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Pollen morphology of rare species of *Linum* L. (Linaceae) from Poland

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Abstract: The aim of the study was to describe pollen morphology and its variability of four *Linum* species: *L. perenne* subsp. *extraaxillare*, *L. hirsutum*, *L. flavum* and *L. austriacum*, all derived from Poland, but occurring in few natural sites within the country. Light and scanning electron microscopy was used and statistical analyses of quantitative pollen traits were performed. All taxa shared pollen grains which were 3-zonocolpate, isopolar, radiosymmetric, spheroidal and medium to large in size. Grains were semitectate, with the sculptural elements of the exine as gemmae or clavae. Additionally, upper parts of gemmae and clavae had a microstriate pattern. Despite great similarity among the investigated *Linum* species in pollen morphology, distinguishing features were determined.

Key words: pollen morphology, *Linum*, Poland, LM, SEM, ANOVA, PCA, LSD test

1. Introduction

Linum L. is the main genus of the Linaceae family with up to 180 species growing in temperate and subtropical regions, especially in the Mediterranean Basin (Hickey & King 1988; McDill *et al*. 2009). There are probably 37 species of the genus in Europe (including 9 provisional, 1 external and 7 endemic) (Ockendon & Walters 2001). In Poland, in turn, it is represented only by 5 species: *L. catharticum* L. (section *Cathartolinum*), *L. flavum* L. (section *Styllinum*), *L. hirsutum* L. (section *Dasyllinum*), *L. austriacum* L. (section *Linum*) and *L. perenne* L. subsp. *extraaxillare* (Kit.) Nyman (= *L. extraaxillare* Kit., section *Linum*) (Mirek *et al*. 2002).

L. catharticum occurs in the wild in almost all of Europe and it is the most common flax species in Polish flora. *L. hirsutum* and *L. flavum* have similar European ranges and they appear from northern and western parts of the continent through central countries, to the Balkans and Turkey in the South. Both species are rare in Poland and can mainly be found in the southern part of the country: *L. flavum* in the Małopolska upland, Lublin upland, Roztocze region and near Przemyśl; *L. hirsutum,* in turn, within Nidziańska basin, as well as in the geobotanical district of Miechów-Pińczów. The extensive range is also characteristic of *L. austriacum*, which, among others, grows naturally in central and southern Europe, in western Asia (Turkey and Iran) and north Africa. However, it is very rare in Poland and occurs naturally exclusively in the environs of the city of Przemyśl. Therefore, its sparse sites in northern Poland and Silesia are considered to be of anthropogenic origin. *L. perenne* subsp. *extraaxillare* is an endemic plant, noted in the Carpathians and mountains of the Balkan Peninsula. In Poland, it is only found in the Tatra Mts., especially above the thickets of the dwarf mountain pine (Piękoś-Mirkowa *et al.* 2000; Kaźmierczakowa *et al.* 2014; Ockendon & Walters 2001; Piękoś-Mirkowa & Mirek 2003; Mirek & Piękoś-Mirkowa 2008).

Since 1950s, a lot of palynological studies on the *Linum* genus both in LM, SEM and TEM have been carried out. Pollen morphology of over 60% flax species has been studied (Erdman 1966; Ockendon 1971;

Rogers & Xavier 1971, 1972; Kuprianova & Alyoshina 1978; Rogers 1980, 1982, 1985; Xavier *et al*. 1980; Dulberger 1981; Punt & De Breejen 1981; Grigoryeva 1988; Moroz & Tsymbalyuk 2005; Perveen & Qaiser 2008; Chestler & Raine 2010; Talebi *et al*. 2012; Lattar *et al.* 2012a, 2012b). As a rule, earlier research focused on a small number of species only and there was no thorough analysis on pollen morphology of the genus. Furthermore, pollen of many species with limited ranges has not been studied yet. There is still no data on variations in pollen morphology of the species already described.

When compared with most angiosperms, pollen grains of *Linum* are rather large. They are usually at least 45 µm in diameter and in section *Linastrum* even 125 µm (Xavier *et al*. 1980). Most often, they are tri-zonocolpate and rarely multiaperturate. The latter have probably evolved from those with triaperturate at least five times within this genus (Rogers & Xavier 1972). According to Yunus & Nair (1988), members of the Linaceae family have a very distinct pattern of pollen surface and specific structural uniqueness dissimilar to other related families. There is also a certain uniformity in the exine sculpture in the *Linum* making pollen grains easily distinguishable for the genus. However, individual species have got diverse sculpture processes, which can take various forms, as bacula, gemma, pila, verrucae, clavae or echinae (Talebi *et al.* 2012). Besides, presence of heterostyly in about 40% of *Linum* species is expressed in variation in pollen morphology. As a rule, pollen surface of short-styled plants comprises almost exclusively monomorphic projections, while in case of long-styled plants, pollen surface consists of clearly dimorphic ones. Then, on average, short-style specimens have got pollen grains of smaller size than long-style specimens (Rogers 1980; Dulberger 1981; Punt & Den Breejen 1981).

With the exception of the study by Stachurska and others (1963) on *L. perenne* subsp. *extraaxillare* (in LM), there are no reports on pollen morphology of the *Linum* genus from Poland.

The aim of the present study was to describe pollen morphology of the following four flax species: *L. perenne* subsp. *extraaxillare*, *L. hirsutum*, *L. flavum* and *L. austriacum*, derived from Poland and occurring in few natural sites within the country. Additionally, pollen variability of the above mentioned taxa was also analyzed to evaluate the potential systematic value of pollen traits in different geographic areas.

2. Material and methods

2.1. Plant material

Pollen samples were gathered from plants cultivated in the collection of the Botanical Garden of Adam Mickiewicz University in Poznań (Poland). The origin of the analyzed *Linum* species is: directly from natural populations (*L. flavum* from Żmudź reserve near Chełm Lubelski, Poland [AMUBG number R_R001_001_0025_7994_R008] and *L. austriacum* from Winna Góra near Przemyśl, Poland [R_R002_002_0012_7994_R009]) or from ex situ collections of botanic gardens (*L. hirsutum* from Arboretum Bolestraszyce, Poland [R_R002_003_0002_7995_R005] and *L. perenne* from the Botanical Garden of M. Curie-Skłodowska University in Lublin, Poland [A_A007_000_0025_7010_A088]). For each species, anthers from 15 flowers were collected. Pollen samples were gathered randomly, without distinction between long- and short-styled forms of flowers. Such a method of pollen collection was applied intentionally to provide mean values of investigated traits for each species. The list of voucher specimens is deposited in the Department of Botany of University of Life Sciences in Poznań (Poland).

2.2. Pollen morphological analyses

Pollen viability was assessed with the standard method (Cresti & Tiezzi 1992). For each investigated species, five anthers per five flowers were removed in the phase of pollination. Then pollen from anthers was transformed onto microscope slides containing a mixture of glycerol and 2% acetic carmine (in the ratio 1:1). On the following day, slides with pollen were analyzed for viability. The percentage of stained pollen grains (viable) in relation to their total number was determined. For each species, 300 pollen grains were investigated.

For light (LM) and scanning electron microscopy (SEM), pollen grains were prepared according to the standard method described by Erdman (1966).

Table 1. Comparison of pollen viability of investigated *Linum* species

Species	Percentage of viable pollen grains	Percentage of nonviable pollen grains
L. perenne subsp. *extraaxillare*	98.73	1.27
L. austriacum	94.12	5.88
L. hirsutum	97.87	2.13
L. flavum	95.33	4.67

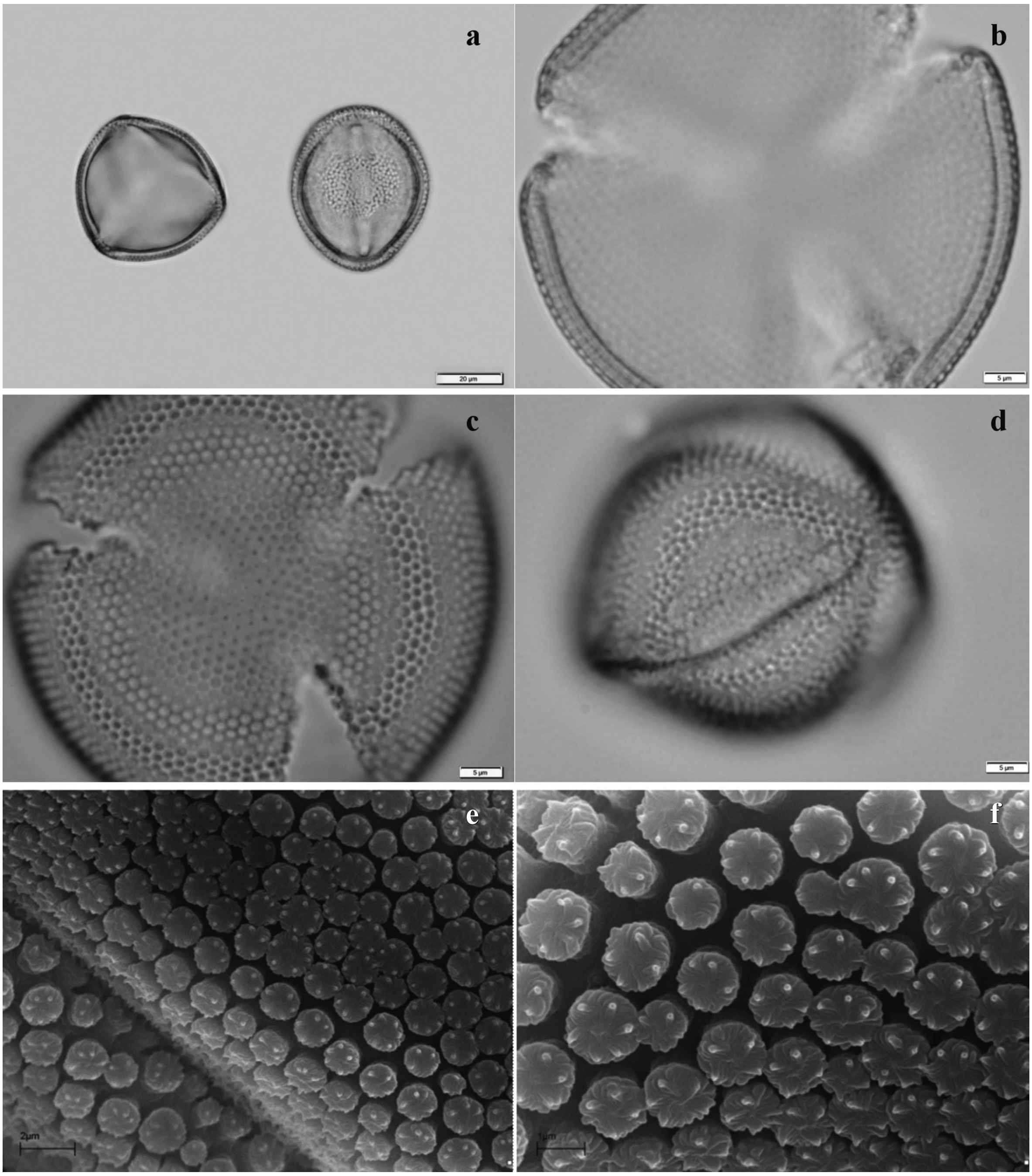

Fig. 1. Pollen morphology of *Linum perenne* subsp. *extraaxillare*
Explanations: a – outline in polar and equatorial views (LM), b – exine structure (LM), c – gemmate sculpture of apocolpium (LM), d – gemmate sculpture of mesocolpia (LM), e – exine sculpture in SEM, f – focus on gemmae (SEM). Scales, a – 20 µm, b-d – 5 µm, e – 2 µm, f – 1 µm

For LM, pollen grains were put into a drop of pure water and observations were made with the Olympus BX 43 microscope.

For SEM, samples of acetolysed grains were mounted on aluminum stubs, coated with gold in a sputter coater and examined with the Hitachi S3000N field emission scanning electron microscope at 5 kV in the Institute of Plant Protection in Poznań (Poland) as well as with the Leo microscope belonging to the Department of Biology AMU in Poznań. Pollen terminology presented by Hesse *et al.* (2009) was used in this study.

2.3. Statistical analyses

Measurements of 30 grains for each specimen were taken and different traits were observed in polar and equatorial views (400 x and 1000 x). Biometric results were statistically elaborated. The arithmetical mean, minimum, maximum, standard deviation (SD), variance and coefficient of variation (CV) were calculated. One factor of variance ANOVA was carried out to examine the significance of differences among mean values of the traits. The Least Significant Difference (LSD) test

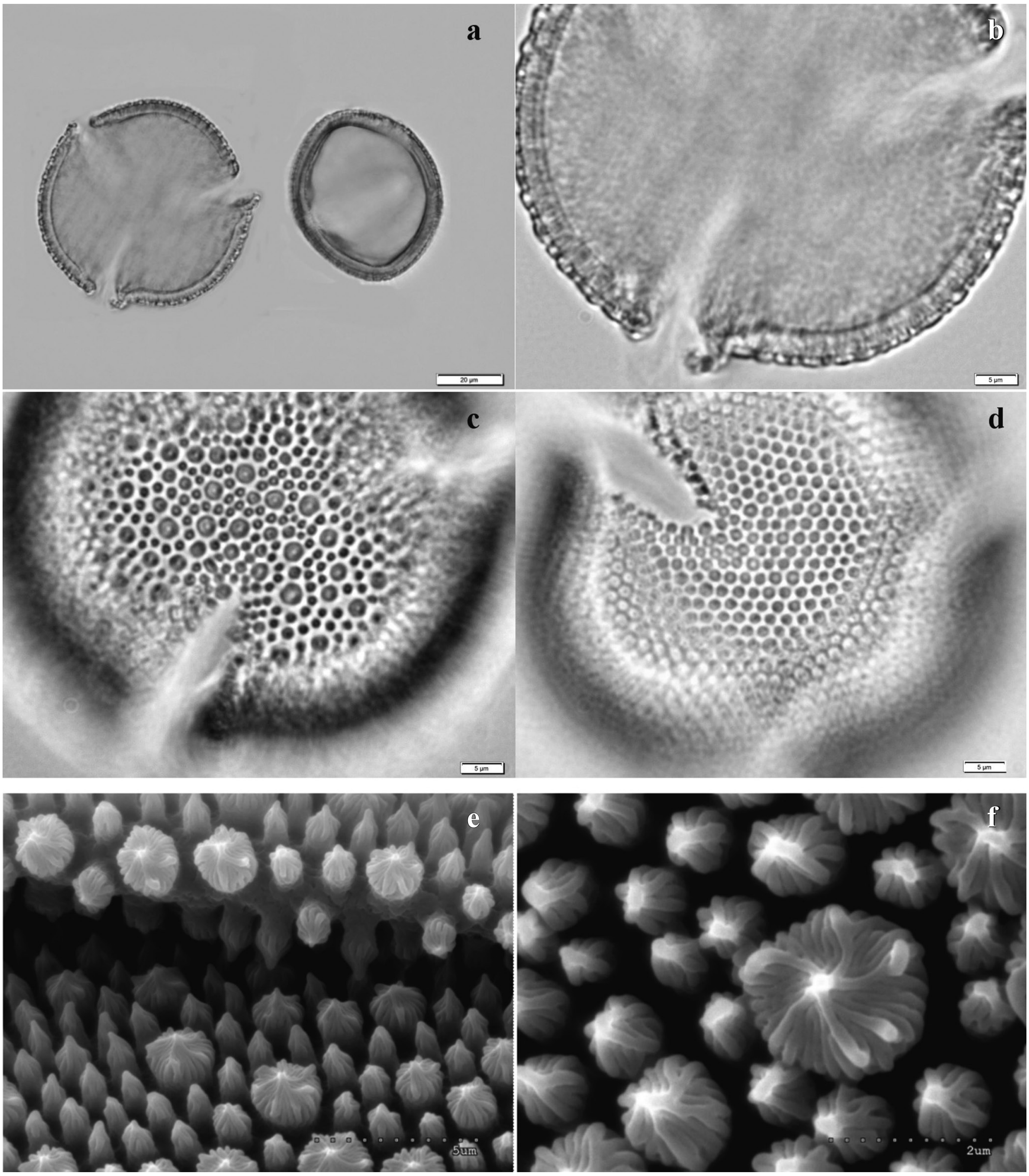

Fig. 2. Pollen morphology of *Linum austriacum*
Explanations: a – outline in polar and equatorial views (LM), b – exine structure (LM), c – dimorphic sexine processes (LM), d – monomorphic sexine processes (LM), e – exine sculpture in SEM, f – focus on gemmae (SEM). Scales, a – 20 µm, b-e – 5 µm, f – 2 µm

was used to investigate the level of significance of the results. Tukey test for unequal numbers of observation was determined to find similar groups. The traits: lengths of P and E axes, P/E ratio and exine thickness were examined in 30 repetitions for each species, while the number of exine processes and apocolpium diameter – in 15. An analysis of the correlation between the measured traits was done. With no prior assumptions, PCA principal component analysis was carried out to examine relationships among individuals from particular samples. Means obtained from the analyses allowed creation of a dendrogram based on Euclidean distance and, thus, to demonstrate the degree of similarity among the investigated species (Sneath & Sokal 1973).

3. Results

3.1. Pollen morphological analyses

In general, all analyzed *Linum* species were characterized by high viability of pollen grains (Table 1). The

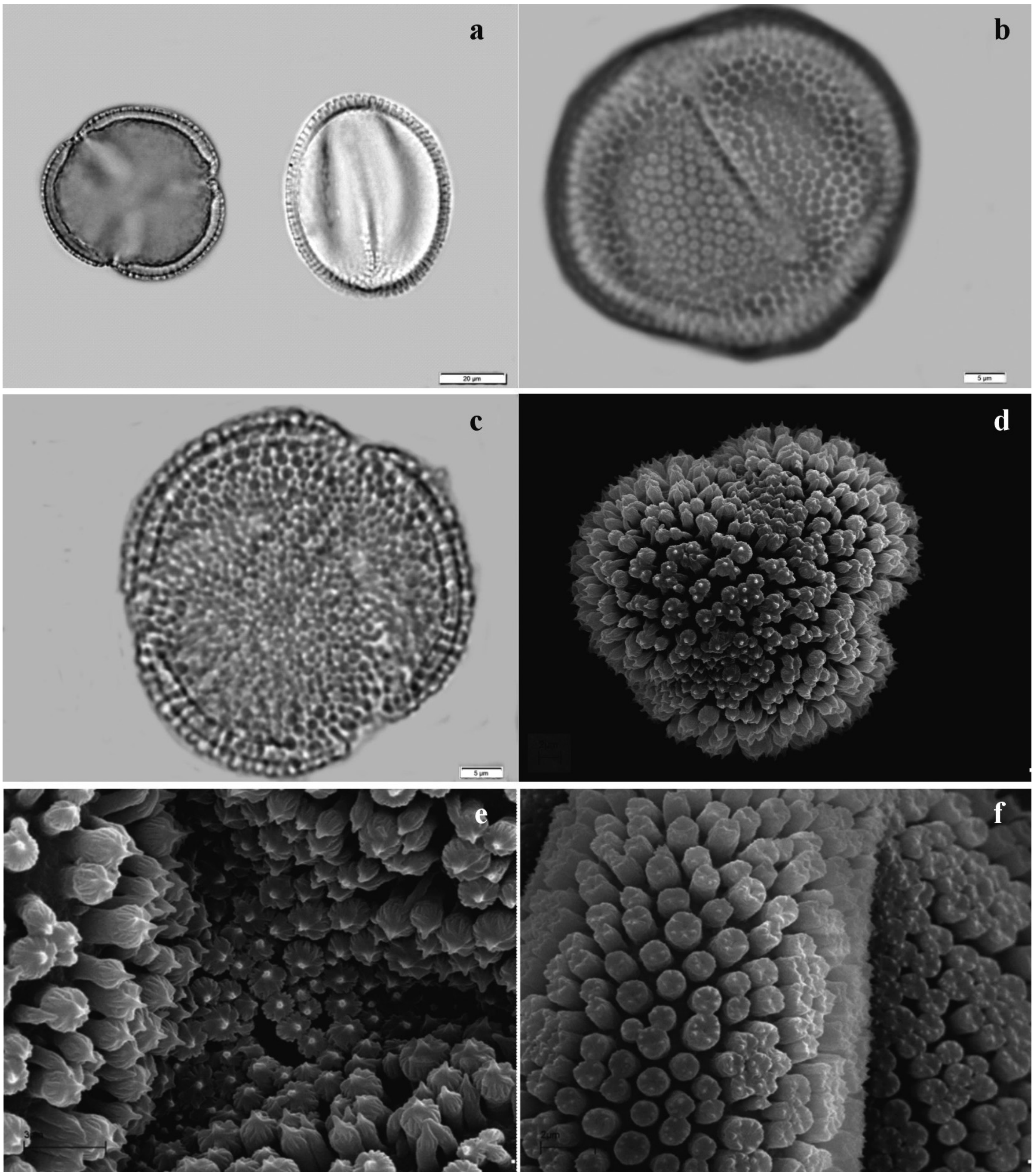

Fig. 3. Pollen morphology of *Linum hirsutum*
Explanations: a – outline in polar and equatorial views (LM), b – monomorphic sexine processes (LM), c – dimorphic sexine processes (LM), d – clavate sculpture in SEM, e – focus on clavae within apocolpium (SEM), f – focus on clavae within mesocolpium (SEM). Scales, a – 20 µm, b-c – 5 µm, d, f – 2 µm, e – 3 µm

lowest percentage of dead grains – about 1.3% was found in the sample of *L. perenne* subsp. *extraaxillare* and the highest, but not exceeding 5.0% – in *L. flavum*. Pollen grains of all investigated species were 3-zonocolpate, isopolar, radiosymmetric and medium (*L. hirsutum*) to large size (*L. austriacum*, *L. flavum* and *L. perenne* subsp. *extraaxillare*). Most often, they were prolate-spheroidal in shape, with circular amb. The length of the colpus amounted to at least ¾ of the length of the polar axis. The exine was 2.8-5.3 µm thick and the sexine and nexine were of similar thickness, or slightly thicker (sexine). Grains were semitectate. With LM, they had gemmate and clavate surface. Pollen grains were both with monomorphic and dimorphic processes (*L. austriacum*, *L. flavum* and *L. hirsutum*) or only monomorphic (*L. perenne* subsp. *extraaxillare*). With SEM, sculpture consisted of gemmae (*L. perenne* subsp. *extraaxillare*) or only exclusively of clavae (*L. austriacum*, *L. flavum* and *L. hirsutum*). Gemmae were ± 0.5-1.3 µm in height and ± 0.8-2.5 µm in diameter (*L.*

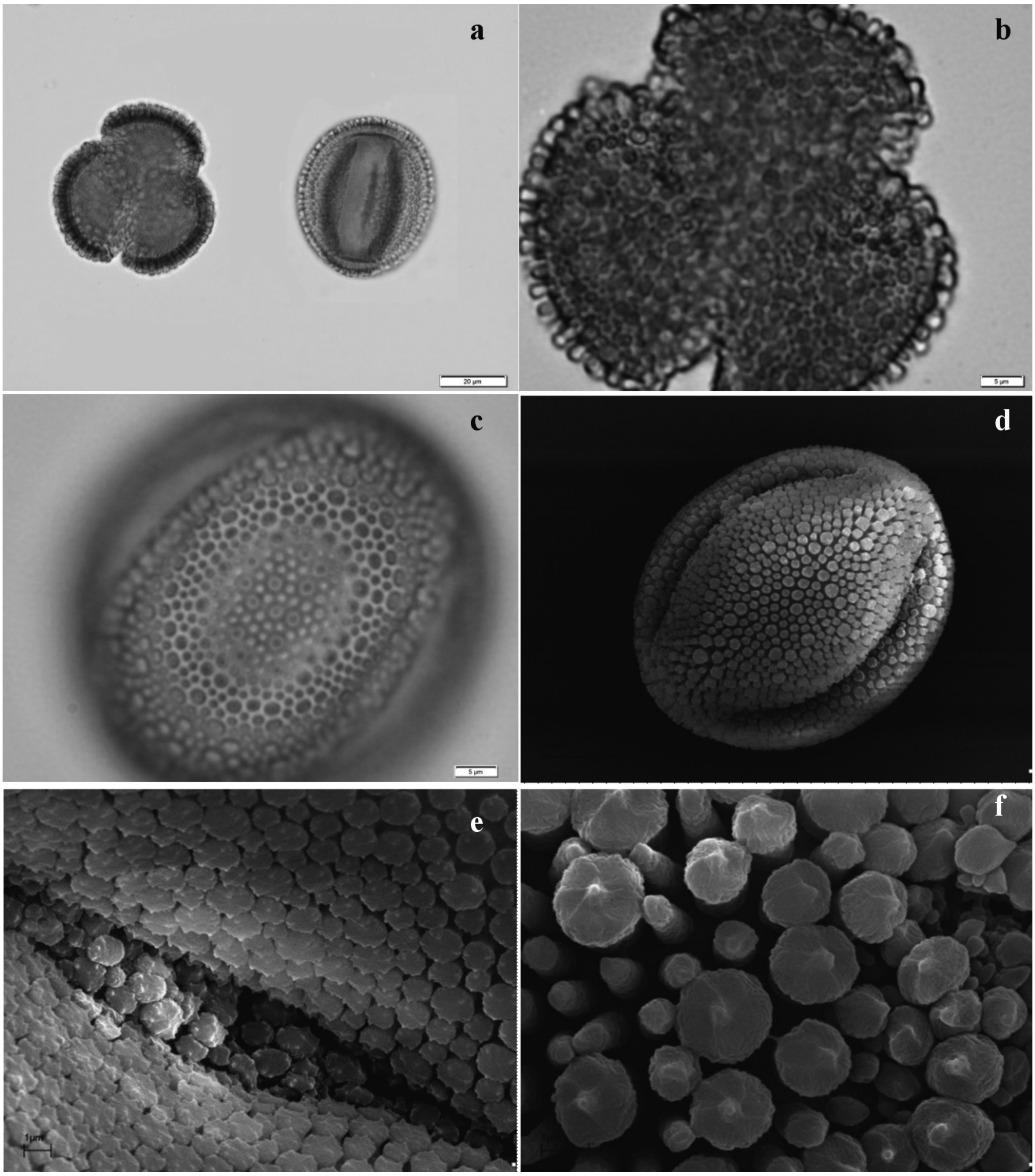

Fig. 4. Pollen morphology of *Linum flavum*
Explanations: a – outline in polar and equatorial views (LM), b – apocolpium with dimorphic sexine processes (LM), c – mesocolpium with dimorphic sexine processes (LM), d – clavate sculpture in SEM, e – zone of colpus (SEM), f – focus on clavae within mesocolpium (SEM). Scales, a – 20 µm, b-c – 5 µm, d – 10µm, e-f – 1 µm

perenne subsp. *extraaxillare*). Clavae were of ± 2 µm (*L. austriacum*) to ± 3 µm (*L. flavum* and *L. hirsutum*) in height and ± 0.7-2.1 µm in diameter. Upper parts of gemmae and clavae showed a striate pattern. Striae were radially spread. Additionally, there were central and 5-6 perispherial rounded granules on tops of gemmae and clavae (*L. perenne* subsp. *extraaxillare* and *L. austriacum*) or only central granules were visible (*L. flavum* and *L. hirsutum*). Margins of colpi were regular, with the same sculpture as the adjacent area of mesocolpi. Specific features of the pollen grains of each investigated species are illustrated in the Figures 1-4.

On average, the largest pollen grains were noted in *L. flavum* and *L. austriacum* (Table 2). The smallest value of P/E ratio was revealed in *L. flavum* (pollen grains most often spheroidal in shape). P/E ratio in other species was usually more than 1.10 (grains mainly prolate-spheroidal). The thickest exine was observed in *L. austriacum* and *L. perenne* subsp. *extraaxillare*. On average, the widest mesocolpia were in *L. perenne*

Table 2. Basic statistical characteristics of analyzed traits of four investigated *Linum* species

Species	Mean	Minimum	Maximum	Standard deviation	Variance	Coefficient of variation
Length of polar axis P (μm)						
L. hirsutum	48.67	35.00	75.00	7.15	51.18	14.70
L. flavum	61.17	40.00	70.00	6.56	42.99	10.72
L. austriacum	61.92	52.50	70.00	4.29	18.40	6.93
L. perenne subsp. *extraaxillare*	51.58	27.50	62.50	8.42	70.90	16.32
Length of equatorial axis E (μm)						
L. hirsutum	43.67	32.50	67.50	6.39	40.83	14.63
L. flavum	56.92	35.00	67.50	7.30	53.31	12.83
L. austriacum	56.33	47.50	65.00	4.39	19.28	7.79
L. perenne subsp. *extraaxillare*	46.67	22.50	60.00	8.31	69.11	17.81
P/E ratio						
L. hirsutum	1.12	1.05	1.29	0.07	0.00	5.93
L. flavum	1.08	1.00	1.40	0.07	0.01	6.56
L. austriacum	1.10	1.04	1.33	0.07	0.00	6.33
L. perenne subsp. *extraaxillare*	1.11	1.00	1.33	0.08	0.01	7.43
Exine thickness (μm)						
L. hirsutum	3.64	2.83	5.32	0.67	0.44	18.32
L. flavum	3.60	3.02	4.27	0.37	0.14	10.24
L. austriacum	3.88	2.94	4.76	0.49	0.24	12.63
L. perenne subsp. *extraaxillare*	3.98	3.19	5.16	0.40	0.16	10.15
Width of mesocolpium (μm)						
L. hirsutum	23.46	19.58	28.79	3.80	14.45	16.20
L. flavum	23.04	19.70	24.94	1.77	3.15	7.70
L. austriacum	20.47	15.89	24.93	2.92	8.53	14.27
L. perenne subsp. *extraaxillare*	32.43	22.97	36.58	5.39	29.10	16.63
Number of exine processes per area of 100 μm^2						
L. hirsutum	35.00	29.00	39.00	3.42	11.67	9.76
L. flavum	41.43	34.00	59.00	8.46	71.62	20.43
L. austriacum	41.86	39.00	48.00	3.13	9.81	7.48
L. perenne subsp. *extraaxillare*	40.86	35.00	47.00	3.98	15.81	9.73
Apocolpium diameter (μm)						
L. hirsutum	15.43	8.17	19.17	4.30	18.45	27.84
L. flavum	19.01	11.84	26.18	5.47	29.91	28.78
L. austriacum	16.98	8.68	22.85	4.73	22.33	27.83
L. perenne subsp. *extraaxillare*	20.04	13.38	23.94	4.02	16.17	20.07

subsp. *extraaxillare* and the shortest - in *L. austriacum*. The smallest number of exine processes per area of 100 μm^2 as well as the smallest apocolpium diameter was noted in *L. hirsutum*. Other species had a similar number of exine processes but their apocolpium area varied slightly in size. The apocolpium diameter was the most variable trait in all species and the most constant was P/E ratio. There were no pollen traits with the largest or smallest values of coefficient of variation for all investigated species (Fig. 5).

3.2. Statistical analyses

The analysis of variance showed that all species were significantly determined by the length of the equatorial and polar axes, the exine thickness and the width of mesocolpium (Table 3). Least significant difference (LSD)

Table 3. The results of variance analysis of 7 dependent variables and factors for the studied *Linum* species; p < 0.001

Traits	F	p	partial eta-squared
Length of P axis	29.40653***	0.000000	0.431984
Length of E axis	29.79436***	0.000000	0.435202
P/E ratio	1.46467	0.227841	0.036497
Exine thickness	4.12368**	0.008082	0.096369
Width of mesocolpium	13.82885***	0.000019	0.633513
Number of exine processes	2.66025	0.071023	0.249549
Apocolpium diameter	1.37016	0.275759	0.146226

test and calculation of partial eta-squared revealed that the species were to the greatest extent explained by the width of mesocolpium and to the smallest – by P/E ratio.

Tukey's test for homogenous groups revealed that the lengths of P and E axes divided pollen of the investigated taxa into two inseparable groups – first with *L. hirsutum* and *L. perenne* subsp. *extraaxillare* and the other one with *L. flavum*, *L. austriacum* and *L. perenne* subsp. *extraaxillare*. The width of mesocolpium marked out two separable groups: one with *L. hirsutum*, *L. flavum* and *L. austriacum* and the other with *L. perenne* subsp. *extraaxillare*. The thickness of exine allowed identification of two inseparable groups: first with *L. hirsutum*, *L. flavum* andi *L. austriacum* and the other with *L. perenne* subsp. *extraaxillare*. According to other quantitative traits – P/E ratio, number of exine processes and apocolpium diameter, all species formed a homogenous group.

Correlation analysis revealed a positive relationship between lengths of P and E axes, and a negative one between P/E ratio and the length of E axis. Other traits were not significantly correlated (Table 4). The analysis of the percentage of explained variance revealed that the first principal component PC1 explained 30.05% of the observed variability and the other principal component PC2 elucidated 19.79% (Fig. 6). A strong correlation, positively associated with PC1 factor, was proved between the lengths of P and E axes. Furthermore, we discovered a weaker and a negative correlation between the length of E and P/E ratio.

Similarly, the dendrogram constructed on the basis of Euclidean distances for the analyzed pollen samples showed close similarity between *L. flavum* and *L. austriacum* (Fig. 7). The second distinct group consisted of *L. hirsutum* and *L. perenne* subsp. *extraaxillare*; however, linkages were distinctly larger than in case of the first pair.

4. Discussion

Pollen quality is an important component of plant reproductive success. Its assessment is critical for many

Table 4. The correlation matrix among 7 analyzed traits for all investigated *Linum* species

Trait	Length of P axis	Length of E axis	P/E ratio	Exine thickness	Width of mesocolpium	Number of exine processes	Apocolpium diameter
Length of P axis	1						
Length of E axis	0.93565***	1					
P/E ratio	-0.07906	-0.42093***	1				
Exine thickness	0.02008	-0.00964	0.07041	1			
Width of mesocolpium	-0.19519	-0.11845	-0.12479	0.09021	1		
Number of exine processes	0.34374	0.29187	0.05934	0.23239	-0.00825	1	
Apocolpium diameter	0.28539	0.34781	-0.15830	0.06977	0.24804	-0.25164	1

aspects of pollination biology, as in studies on genetics, pollen-stigma interaction, gene bank or pollen biochemistry (Cresti & Tiezzi 1992; Dafni & Firmage 2000). Until now, pollen viability studies within Linaceae family have only referred to commonly cultivated *L. usittatisimum* (Kumar *et. al.* 2013). According to Dufay *et al.* (2008), pollen viability is positively correlated with the size of pollen grains. Our study only partially confirmed this observation, since we recorded the lowest percentage of dead grains both in species with the largest (*L. perenne* subsp. *extraauxilare*) as well as with

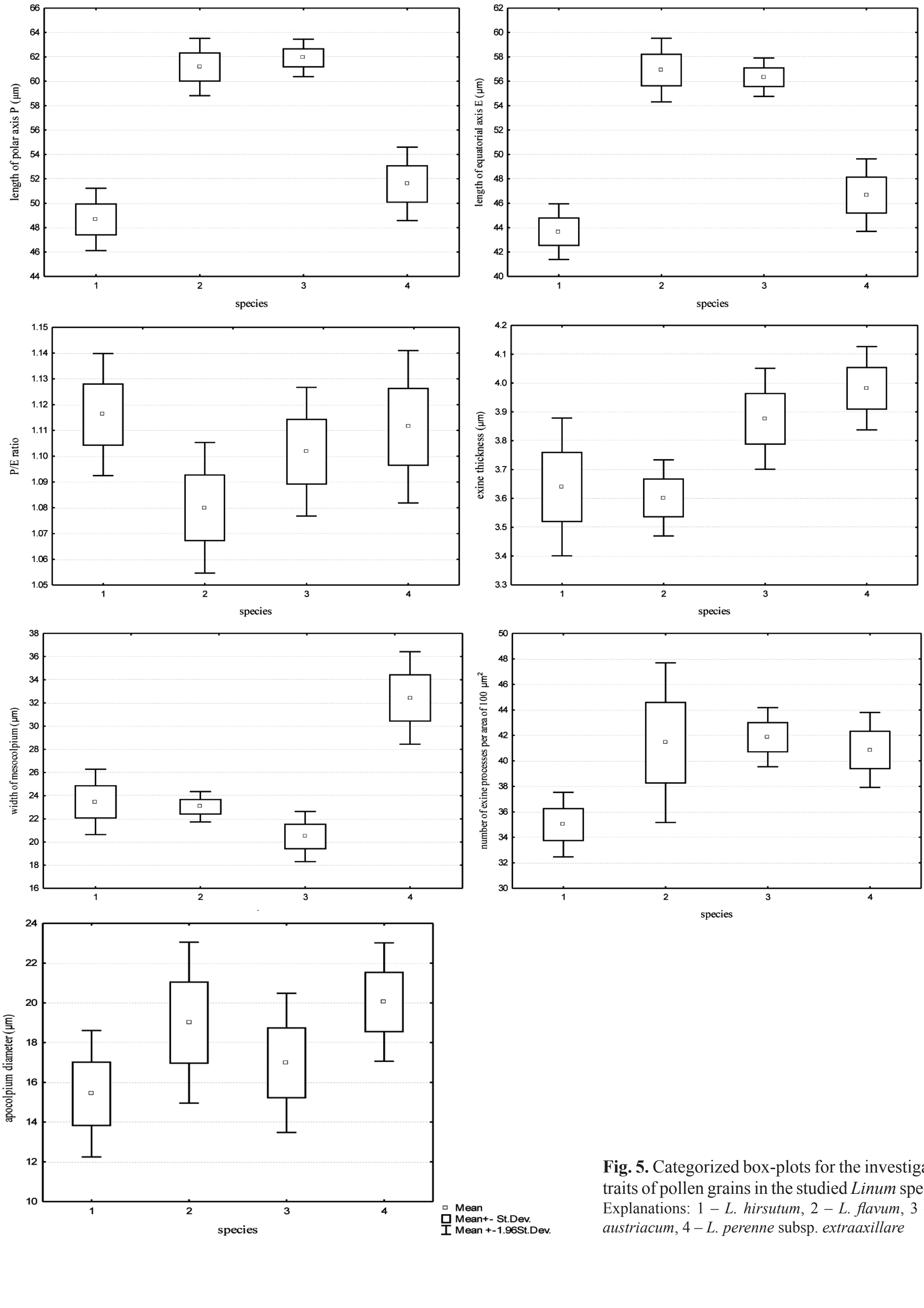

Fig. 5. Categorized box-plots for the investigated traits of pollen grains in the studied *Linum* species
Explanations: 1 – *L. hirsutum*, 2 – *L. flavum*, 3 – *L. austriacum*, 4 – *L. perenne* subsp. *extraaxillare*

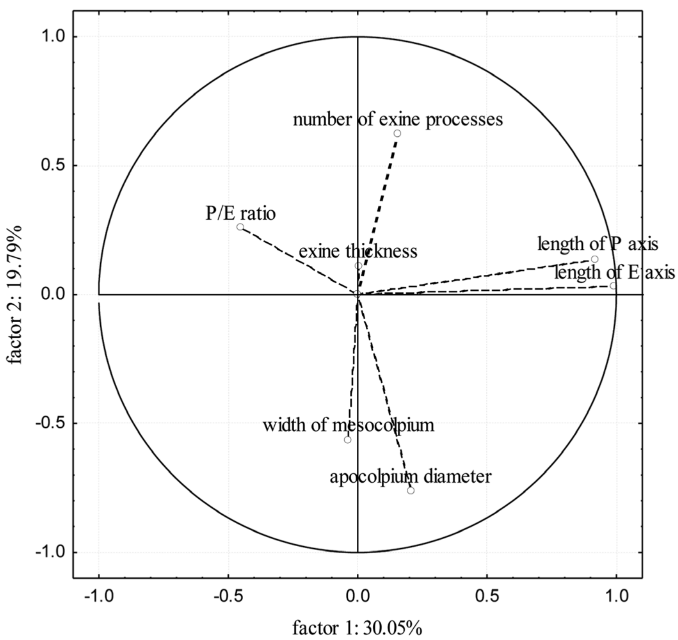

Fig. 6. Factor loadings of 7 analyzed traits of the studied *Linum* species

the smallest grains (*L. hirsutum*). In current research, however, all analyzed *Linum* taxa were characterized by very high viability of pollen grains.

Acccording to Punt & Den Breejen (1981), several *Linum* species are characterized by compound apertures. They described pollen grains of *L. austriacum* with well-marked lolongate endocolpi, longer and wider than ectocolpi. Besides, the authors considered endoaperture to be sometimes faintly visible in pollen grains of *L. flavum*. The same opinion was expressed by Chestler & Raine (2010), who analyzed pollen grains of *L. austriacum* and *L. flavum* from north-western Greece. However, those observations were not confirmed in any other study on these species (Grigoryeva 1988; Moroz & Tsymbalyuk 2005; Talebi *et al.* 2012). Similarly, we observed grains of *L. austriacum* and *L. flavum* with only simple apertures.

In our current work, all analyzed species were distylous. Hence, their pollen should be differentiated morphologically, depending on participation level of long- and short-styled specimens in a given population (Rogers 1980; Dulberger 1981). Based on the works carried out so far, the most distinct differences in pollen size were observed in *L. perenne* subsp. *extraaxillare,* where polar axis of the grains was up to 10 µm longer in long-styled specimens, than in those with short styles (Moroz & Tsymbalyuk 2005). However, Punt & Den Breejen (1981) assessed differences in the length of P axis between both forms of flowers of this species to be only about 5 µm. According to the cited authors, for the other three species, differences in P length between two forms of flowers did not exceed 3-6 µm. Furthermore, axis length ranges of both forms in all analyzed species often overlapped. Therefore, the size of pollen grains does not seem to be an unambiguous diagnostic feature. On the basis of literature, average length of P axes in all

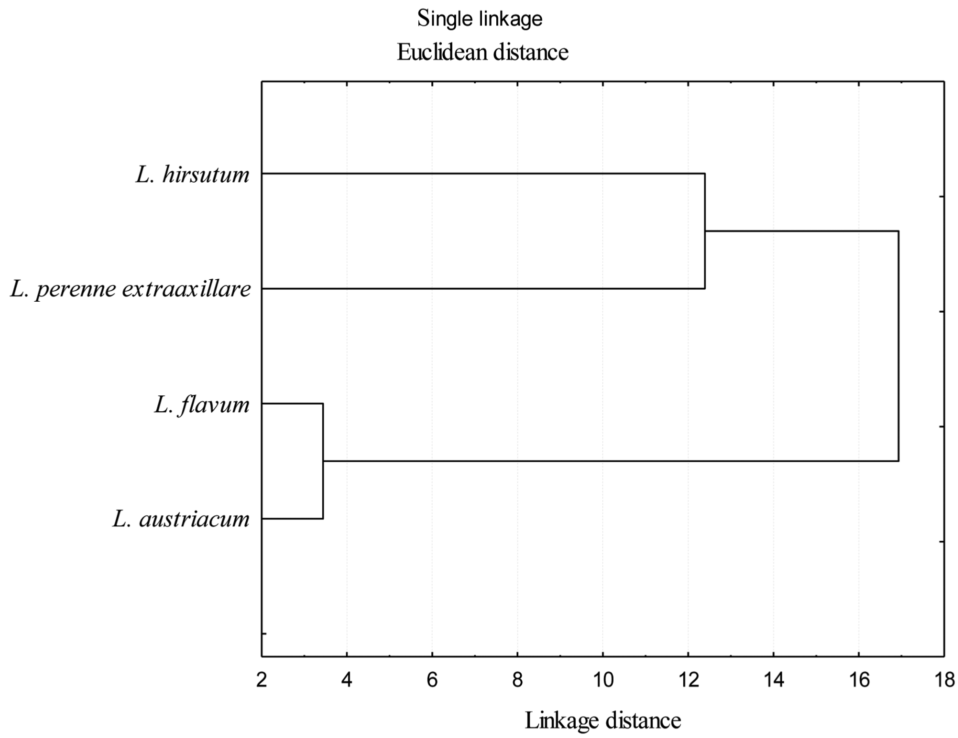

Fig. 7. Dendrogram constructed on the basis of the shortest Euclidean distances according to the single linkage method using 7 morphological traits of the studied *Linum* species

investigated species in our current work corresponded to pollen grains from long-styled flowers.

It is widely believed that heterostyly is also reflected in a variation of exine sculpture of pollen grains (Kuprianova & Alyoshina 1978; Rogers 1980). According to literature, this phenomenon was noticed in all currently analyzed species (Punt & Den Breejen 1980; Grigoryeva 1988). In our present study, we observed distinct diversity of exine sculpture among pollen grains in the samples of *L. hirsutum* and *L. austriacum*, while pollen samples of *L. flavum* and *L. perenne* subsp. *extraaxiliare* were morphologically uniform. Based on properties of exine processes, pollen grains of *L. flavum* corresponded to pollen of long-styled flowers and of *L. perenne* subsp. *extraaxillare* – to pollen of short-styled flowers.

In palynological literature, exine processes of *L. austriacum* are both described as gemmae and clavae, in *L. hirsutum* – as verrucae and clavae and in *L. perenne* s.l. – as bacula and gemmae (Erdman 1966; Perveen & Qaiser 2008; Chestler & Raine 2010). On the other hand, in studies on pollen of some *Linum* species from Eastern Europe, individual terms were used without direct reference to universally recognized palynological dictionaries (Grigoryeva 1988; Moroz & Tsymbalyuk 2005). On the basis of the current study, in pollen grains of the investigated species, we distinguished 2 types of exine processes: gemmae and clavae. Both exine elements were very similar in shape; however, they varied in size (Punt *et al.* 2007; Hesse *et al*. 2009).

The present study revealed some variation in pollen morphology of 4 *Linum* species. However, the investigated taxa exhibited many features in common, especially those related to the shape, amb, exine structure and apertures. Pollen of *L. hirsutum* was distinguished by smaller size and lower density of exine processes. Due to the sculpture, it resembled pollen of *L. austriacum* the most, but without distinct peripheral granules on the capita of exine processes. On the other hand, *L. perenne* subsp. *extraauxilare* was most similar to *L. flavum*, but its exine processes were shorter and took the form of gemmae (not clavae like in the other species). So, the current study revealed a small morphological similarity between two related species – *L. austriacum* and *L. perenne* subsp. *extraauxilare* – of the section *Linum.* This observation does not confirm Ockendon's opinion (1971), who, based on pollen morphology, distinguished *L. perenne* group consisting of very closely related species with, among others, *L. austriacum* and *L. perenne* subsp. *extraauxilare*.

This comprehensive study on pollen morphology of four *Linum* species is of practical importance for the use in dating fossil and subfossil materials from the area of Central Europe. More studies concerning differences in pollen in heterostylous species of the *Linum* genus in Poland are currently under way.

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