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CHARACTERIZATION OF FRUIT TREES POLLEN

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ABSTRACT
One of the options to determine botanical origin of trees or honey is the analysis of pollen grains. The characteristics of pollen grains in Czech flora has not been sufficiently described yet. Within this work, fruit trees pollen of Czech origin was characterized on the basis of morphological and spectral description of pollen grains produced by fruit species of *M. domestica, P. armenica, P. persica, P. domestica, P. avium* and *P. cerasus*. The morphological characterization results of the studied fruit species are consistent with results by other authors, but certain differences between the pollen grains of some fruit trees were confirmed. Most morphological differences were confirmed among the *Malus* and *Prunus genera*. Results of morphological and spectral analyzes further confirmed the differences between some types of fruit trees, but homogeneity remained for individual species even in mixed samples. Morphological and spectral analysis can therefore be used for botanical identification of pollen. If this knowledge is applied to pollen analysis in honey, these methods can also be used to verify the botanical origin of honey.

Keywords: spectral characteristic; image analysis; palynology; honey; authenticity; melissopalynology

INTRODUCTION
Pollen grains have their typical characteristics according to botanical plant species. Differences are described between plant species **(Evrenosoğlu and Misirli, 2009)** as well as between individual varieties **(Hebda and Chinnappa, 1994; Geraci et al., 2012)**. In their study, **Radice et al. (2004)** demonstrated morphological differences in the size of pollen grains in one Forastero variety of *P. persica* (L.) Batsch sp. grown on different rootstocks. Although there are certain differences between plant species and varieties, application of their morphological characteristics is significant in terms of research on plant pollination **(Kermani et al., 2003; Asma, 2008)** as well as in forensic diagnostics **(Milne et al., 2004; Morgan et al., 2014; Arguelles, Reinhard and Shin, 2015; Bell et al., 2016)**. In addition to suitability of palynology for forensic diagnostics, the above authors describe some factors (climatic, time period, temperature effects) which alter the morphological characterization of pollen grains to a certain extent; but even with regard to the morphological change, such pollen grains can still be identified and characterized. Pollen grains are also used for authentic honey demonstration – this scientific discipline is called melissopalynology. One parameter is the presence of pollen grains itself. Another option is qualitative or quantitative palynology **(Louveaux, Maurizio and Vorwohl, 1978)**. Morphology of pollen grains is also used to prove the geographical origin of honey **(Rodopoulou et al., 2017; Carreck, 2018)**.

Scientific hypothesis
This article presents fundamental morphological and spectral characteristics of fruit trees pollen and verifies suitability of the obtained characteristics for the creation of mathematical models to discriminate fruit species pollens from each other.

MATERIAL AND METHODOLOGY
For pollen analysis, fruit trees pollen from southern Moravia was collected in 2018. Pollen collection and processing was performed in compliance with methods for preparation of comparative preparations under the Czech standard norm **(ČSN 570190:1974)**. Pollen was collected from fruit trees, namely from apple trees, apricot trees, peach trees, cherry trees, plumb trees, and sour cherry trees (*M. domestica, P. armenica, P. persica, P. domestica, P. avium,* and *P. cerasus*). Mixed samples of all examined varieties were produced for each species. Description of fruit species included in the collection is shown in Table 1.

Morphological and spectral mesurement
Morphological and spectral parameters were evaluated for pollen grains. A specified number of pollen grains (n = 10) was examined. Scanning for both analyzes was performed using the Eclipse Ci microscope (Nikon, JPN). 60x/0.80 lens (Nikon, JPN) was used, real magnification for morphometry was 600x. The pollen grains were captured by DFK 23U274 camera (The Imaging Source, GB). Morphometry was performed using NIS Elements ver. 6.5 (Laboratory Imaging, CZE). The measured morphological

parameters included Area, EqDiameter, VolumeEqSphere, VolumeEqCylinder, Perimeter, Perimeter Contour, MeanChord, Length, Width, MaxFeret, MinFeret, MaxFeret90, Circularity, Elongation, Orientation, LineLength, ShapeFactor, Convexity, Roughness, RoughnessInf.

Spectral analysis was performed using the USB4000-UV-VIS-ES spectrophotometer (Ocean Optics Inc., USA). The spectrum within the range of 420-750 nm was analyzed.

Statistic analysis

Correlation analysis, regression analysis, ANOVA, factor analysis, Cochrane-Orcutt estimation, and normal homogeneity test were carried out using the XLSTAT software ver. 2014.5.03 (Addinsoft SARL, New York, NY, USA).

RESULTS AND DISCUSSION

Melissopalynology is a scientific area that is demanding even on experienced evaluators who are able to interpret the origin of honey through characterization of pollen grains. The analysis principle lies in counting the pollen grains and their categorization into species. Pollen analysis allows for both, the geographical and botanical origin determination of honey **(Von Der Ohe, 2004)**. Only a few organizations in the world usage melissopalynology for botanical and geographical honey identification. This is mainly due to the high demands of the method on human resources. Using new imaging techniques and primarily correlation microscopic techniques, however, it is possible to better characterize the pollen grains and then use the characteristic parameters for automatic or semi-automatic systems to determine the origin of honey. In order to determine the origin and the botanical source of honey, characterization of pollen grains in the given regions must be available. Nonetheless, characterization of pollen from Czech honey-producing plants is not available and subsequent diagnosis of the authenticity of honey is therefore difficult. Table 2 shows the obtained morphological characteristics of pollen grains of fruit trees in southern Moravia.

The morphological characteristics demonstrate that the length and width of *M. domestica* pollen grains is scattered in harmony with **Nazeri (2008) and Evrenosoğlu and Misirli (2009)**. These parameters for *P. armeniaca, P. domestica*, are consistent with the results published by **Evrenosoğlu and Misirli (2009)** as well as **Geraci et al., (2012)**. Length of *P. persica* pollen grains is in line with **Evrenosoğlu and Misirli (2009)** and with the Spaccareli variety described by **Geraci et al. (2012)**, but the width is out of their reported range. On the other hand, our results are also consistent with the study **(Radice et al., 2004)** comparing diamether of pollen grains of forastero on various rootstocks. The diameter in this study was compared to the max Feret diameter which is defined as the distance between two parallel tangential lines rather than planes. Therefore, differences in pollen grains of Turkish varieties compared to Czech varieties can be expected. This finding can also be used to determine the geographical origin of honey. *P. Avium* described in an Italian study **(Geraci et al., 2012)** is in line with the Czech varieties. Length of *P. cerasus* pollen grains was in agreement with the study by **Sótonyi et al. (2000)**, in contrast to the width and diameter that did not overlap within the variance of the measured values in most of the studied varieties, except for the Germersdorfi óriás variety. Besides the different size of pollen grains of individual botanical species of fruit trees, certain differences between individual varieties were also confirmed. Pollen grain differences can therefore be utilized to identify pollen origin used in both forensic diagnostics **(Milne et al., 2004; Morgan et al., 2014; Arguelles, Reinhard and Shin, 2015; Bell et al., 2016)** as well as in melissopalynology **(Von Der Ohe, 2004)**. The characteristic microimages of fruit tree pollen grains are shown in Figure 1.

According to our measured results, some botanical species of fruit trees can be distinguished from each other based on the measured parameters (Table 2). The most diverse morphological parameters were found among the *Malus* genus (*M. domestica*) compared to *Prunus (P. domestica, persica, and cerasus*). The Malus genus was statistically significantly different from some prunus specimens in the parameters of length, circularity, convexity, perimeter, line length, roughness, max. feret, and perimeter contour (p <0.05). Cylindrical and tricolporate shape of *M. domestica* was in agreement with **Evrenosoğlu and Misirli (2009)** and **Nazeri (2008)**. Even with regard to identical shape of *P. persica* pollen grains **(Evrenosoğlu and Misirli, 2009)** described by several authors, a statistically significant difference in the circularity parameter was found between *M. domestica* and *M. Persica*. This result may be due to the high shape variability of *P. Persica* pollen **(Sótonyi et al., 2000)** or due to the transverse pollen grains position during image capturing. The most common morphological differences were reported between *M. domestica* and *P. domestica*. Differences can be attributed in particular to the shape characteristics that are described for *P. domestica* as triangular-obtuse-convex in the polar view and elliptic-acuminate-acute in the equatorial view **(Ćalić et al., 2013)**. The possibility to distinguish between *P. domestica and M. domestica* is an important finding especially of the great morphological variability of *P. domestica* **(Geraci et al., 2012)**.

A statistically significant difference was also demonstrated between *M. Domestica and P. cerasus* in the convexity parameter (p <0.05). A statistically significant difference in only one parameter can be attributed to the low homogeneity of *P. cerasus* pollen grains **(Anvari, 1977)** which is in morphological characteristic in ranges between 78 – 91% **(Bolat and Pirlak, 1999)**. Morphological differences, however, were also demonstrated within the *Prunus* genus. Primarily between *P. persica and P. domestica* in the parameters of area, perimeter, line length, max feret, min feret, max feret 90, eq diameter, perimeter contour. and volumetric parameters of volume eq sphere and volume eq cylinder. This result is in line with **Evrenosoğlu and Misirli (2009)** who described the shape of *P. persica* and *P. domestica* as cylindrical and tricolporate in our study best characterized by the parameter of circularity and convexity where no statistically significant difference was found.

Table 1 Characteristics of the analyzed samples of fruit trees.

Species	***Malus domestica* (Borkh.)**	***Prunus armeniaca* (L.)**	***Prunus persica* (L. Batsch)**	***Prunus domestica* (L.)**	***Prunus avium* (L.)**	***Prunus cerasus* (L.)**
Variety	Biogolden (Czech breed)	Leskora (Czech breed)	Red Haven	Wangenheim (GER import)	Hurlat (Czech breed)	Érdi Bötermö (Hungary breed)
Rootstock	A-2, reg. no. 584	M-VA-2, reg. no. 584	B-SB-1, reg. no.:1142	Mirobalan, reg. no.:1142	Mahaleb, reg. no.: 584	Mahaleb, reg. no.: 584
Variety	Goldstar	Harogem (Canada import)	Suncrest (Czech breed)	Althanova	Kordia (Czech breed)	Újfehértoi FürtösS (Hungary breed)
Rootstock	M25, reg. no.: 1216-09/17	B-VA-2 reg. no.: 1142	BM-VA-1, reg. no.: 584	Myrobalan, reg. no.: 584	Mahaleb, reg. no.: 584	MAN25, reg. no.: 1061
Variety	Rubin	Lebela (Czech breed)	Flamingo (Slovak breed)	Tuleu gras (RO import)	Horka (Czech breed)	Moravia (Czech breed)
Rootstock	B-VA-2 reg. no.: 1142	M-VA-2, reg. no. 584	B-SB-1, reg. no.:1142	Myrobalan, reg. no.: 584	Mahaleb, reg. no.: 584	Mahaleb, reg. no.: 584
Amount of nectar mg	1.12	1.19	1.65	3.4	1.9	N/A
Nectar sugar content %	41	27	38	13	29.9	N/A
Nectar sugar amount mg / 24hr	0.45	0.32	0.63	0.44	0.57	N/A

Table 2 Morphological characteristics of fruit trees pollen.

	Length	SD	Width	SD	Circularity	SD	Elongation	SD	ShapeFactor	SD	Convexity	SD	Area	SD	Perimeter	SD	LineLength	SD	Roughness	SD
M. domestica	32.17[a]	8.88	22.78	7.20	0.92[a]	0.08	1.12	0.09	0.97	0.03	0.99[a]	0.01	783.36	453.52	101.05[b]	34.05	50.91[a]	17.20	0.97[a]	0.04
P. armeniaca	40.53	11.78	21.87	4.33	0.76	0.09	1.14	0.05	0.93	0.04	0.97	0.01	922.48	366.36	122.25	33.23	60.95	16.35	0.90	0.05
P. persica	36.00	14.38	17.11	3.30	0.73[b]	0.17	1.08	0.03	0.93	0.05	0.95	0.03	594.28[a]	177.69	103.53[b]	27.41	51.46[a]	12.64	0.87	0.09
P. domestica	55.15[b]	17.00	22.45	6.94	0.64[b]	0.17	1.14	0.07	0.91	0.05	0.95[b]	0.02	1170.39[b]	331.58	154.08[a]	28.02	77.55[b]	14.18	0.83[b]	0.11
P. avium	44.11	20.66	20.78	5.03	0.73	0.19	1.12	0.06	0.95	0.05	0.97	0.01	879.60	300.81	126.58	40.69	63.56	21.00	0.87	0.12
P. cerasus	48.89	12.15	19.97	3.00	0.67	0.14	1.15	0.08	0.91	0.06	0.95[b]	0.03	952.69	131.62	136.09	21.86	68.38	11.70	0.85	0.07

	MaxFeret	SD	MinFeret	SD	MaxFeret90	SD	Orientation	SD	RoughnessInf	SD	EqDiameter	SD	VolumeEq Sphere	SD	VolumeEq Cylinder	SD	Perimeter Contour	SD	MeanChord	SD
M. domestica	32.67[a]	9.71	29.12	8.65	29.82	8.86	77.00	52.91	1.03	0.04	30.51	8.82	18210.5	15414.1	12733.7	11625.1	101.05[a]	34.05	22.78	5.59
P. armeniaca	37.32	9.75	32.66	7.82	33.21	8.04	53.80	60.11	1.11	0.06	33.51	8.05	22174.6	11083.0	13820.5	6805.7	120.43	32.08	22.71	4.88
P. persica	29.37[a]	5.05	27.16[a]	4.58	28.10[a]	4.82	94.75	47.26	1.16	0.13	27.21[a]	4.31	11226.4[a]	4742.0	6572.5[a]	2437.2	101.93[a]	24.80	17.94	2.73
P. domestica	42.77[b]	5.64	37.75[b]	5.63	38.69[b]	6.26	102.20	56.49	1.23	0.17	38.26[b]	5.45	30929.9[b]	13076.4	18029.6[b]	8940.3	154.08[b]	28.02	23.90	5.21
P. avium	35.81	6.21	32.19	6.17	32.92	6.34	69.80	54.24	1.17	0.20	32.99	5.91	20407.7	10091.0	12073.0	5122.2	126.58	40.69	21.71	3.57
P. cerasus	39.25	4.79	34.22	2.95	35.53	2.94	85.83	56.40	1.18	0.09	34.76	2.46	22254.2	4490.4	12684.1	2097.5	134.97	23.19	22.11	1.99

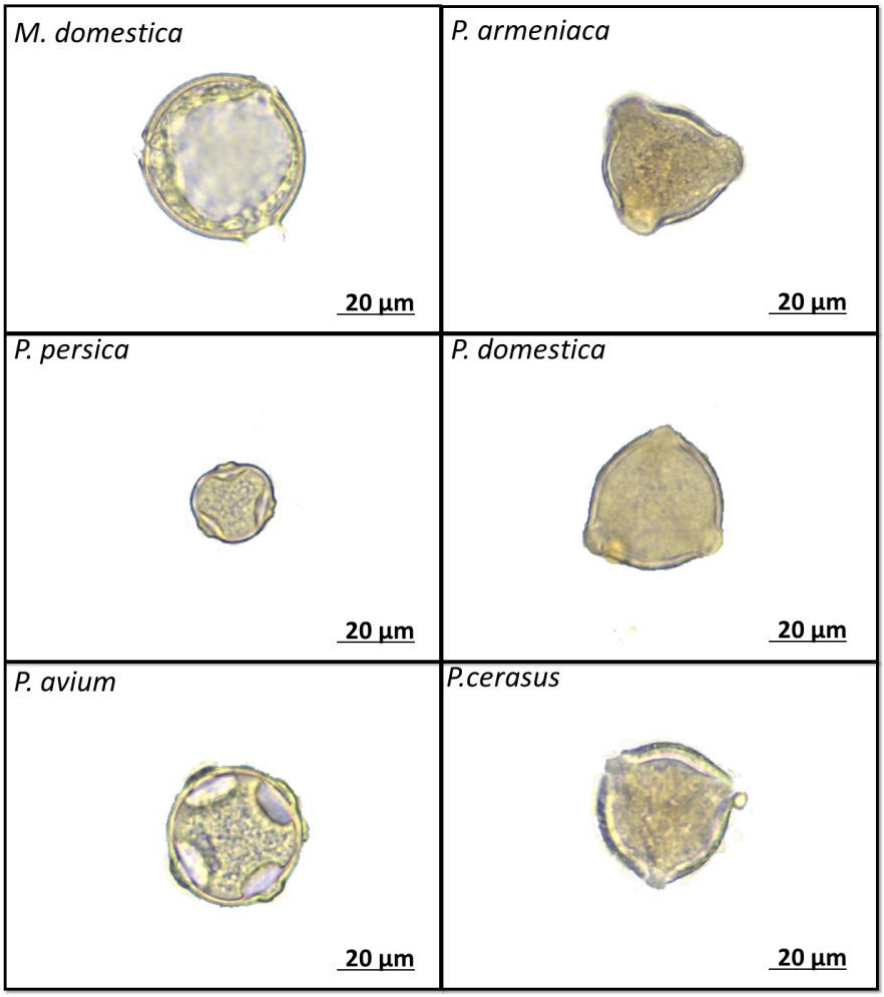

Figure 1 Pollen shape in parallel view.

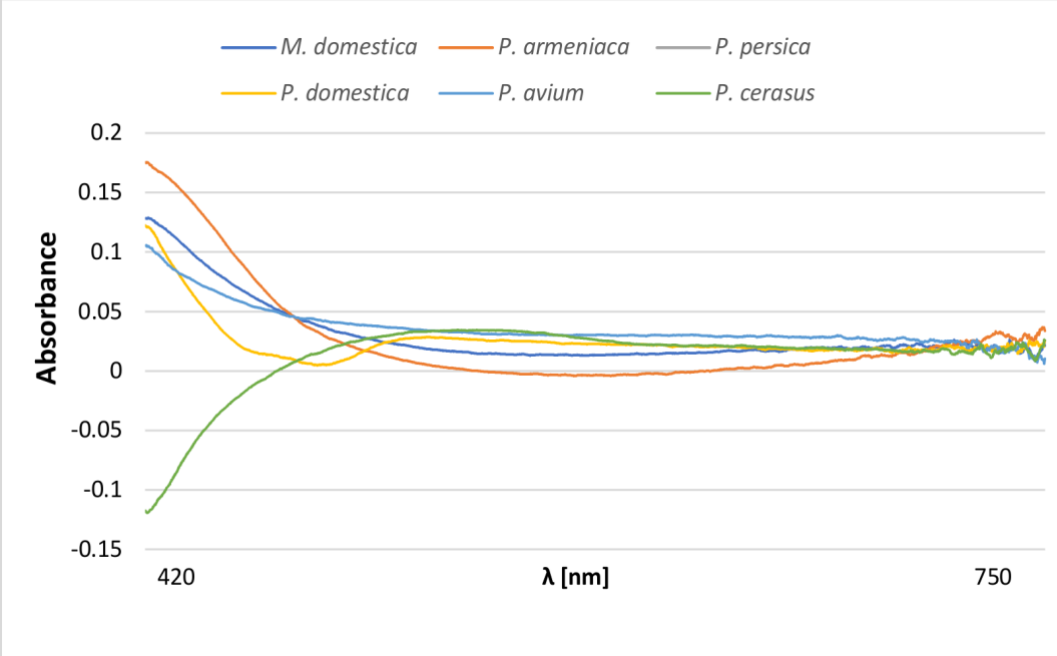

Figure 2 UV-VIS spectra plot of fruit tree species.

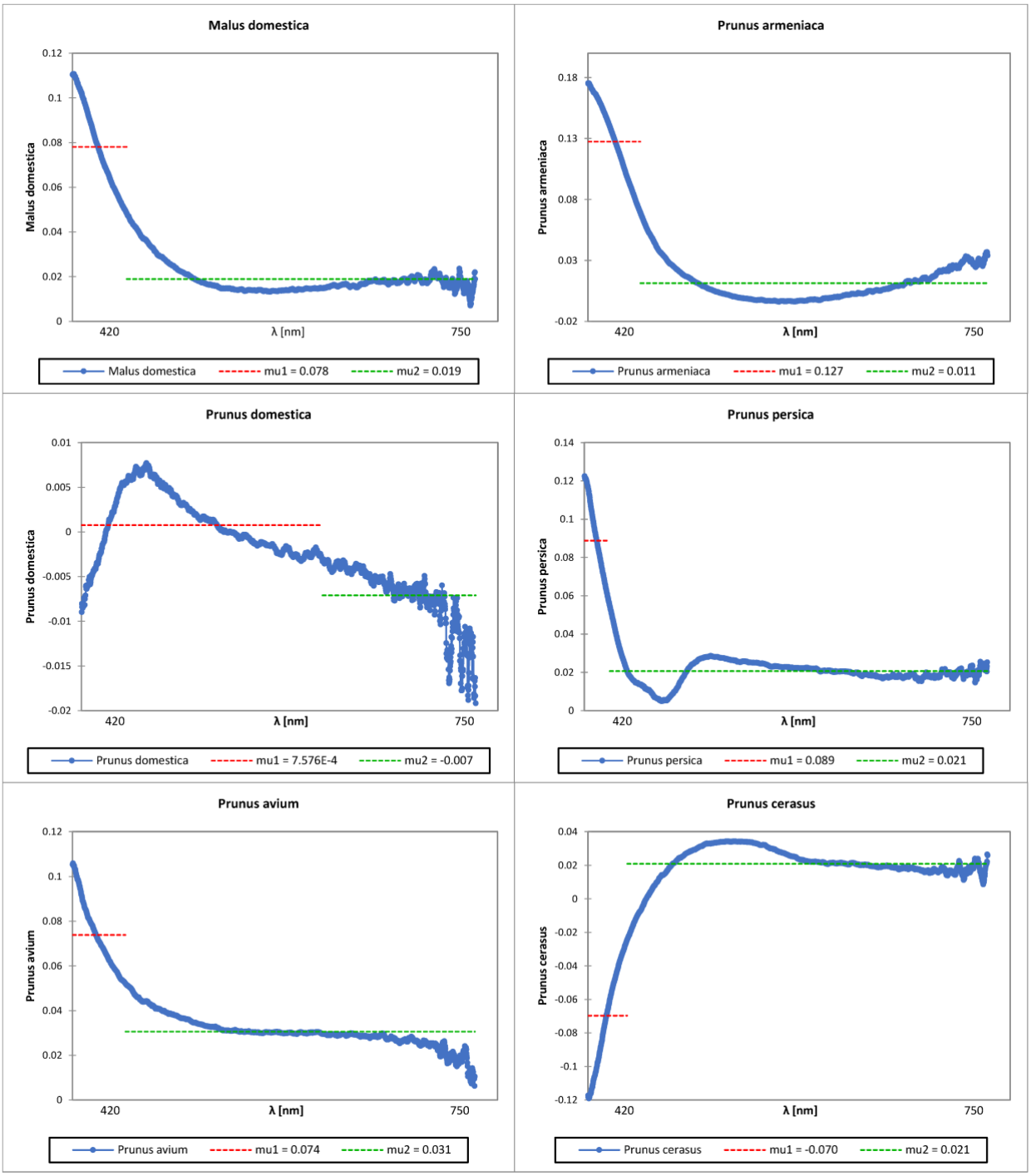

Figure 3 Standard normal homogeneity test (p <0.05).

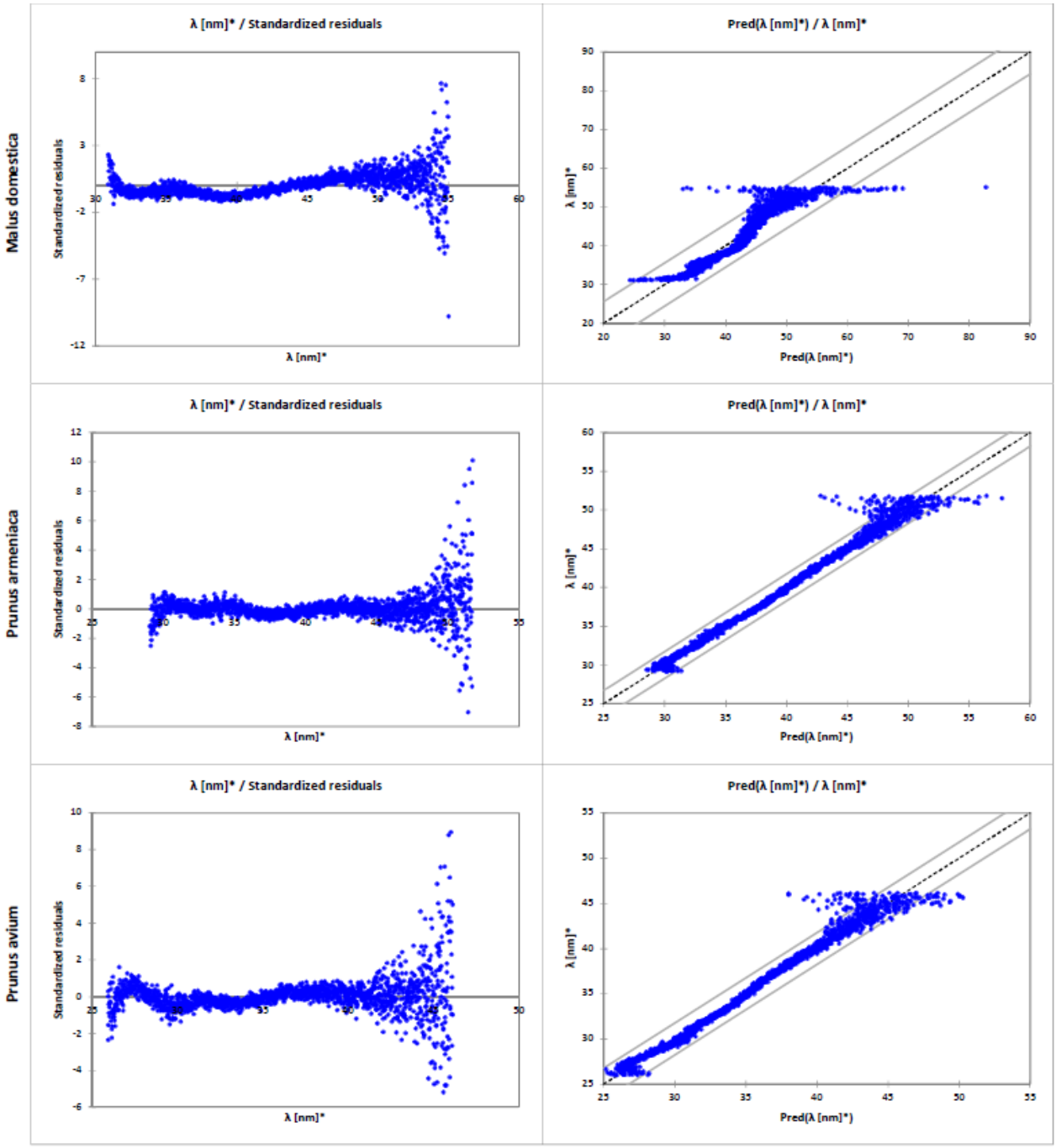

Figure 4 Cochrane-Orcutt prediction model of fruit tree pollen.

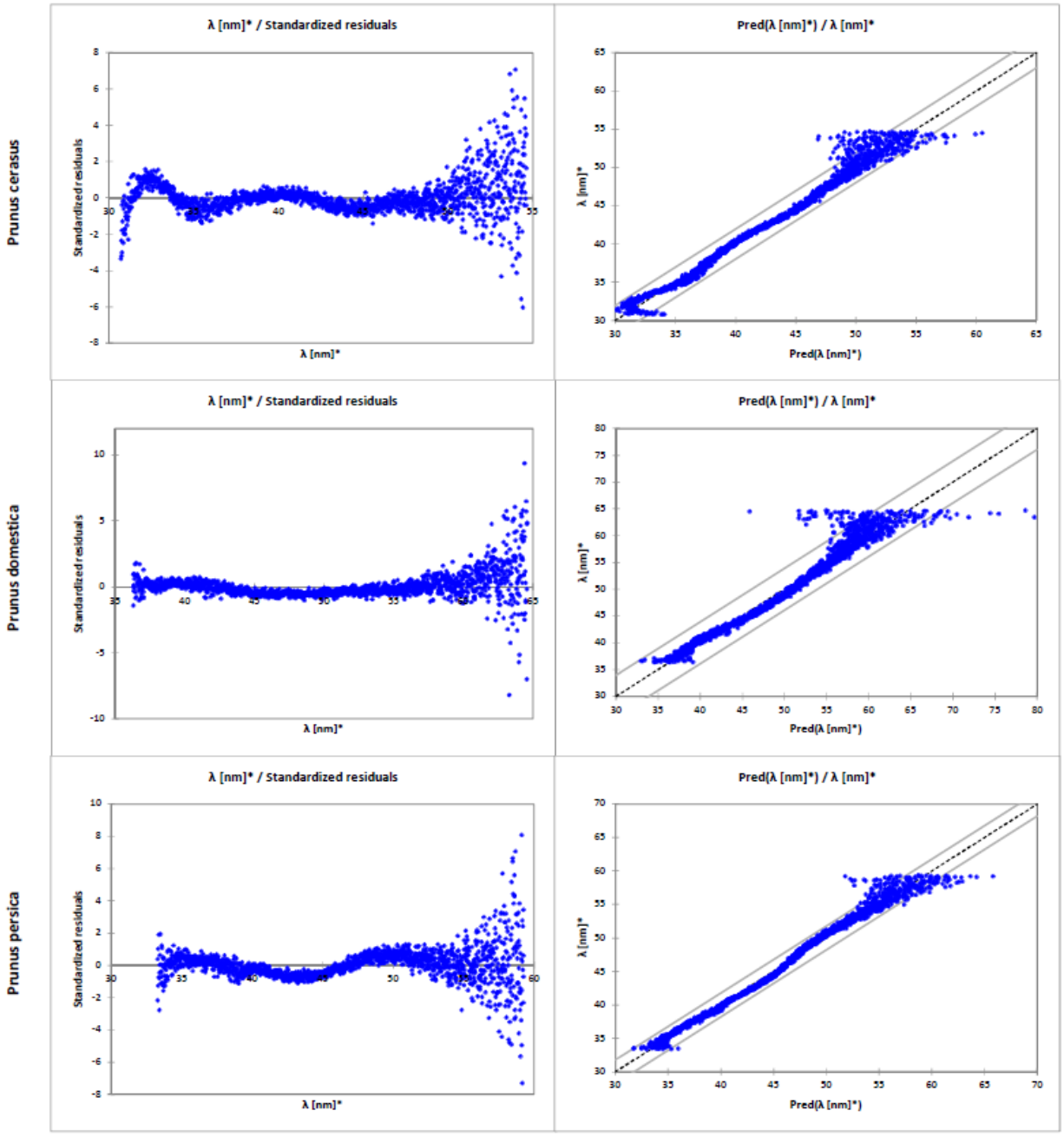

Figure 5 Cochrane-Orcutt prediction model of fruit tree pollen.

Although there was a statistically significant difference (p <0.05) in the min, max feret and max feret 90 parameters, which indicates that there are shape differences between the species, the pollen grains are rather irregular bodies and therefore they are better described by feret parameters that are more suitable for the description of irregular bodies than simple circularity or convexity. Circularity and convexity are values calculated of the object circumference and any roughness skews the results. Further, **Evrenosoğlu and Misirli (2009)** report differences in pollen grain surface which could also be reflected in the parameters we measured, namely in the perimeter and perimeter contour, indicating that the *P. domestica* surface (154.08 and 154.08) is more wrinkled than that of *P. persica* (103.53 and 101.93).

The statistical differences found within the *Prunus* genus are in agreement with **Wrońska-Pilarek and Jagodziński (2011)** who confirmed that the morphology of pollen grains is an important feature for distinguishing species within the Rosaceae family where the *Prunus* genus belongs. Taking into account the large number of morphological parameters described in the work, factor analysis of morphological parameters was also made, which outlined the most different morphological criteria of max feret 90 and circularity (Factor 1). Volumetric factors are preferably excluded with respect to the unequal longitudinal and transverse shape of the pollen grains, which could interpret the result with great error. As a secondary factor (Factor 2), roughness (convex hull perimeter/perimeter) and roughness inf (1/roughness) were selected. Given the reversed value of these two factors, in case of reducing the number of the

measured factors, it would be preferable to take a factor with lower correlation into account, namely the shape factor (R = -0.06 for Factor 1 and R = -0,21 for Factor 2, respectively). Factor analysis further showed that Factor 1 represents 58.9% and Factor 2 represents 24.7% of the decision criterion.

On the other hand, shape characteristics are not the only parameter that can be used to discriminate pollen of fruit species from each other. Another option is to evaluate the spectral characteristics of pollen grains. With conventional spectroscopic techniques, color evaluation within the visible spectrum of light can be considered. However, with regard to the availability of spectrophotometric techniques, it is not a problem to analyze also regions outside of visible spectrum of light. An important fact is that spectral analysis, especially in UV and NIR, also provides information about natural substances contained. In the UV-VIS region, spectral characteristics are often used to evaluate the content and amount of polyphenols **(Paradiso et al., 2016)**. Polyphenols represent a large group of natural photoactive substances presented throughout the plant kingdom. Their color ranges from red to purple and is determined by the number of OH-phenol groups reaching from 100 to 3000 daltons **(Anouar et al., 2012)**. Polyphenols achieve maximum absorption primarily in the UV region, because they are designed specifically to protect against UV light. In addition to OH groups, OCH_3 and glycoside groups are likewise responsible for UV absorption **(Harborne and Mabry, 2013)**. Though, the content of polyphenols is not represented only in vegetative plant forms. Polyphenols are also present in generative parts including pollen **(Serra Bonvehi et al., 2001; Almaraz-Abarca et al., 2007; Tian et al., 2007; Rzepecka-Stojko et al., 2015)**. In view of the yet unknown presence of polyphenols in pollen from Czech plants, the spectral analysis was used as a fingerprint methods. UV-VIS fingerprinting has been successfully used, for example, to compare grapes of red varieties in the spectral range of 160 – 600 nm **(Pop, Babeş and Bunea 2008; Casale et al., 2010)**. Average spectrum of fruit trees pollen is shown in Figure 2.

Figure 2 shows that individual UV-VIS spectra of fruit tree pollen differ from each other. Differences are mainly due to the different content of polyphenols **(Almaraz-Abarca et al., 2004)**. Differences were not observed only among the different types of fruit trees. Relatively large variability was also demonstrated between individual pollen grains. The variability is given both by differences in the pollen grains of individual flowers, as well as between cultivar variability **(Daoud et al., 2015)**. The UV-VIS absorbance variability of individual pollen grains is shown in Figure 4 and Figure 5. The Cochrane-Orcutt model was used to predict variability of the spectrogram, predicting the probability of occurrence of values in the 95% interval for further measurements. The chart illustrates that abnormal number is outside the interval only in certain wavelengths. Even considering a rather small group of pollen used for the analysis, it can be assumed that the whole spectrum analysis is a suitable method to determine botanical origin of pollen. Conformance of spectrograms in individual fruit species was also demonstrated by linear regression *M. domestica* (R^2 = 0.84), *P. armeniaca* (R^2 = 0.98), *P. persica* (R^2 = 0.99), *P. domestica* (R^2 = 0.94), *P. avium* (R^2 = 0.98), *P. cerasus* (R^2 = 0.98).

Abnormal numbers outside the interval were observed only in low and the highest wavelengths. It can be assumed that interference with glass optics used in the microscopes occurs particularly in the upper wavelength range. The measured absorbance was also tested for homogeneity within wavelengths. The test results showed that the absorbance across the wavelength spectrum is not homogeneous (p <0.05). Differences between individual varieties of fruit tree pollen were demonstrated (Figure 3).

The homogeneity test showed similarity between species of *M. domestica* (λ = 466), *P. armeniaca* (λ = 465), P. *persica* (λ = 441),), *P. avium* (λ = 465), *P. cerasus* (λ = 453). It can be assumed that the reason is a high content of polyphenols having a maximum absorbance between 250 – 410 nm. In the 270 – 280 range, phenolic acid and flavanones are involved **(Pop, Babeş and Bunea 2008; Anouar et al., 2012)**, in the 300 – 410 nm range, flavones and flavonols are involved **(Anouar et al., 2012)**. For *P. domestica*, the absorbance of the homogeneity test was different from the wavelength (λ = 620) which indicates a different representation of polyphenols. It can be assumed that *P. domestica* pollen contains a rather large amount of anthocyanins. Anthocyanins have absorbance between 520 – 546 **(Pop, Babeş and Bunea, 2008; Anouar et al., 2012)**. The above data show spectral variability between the pollen grains of the individual fruit trees, with variations within cultivars, and individual pollen grains are within the framework of predictive models. The spectral analysis can therefore be applied for fingerprinting of pollen grains similarly to studies performed on other analytes within the UV-VIS spectroscopy **(Pons, Le Bonté and Potier, 2004; Van Den Broeke, Langergraber and Weingartner, 2006; Pop, Babeş and Bunea, 2008; Casale et al., 2010; Zavoi et al., 2011)**.

CONCLUSION

Based on the morphological criteria of pollen grains, individual species of fruit trees can be discriminated from each other. Morphological differences of pollen grains within the *Prunus* genus are smaller. Comparison to the literature reveals that the morphological characteristics of pollen grains of fruit trees in the Czech Republic do not differ significantly from other countries. Exceptions are *P. persica, P. avium, and P. cerasus*, where Czech varieties can be assumedly distinguished from foreign varieties of these fruit trees based on the morphological characteristics. The Cochrane-Orcutt prediction model shows that variability in microspectrophotometric measurements of individual species within the wavelength range of 230 – 780 nm is low, but variations were found between individual spectrograms. It can therefore be assumed that the microspectrophotometric method can be used as a fingerprinting method to identification of pollen grains. It was also confirmed in the work that the pollen of individual fruit trees has a different content of photoactive substances, most likely polyphenols. Based on these results, it was confirmed that microspectrophotometric analysis is a suitable complementary method for determining botanical origin of pollen and, together with the morphological criteria, it can also be used as a fingerprinting method to determine botanical origin of honey based on pollen identification.

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