among the 58 examined species, 12 had two types of exine ornamentation (Fig 3, Table 4).

In most of the species (56 of the 58), elliptic or circular perforations of different diameters (0.05–0.4 μm) were found at the bottom of the grooves (Fig 3). The perforations were not found in *R. canadensis* and *R. czarnunensis*. In the majority of the species studied the perforations were small, with similar diameters (0.1–0.2 μm) and more or less numerous, with the exception of *R. bifrons*, *R. capitulatus*, *R. constrictus*, *R. gracilis*, *R. hercynicus*, *R. lamprocaulos*, *R. odoratus*, *R. opacus*, *R. orthostachys*, *R. ostroviensis*, *R. pedemontanus*, *R. perrobustus* and *R.*

Table 4. Striate exine ornamentation types and subtypes of studied *Rubus* species (according to Ueda [47] classification).

Striate exine ornamentation type or subtype	Species
IA	*R. chaerophylloides*, *R. corylifolius*, *R. fasciculatus*, *R. henrici-egonis*, *R. hercynicus*, *R. lamprocaulos*, *R. pfuhlianus*, *R. posnaniensis*
IA/IIA	*R. plicatus*
IIA	*R. acanthodes*, *R. allegheniensis*, *R. angustipaniculatus*, *R. camptostachys*, *R. circipanicus*, *R. constrictus*, *R. grabowskii*, *R. gracilis*, *R. hevellicus*, *R. koehleri*, *R. macrophyllus*, *R. marssonianus*, *R. nessensis*, *R. ostroviensis*, *R. parthenocissus*, *R. sprengelii*, *R. wimmerianus*, *R. xanthocarpus*
IIA/IIB	*R. apricus*, *R. bavaricus*, *R. bifrons*, *R. capitulatus*, *R. clusii*, *R. micans*, *R. pyramidalis*, *R. spribillei*, *R. chlorothyrsos*, *R. schleicheri*, *R. seebergensis*
IIB	*R. caesius*, *R. dollnensis*, *R. glivicensis*, *R. gothicus*, *R. idaeus*, *R. mollis*, *R. orthostachys*, *R. siemianicensis*
IIIA	*R. canadensis*, *R. czarnunensis*, *R. divaricatus*, *R. fabrimontanus*, *R. opacus*, *R. pedemontanus*, *R. perrobustus*, *R. radula*, *R. scissus*
IIIB	*R. montanus*
striate-verrucate	*R. odoratus*
V	*R. saxatilis*

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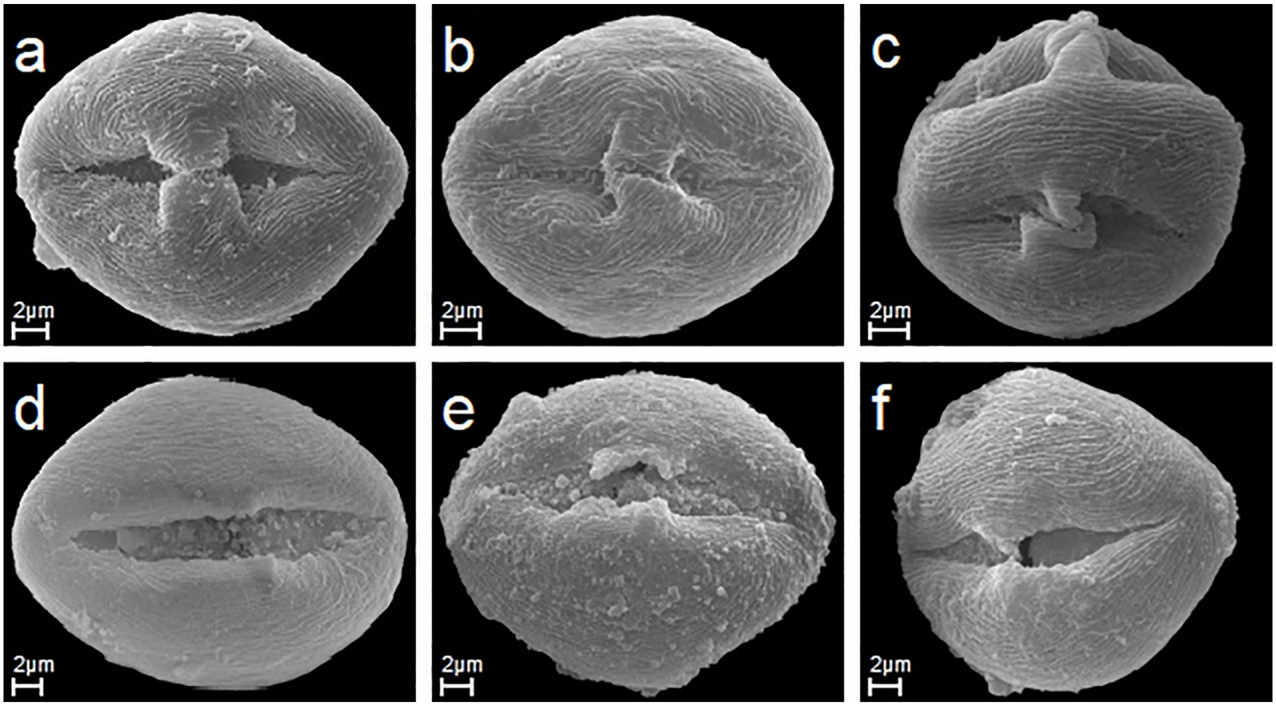

Fig 4. The bridge and apertures of studied species. A-C. *R*. *macrophyllus*, *R*. *circipanicus*, *R*. *angustipaniculatus* the bridge (exine connection between the margins of an aperture—colporus) in three pollen grains in equatorial view. D-F. *R*. *gothicus*, *R*. *scisus*, *R*. *nessensis* colporus with rugulate membrane in three pollen in equatorial view.

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radula, where they were relatively few. The single perforations were observed in *R*. *corylifolius*, *R*. *czarnunensis*, *R*. *henrici-egonis* and *R*. *pyramidalis*.

Pollen grains usually had three apertures—colpori. Ectoapertures—colpi were arranged meridionally, regularly, they were more or less evenly spaced and long, at a mean length of 21.23 (14–32) μm (Table 3; Fig 4D–4F). On average, the length of colpi constituted 83% (from 60 to 100%) of the polar axis length, with the shortest colpi found in *R*. *xanthocarpus* (16.2 μm) and the longest in *R*. *corylifolius* (25.3 μm). Colpi were fusiform in outline. Their width was variable and usually greatest in the equatorial region. Sculpturing of ectocolpus membranes approached rugulate, rarely partly psilate (Fig 4D–4F). Colpus margins frequently had small undulations (Fig 4D–4F).

In all of the species studied the colpus was crossed at the equator by a bridge dividing it into two parts, formed by two bulges of the ectexine that meet in the middle (Fig 4A–4C). The bulges were of the same or unequal length.

The polar area index (PAI) or the apocolpium index (d/E ratio) averaged 0.20 (0.08–0.45). The lowest mean values of this index were recorded in *R*. *canadensis* (0.14), while the highest—in *R*. *odoratus* (0.29) (Table 3).

Endoapertures were usually located in the middle of colpi, less frequently asymmetrically, usually singly and very rarely in pairs. They were circular or elliptic in outline with irregular margins (Fig 4D–4F).

Pollen key

Pollen key can be seen as a summary of the outcome of our study thus it has been placed at the very end of this chapter.

1 Exine ornamentation striate-verrucate with microgranules and small perforations. . ..*R. odoratus*

1* Exine ornamentation striate. 2

2 Exine ornamentation striate without perforations. 3

2 *Exine ornamentation striate with perforations. 4

3 Pollen grains small; P on average from 10 to 25 μm. . . . .*R. canadensis*

3*Pollen grains medium; P on average from 25.1 to 50 μm. . . . .*R. czarnunensis*

4 Exine subtype IA (grooves distinct with medium width, striae narrow; perforations few or absent to numerous, small. 5

4* Exine type II (grooves distinct, with medium, similar width like striae; perforations numerous, medium or large). 7

4** Exine type III (grooves very distinct and width, striae narrow to wide; perforations few, small). 12

4*** Exine type V (grooves flat and blurred; perforations numerous, large to small). . . *R. saxatilis*

5 Perforations numerous. . . . .*R. chaerophylloides*, *R. fasciculatus*, *R. pfuhlianus*, *R. posnaniensis*, *R. plicatus*

5* Perforations few. . . . .*R. hercynicus*, *R. lamprocaulos*

5** Perforations single. 6

6 Pollen grains small. . . . .*R. henrici-egonis*

6*Pollen grains medium. . . . .*R. corylifolius*

7 Striae narrow. 8

7* Striae wide. 10

8 Perforations numerous. 9

8* Perforations few. . . . .*R. bifrons*, *R. capitulatus*, *R. constrictus*, *R. gracilis*, *R. ostroviensis*

8** Perforations single. . . . . *R. pyramidalis*

9 Pollen grains small. . . . .*R. allegheniensis*, *R. camptostachys*, *R. circipanicus*, *R. grabowskii*, *R. hevellicus*, *R. micans*, *R. nessensis*, *R. parthenocissus*, *R. plicatus* *R. xanthocarpus*

9* Pollen grains medium. . . . .*R. acanthodes*, *R. angustipaniculatus*, *R. apricus*, *R. bavaricus*, *R. chlorothyrsos*, *R. clusii*, *R. koehleri*, *R. macrophyllus*, *R. marssonianus*, *R. schleicheri*, *R. seebergensis*, *R. sprengelii*, *R. spribillei*, *R. wimmerianus*

10 Perforations numerous. 11

10* Perforations few. . .*R. bifrons*, *R. capitulatus*, *R. orthostachys*

10** Perforations single. . . . . *R. pyramidalis*

11 Pollen grains small. . . . .*R. idaeus*, *R. micans*, *R. plicatus*

11* Pollen grains medium. . . . .*R. apricus*, *R. bavaricus*, *R. caesius*, *R. chlorothyrsos*, *R. clusii*, *R. dollnensis*, *R. glivicensis*, *R. gothicus*, *R. mollis*, *R. schleicheri*, *R. seebergensis*, *R. siemianicensis*, *R. spribillei*

12 Grooves wide, striae narrow 13

12* Grooves very wide, striae medium. . . . .*R. montanus*

13 Perforations numerous. 14

13* Perforations few. 15

13** Perforations single. . . . .*R. czarnunensis*

14 Pollen grains small. . . . .*R. canadensis*, *R. divaricatus*

14*Pollen grains medium. . . . .*R. fabrimontanus*, *R. scissus*

15 Pollen grains small. . . . .*R. opacus*, *R. pedemontanus*, *R. perrobustus*

15*Pollen grains medium. . . . .*R. radula*

Intrageneric and interspecific variability of pollen grains

The results of the MANOVA indicated that all the species were significantly different with regard to all of the 11 quantitative traits (Wilk's $\lambda = 0.04048$; $F_{627,18111} = 9.98$; $P<0.0001$). The results of analysis of variance for the 11 quantitative traits [P ($F_{57,1682} = 40.42$), E ($F_{57,1682} = 33.51$), Le ($F_{57,1682} = 32.48$), d ($F_{57,1682} = 12.41$), Exp ($F_{57,1682} = 11.26$), Exe ($F_{57,1682} = 12.11$), P/E ($F_{57,1682} = 9.87$), Le/P ($F_{57,1682} = 3.89$) d/E ($F_{57,1682} = 9.24$), Exp/P ($F_{57,1682} = 15.35$) and Exe/E ($F_{57,1682} = 15.29$)] showed variability of the tested species at a significance level $\alpha = 0.001$. The mean values and standard deviations for the observed traits indicated a high variability among the tested species, for which significant differences were found in terms of all the analysed morphological traits (Table 3).

The correlation analysis indicated statistically significant correlation coefficients for 25 out of 55 coefficients (Table 5). A total of 16 out of 25 significantly correlated pairs of traits were characterised by positive correlation coefficients. In the case of 30 pairs of traits, no significant correlation was established.

In the presented dendrogram, as a result of agglomeration grouping using the Euclidean distance method, all the examined *Rubus* species were divided into four groups (Fig 5). The first group (I) comprised one species—*R. czarnunensis*, while the second one (II) four species (*R. dollnensis*, *R. corylifolius*, *R. chaerophylloides* and *R. phuhianus*). The third group was divided into two subgroups: III A—*R. camptostachys*, *R. xanthocarpus*, *R. clussi*, *R. odoratus*, and III B—including all the other species from this group. The fourth group (IV) comprised *R. canadensis*, *R. capitulatus*, *R. acanthoides* and *R. spribillei*.

Individual traits were of varying importance and had different shares in the joint multivariate variation. A study on the multivariate variation for species includes also identification of the most important traits in the multivariate variation of species. Analysis of canonical variables is a statistical tool making it possible to solve the problem of multivariate relationships. Fig 6 shows the variability of the pollen grain features in 58 studied *Rubus* species in terms of the first two canonical variables. In the graph the coordinates of the point for particular shrubs were the values for the first and second canonical variable, respectively. The first two canonical

Table 5. Correlation coefficients between all pairs of observed traits.

Trait	P	E	Le	d	Exp	Exe	P/E	Le/P	d/E	Exp/P	Exe/E
P	1										
E	0.820***	1									
Le	0.975***	0.799***	1								
d	0.575***	0.614***	0.477***	1							
Exp	0.015	0.015	-0.014	0.186	1						
Exe	-0.034	-0.028	-0.045	0.156	0.937***	1					
P/E	0.322*	-0.275*	0.310*	-0.026	0	-0.012	1				
Le/P	0.169	0.141	0.380**	-0.285*	-0.139	-0.075	0.028	1			
d/E	0.238	0.17	0.124	0.878***	0.226	0.207	0.143	-0.454***	1		
Exp/P	-0.632***	-0.520***	-0.641***	-0.22	0.757***	0.730***	-0.201	-0.236	0.033	1	
Exe/E	-0.533***	-0.635***	-0.537***	-0.245	0.710***	0.779***	0.157	-0.184	0.07	0.892***	1

* P<0.05

** P<0.01

*** P<0.001

P—the length of polar axis, E—the length of equatorial axis, Le—the length of ectocolpi, d—the distance between the apices of two ectocolpi, Exp—the thickness of exine along polar axis, Exe—the thickness of exine along equatorial axis

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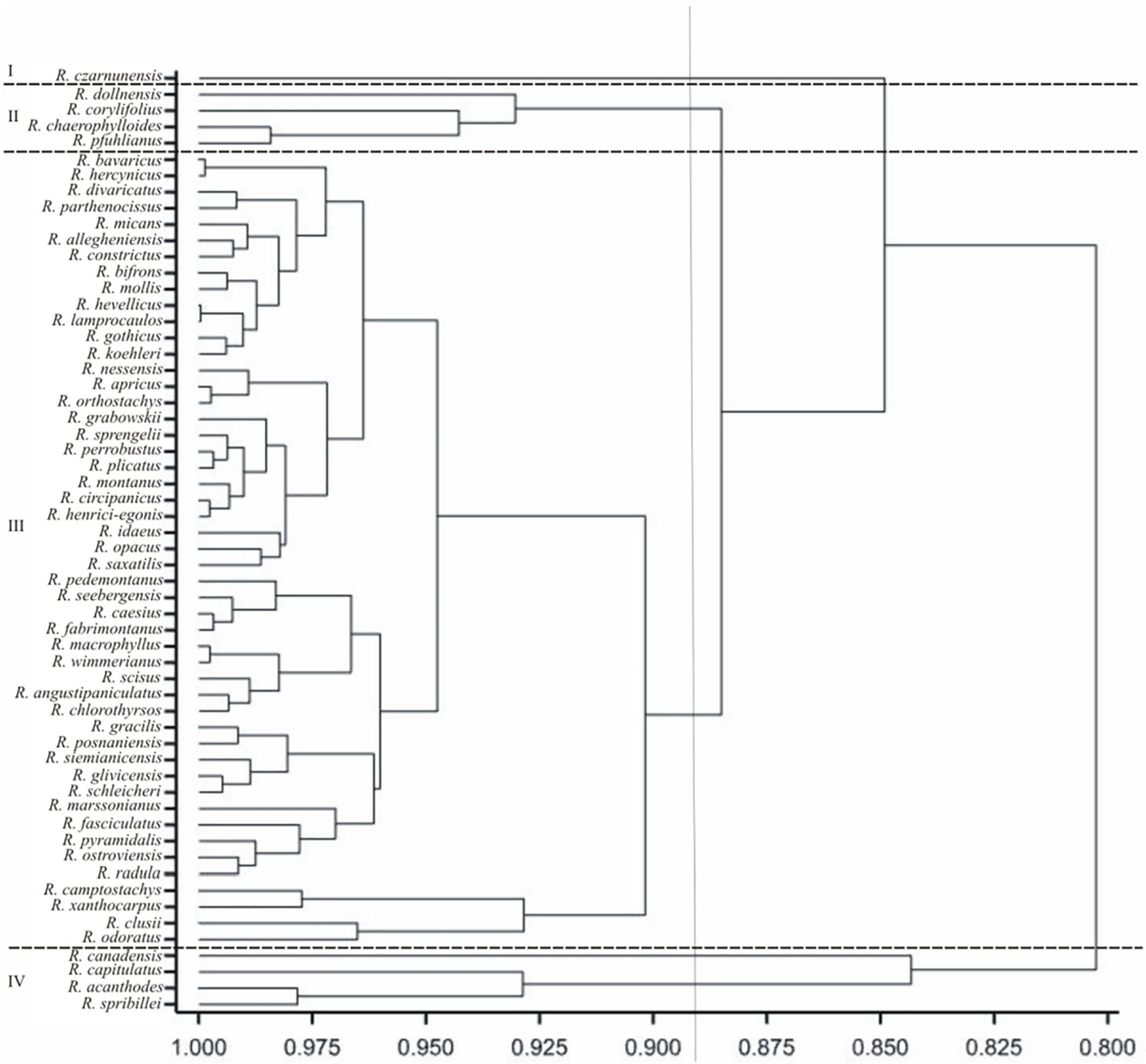

Fig 5. Dendrogram of cluster groupings of *Rubus* species based on all 11 morphological traits.

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variables accounted for 56.75% of the total multivariate variability between the individual species. Five groups of species were distinguished (Fig 5). A majority of the examined species were found in the first group (I), which means that they had more or less similar pollen features. Only one up to maximum three species (II—*R. capitulatus*, III—*R. xantocarpus*, IV—*R. acanthoides* and *R. spribillei*, and V—*R. corylifolius*, *R. dollnensis*, and *R. czarnunensis*) fell into the other four groups (Fig 6). Pollen grains of *R. capitulatus* were the most different from those of the other species (large, with a thin exine and the P/E ratio usually prolate-spheroidal).

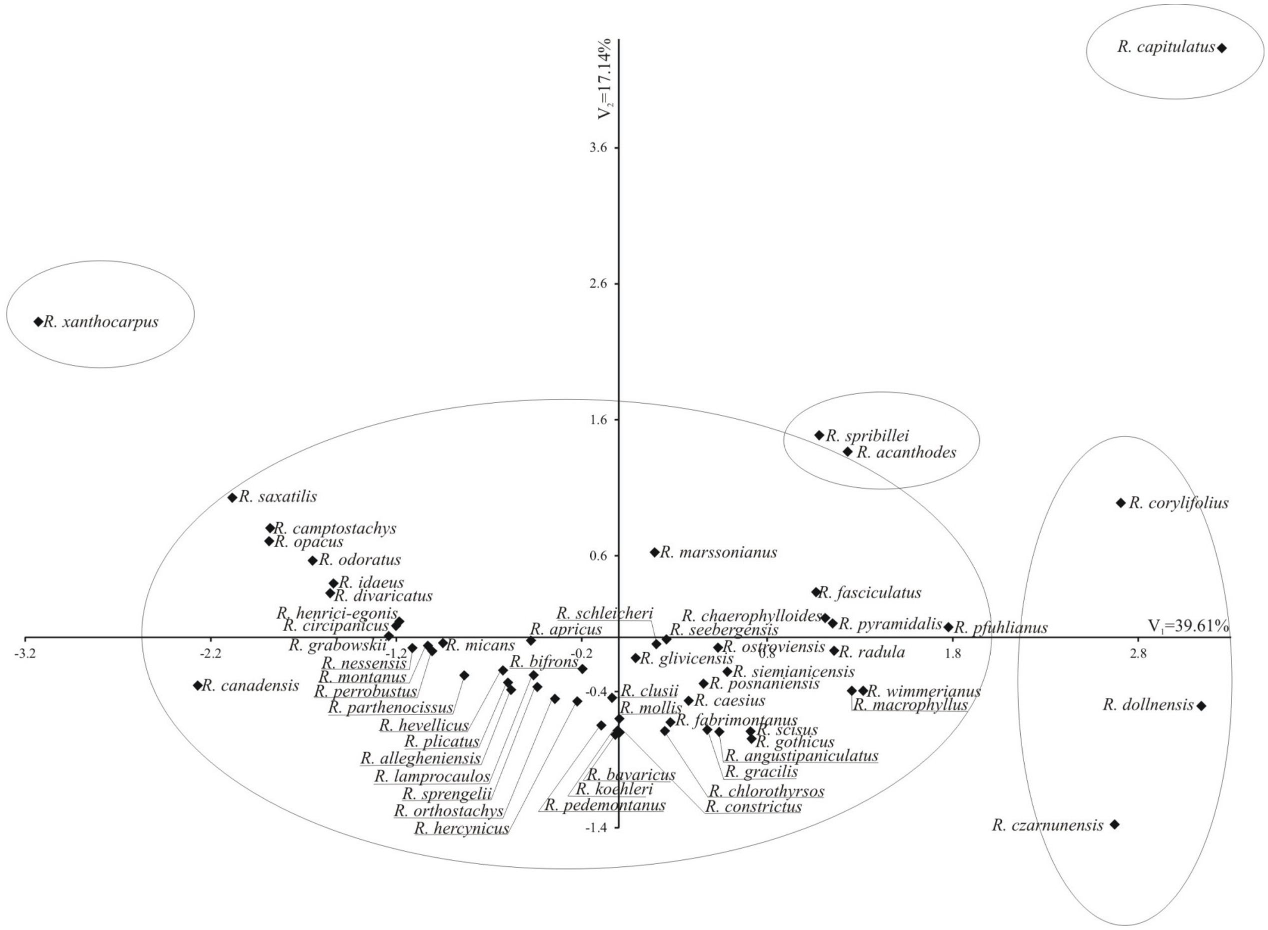

Fig 6. Distribution of the studied *Rubus* species in the space of the first two canonical variables.

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Species from groups IV and V had the largest pollen grains and *R. xantocarpus* (group III)—the smallest ones.

The most significant, positive, linear relationship between the first canonical variables was found for P, E, Le and d, while it was negative for Exp/P and Exe/E (Table 6). The second canonical variable was significantly negatively correlated with Exp, Exe, Exp/P and Exe/E (Table 6). The greatest variation in terms of all the traits jointly (measured Mahalanobis distances) was found for *R. canadensis* and *R. capitulates* (the Mahalanobis distance between them amounted to 8.24). The greatest similarity was found for *R. lamprocaulos* and *R. hevellicus* (0.313).

Discussion

Similarly to a majority of palynologists, the authors of this study maintain that exine ornamentation features were diagnostic, that means they allow for differentiate species within the genus *Rubus* [24, 25, 27–31, 33, 34, 38, 39, 42, 46, 59]. The most important exine ornamentation traits include the width, number and course of grooves (muri) and the width of the striae as well as the number and diameter of perforations [31, 33, 34, 42, 46, 59–61]. Some authors considered pollen size and shape as potentially important features in the diagnosis of the analysed *Rubus* species [27, 28, 33], while others claim that they have no diagnostic significance [31, 45, 46].

Table 6. Correlation coefficients between the first two canonical variables and original traits.

Trait	First canonical variable	Second canonical variable
P	0.9634***	-0.0536
E	0.9353***	-0.0382
Le	0.9427***	-0.0812
d	0.5995***	-0.1054
Exp	-0.0477	-0.5907***
Exe	-0.0993	-0.6587***
P/E	0.0751	-0.0254
Le/P	0.1822	-0.1743
d/E	0.1939	-0.087
Exp/P	-0.6568***	-0.3354*
Exe/E	-0.6497***	-0.3919**
Percentage of explained multivariate variability	39.61%	17.14%

* $P<0.05$

** $P<0.01$

*** $P<0.001$

P—the length of polar axis, E—the length of equatorial axis, Le—the length of ectocolpi, d—the distance between the apices of two ectocolpi, Exp—the thickness of exine along polar axis, Exe—the thickness of exine along equatorial axis

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Based on our results, we partially agree with the opinion of these former, because the length of the polar axis (P) has been an important feature.

In a study by Li et al. [42] the 103 examined *Rubus* species from China belonged to four types of exine ornamentation (rugulate, striate, cerebroid and reticulate-perforate), which were further divided into 11 subtypes. Other palynologists distinguish in blackberries mainly striate or striate-perforate exine ornamentation [24, 25, 28, 29, 31, 33, 34, 38–40, 46, 59]. Except for the typical striate ornamentation, also striate-scabrate, striate-rugulate or rugulate [31, 46], echinate or gemmate [29], verrucate [29, 38, 39], baculate and clavate [24, 25] or reticulate ornamentation [59] have been rarely observed. According to current palynological studies, European blackberry species are slightly less variable in terms of this feature than Asian ones. Our results confirm this thesis, because in the examined pollen grains only two types of exine ornamentation (striate and striate-verrucate with microgranules) were found.

Ueda & Tomita [61] and Ueda [47] distinguished six types and six subtypes of exine ornamentation in species and other taxa from the genus *Rosa* and the family Rosaceae, including the genus *Rubus*. In the current study they were classified into four types (types IV and VI were not identified) and five subtypes (I A, II A, B, III A, B). Our results were similar to the cited authors, since most of the examined pollen belonged to the IIA and IIIA subtypes and no grains were found in the very rarely represented types IV and VI or subtype IB. The only species described both by Ueda [47] and in our study was *R. odoratus*. Ueda [47] described it as a type VI and we as type V.

The research results obtained in this study confirmed the diagnostic significance of the number and diameter of perforations, found by Hebda & Chinnappa [38, 39], Monasterio-Huelin & Pardo [28], Tomlik-Wyremblewska [31], Li et al. [42], Wrońska-Pilarek et al. [33] or Ghosh & Saha [59], because these traits allowed to distinguish certain *Rubus* species (see: pollen key). On the other hand, groups of species from different sections possess similar numbers of perforations (e.g. *R. opacus* from the series *Rubus*, *R. canadensis* from the series *Canadenses* or *R. henrici-egonis* from the series *Discolores*). However, also species from many different sections (e.g. *Rubus*, *Alleghenienses*, *Sylvatici* or *Micantes*) representing the subgenus *Rubus* were characterised by high numbers of small perforations with similar diameters. Hebda and Chinnappa [38] distinguished two types of perforations in the family Rosaceae (striate—macroperforate and non-striate—macroperforate, each with six subtypes) possibly indicating different evolutionary lines. According to the above cited study, pollen of *Rosa* (with *Prunus*, *Rubus* and *Spiraea*) belongs to the subcategory with striae separated by grooves, containing larger perforations (0.1–0.2 μm in diameter). The current data corroborated this latter thesis, with the reservation that some of the species were characterised by ornamentation different than striate (*R. odoratus*—striate-verrucate with microgranules), and that perforation diameters in *Rubus* ranged from 0.05 to 0.4 μm. In turn, Hebda and Chinnappa [39] classified pollen types in Rosaceae into six main categories: 1—striate and macroperforate, 2—striate and microperforate, 3—tuberculate and perforate, 4—microverrucate, 5—verrucate and 6—perforate, without supratectal features. They included species from the *Rubus* genus, similarly to the study from 1990, in type 1 (striae long and parallel to colpus). Our studies demonstrated that the inclusion of the *Rubus* genus into one type is too general because, firstly, there were blackberry species with the striate-verrucate exine ornamentation with microgranules (e.g. *R. odoratus*), with perforations sometimes being large, but also small (type 2—striate and microperforate). Additionally, in some species perforations were very scarce or did not occur at all (e.g. *R. corylifolius*, *R. henrici-egonis*, *R. canadensis*, *R. czarnuensis*). Consequently, species from the *Rubus* genus also belong to other types mentioned above, as well as types not mentioned by Hebda & Chinnappa [39].

Many studies reported that the bridges are located in the most of studied Rubus species. [28, 31, 33, 46]. They were wide, well-developed and with margins. In blackberries Tomlik-Wyremblewska [31] distinguished two bridge types, with margins stretched or constricted at the equator. In our study, bridges were observed in all the analysed blackberry species and this structure was not used as a basis for the identification of species, because its characteristics were too similar. Besides, it usually appeared in mature pollen grains, so it could not be noticed when analysing pollen at other developmental stages.

The presented results shows that studied pollen grains, were small (43.3%) or medium (56.7%). Similar results regarding pollen size were obtained by all other researchers [24, 25, 27, 28, 32–34, 42, 46, 59].

In the opinion of Li et al. [42] pollen shape varied from spheroidal, subspheroidal, prolate and perpolate, to occasionally rhomboid and hexagonal. In turn, Monasterio-Huelin & Pardo [28] stated that they were just prolate or spheroidal, while other authors distinguished several pollen shape types—subprolate, prolate spheroidal, prolate or perprolate [31, 33, 34, 40, 46, 59]. We agree with Tomlik-Wyremblewska [31, 46] opinion, that pollen shape turned out to be a poor criterion in identifying blackberry species, because most pollen grains (81.6%) have a similar shape—subprolate or prolate-spheroidal.

The arrangement of the investigated species on the dendrogram (Fig 5) does not corroborate the division of the genus *Rubus* into subgenera, sections and series [16], currently adopted in taxonomy.Species from three different subgenera (*R. saxatilis* and *R. xanthocarpus* from the subgenus *Cylactis*, *R. odoratus* from the subgenus *Anoplobatus* and *R. idaeus* from the subgenus *Idaeobatus*) were found in the same group III, with most of the species from a large subgenus *Rubus*. Similar results were obtained for the three sections from the subgenus *Rubus* (*Rubus*, *Corylifolii* and *Caesii*). Thus, *R. caesius* from the section *Caesii* and *R. gothicus*, *R. camptostachys*, *R. mollis* or *R. fabrimontanus* from the section *Corylifolii* were found in group III, with the species representing the most numerous third section of *Rubus*. Also in the case of the series it were not observed that species belonging to these taxa formed separate groups (Figs 5 and 6). Other genera of the family Rosaceae (e.g. *Spiraea*, *Rosa*, *Crataegus*) showed a correlation between pollen morphology and intrageneric taxonomic classification [62–64]. In *Rubus* the lack of dependence could be the result of apomixis, defined as the replacement of the normal sexual reproduction by asexual reproduction, without fertilisation, which could reduce natural variability.

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