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Chemical Characterization and Classification of Pollen

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We report on the in situ characterization of tree pollen molecular composition based on Raman spectroscopy. Different from purification-based analysis, the nondestructive approach allows (i) to analyze various classes of molecules simultaneously at microscopic resolution and (ii) to acquire fingerprint-like chemical information that was used for the classification of pollen from different species. Hierarchical cluster analysis of spectra from fresh pollen samples of 15 species partly related at the genus level and family level indicates separation of species based on the complete Raman spectral signature and yields classification in accord with biological systematics. The results have implications for the further elucidation of pollen biochemistry and also for the development of chemistry-based online pollen identification methods.

The chemical composition of pollen, the physiological containers that produce the male gametes of seed plants, has been a subject of research of plant physiologists, biochemists,[1,2] and lately even materials scientists[3,4] for various reasons. In spite of a multitude of applied analytical approaches it could not be elucidated in its entirety yet. Nevertheless, it has become clear that pollen grains, owing to their biological function, consist of a broad variety of molecules. These range from an extremely stable biopolymer of largely unknown composition and structure, the sporopollenin of their outer layer, to flavonoid and carotenoid pigments, lignin, pectin, and the constituents of the cells in the pollen interior, namely, proteins, lipids, carbohydrates, and nucleic acids.[1,2,4,5] Many phenomena related to pollen are not well understood yet, among them the composition of the aforementioned sporopollenin capsules and the local distribution of molecules. To date, approaches to pollen chemical characterization involve purification, the latter implying possible modification or extraction of selected pollen constituents (allergens, carotenoids, lipids).[6,7]

Different from identification of other biological entities, e.g., in bacterial typing, current pollen identification does not rely on molecular parameters but mainly on morphological information obtained through optical or electron microscopies. Morphological information from light microscopy and image analysis is also the basis of current allergy warning systems. In the light of the urgent need for more efficient pollen identification and warning systems due to an increased prevalence of pollen allergies,[8] a fast identification method based on objective chemical information is needed.

As we report here, individual fresh pollen grains and their morphological constituents can be characterized and also classified in situ without prior preparation. Classification of pollen is based on their biochemical fingerprint revealed in their Raman spectrum. Raman spectroscopy is nondestructive and can be carried out with single pollen grains or fragments. In this inelastic scattering process, photons from a laser source interact with the molecular vibrations of a sample, yielding scattered photons with a changed energy. The energy difference corresponds to the energy of the molecular vibration the photons interacted with. The resulting spectra provide superimposed vibrational information on structure, composition and interactions from all classes of molecules.[9,10] Thereby, information obtained in Raman spectra of pollen naturally integrates the genomic, proteomic, and metabolic status of the tissue. Other types of complex biological materials that have been studied by Raman spectroscopy include prokaryotes, fungi, animal cells, and foodstuffs.[11−18]

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Table 1. Tree Species Analyzed in This Study, Together with Their Respective Plant Family and the Number of Samples

species		family	no. of samples
sallow	*Salix caprea*	*Salicaceae*	5
silver birch	*Betula pendula*	*Betulaceae*	7
hophornbeam	*Ostrya carpinfolia*	*Betulaceae*	8
ash leaf maple	*Acer negundo*	*Sapindaceae*	6
Norway maple	*Acer platanoides*	*Sapindaceae*	5
sycamore maple	*Acer pseudoplatanus*	*Sapindaceae*	5
horse chestnut	*Aesculus hippocastanum*	*Sapindaceae*	6
sycamore tree	*Platanus acerifolia*	*Platanaceae*	4
wild cherry	*Prunus avium*	*Rosaceae*	4
bird cherry	*Prunus padus*	*Rosaceae*	4
pedunculate oak	*Quercus robur*	*Fagaceae*	13
northern red oak	*Quercus rubra*	*Fagaceae*	5
large-leaved linden	*Tilia platyphyllus*	*Malvaceae*	5
tree of heaven	*Ailanthus altissima*	*Simaroubaceae*	9
silverberry	*Eleagnus angustfolia*	*Elaeagnaceae*	5
	total number of samples:		91

MATERIALS AND METHODS

Sample Preparation. Pollen of 15 different species were collected during their flowering season. Their full names, plant family, and the number of samples that were used are listed in Table 1. These pollen samples were snap-frozen in liquid nitrogen and stored at −20 °C. We considered snap-freezing the method of choice if a sample is to be studied unaltered. Morphological structures are fully preserved due to prevention of ice crystal formation, and also the majority of important biological molecules including sensitive species such as RNA should be preserved. The pollen grains were snap-frozen within very short times upon removal from their flowers. At a time 45 min prior to the Raman experiments, the samples were thawed in a desiccator on CaF_2 slides to avoid accumulation of condensing water and possible hydration.

Rye pollen was purchased from Sigma, mounted for cryosectioning in a drop of Jung Tissue Freezing Medium (Leica Microsystems, Germany) and snap-frozen. After equilibration to −20 °C, cryo-sections were cut at a thickness of 10 μm using a cryostat (Leica, Germany). The sections were thaw-mounted onto polished CaF_2 crystals for Raman mapping experiments.

Raman Spectra. The measurements were performed using a LabRam HR800 Raman setup (Horiba Jobin Yvon, Bensheim, Germany). A BX41 microscope (Olympus, Hamburg, Deutschland) with a 100× objective was coupled to the 800 mm focal length spectrograph. To reject the Rayleigh scattered light, notch filters were used. The spectrometer was equipped with a nitrogen-cooled CCD (1024 × 256 pixels, Horiba). For excitation, a HeNe laser (Horiba Jobin Yvon, Bensheim, Germany) at 633 nm and a diode laser (Toptica, Germany) providing 785 nm were used. The laser power on the samples was 10 and 18 mW at a spot diameter of ~1 μm, corresponding to $\sim 1 \times 10^6$ W/cm^2 and 1.8×10^6 W/cm^2 irradiance, respectively. The 633 nm laser was also used for

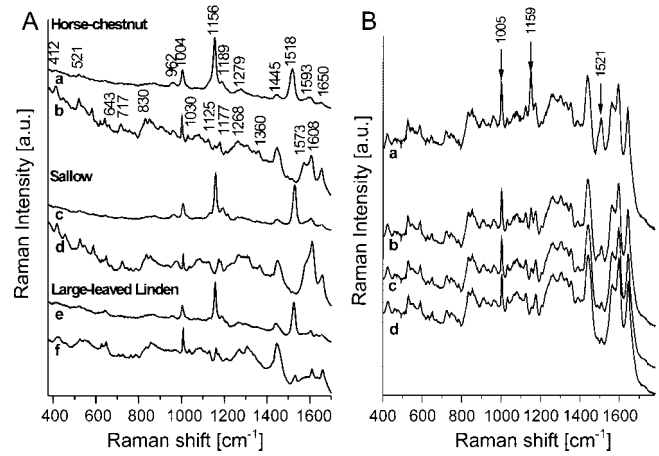

Figure 1. Raman spectra of tree pollen. (A) Spectra of pollen from horse-chestnut (a,b), sallow (c,d), large-leaved linden (e,f) before irradiation with laser light of 633 nm wavelength for photodestruction of carotenoids (traces a,c,e) and after 1 h photodestruction with 633 nm. All spectra were excited with 785 nm, 10 s accumulation time, and laser power of 18 mW. (B) Series of spectra of pollen from horse-chestnut, indicating the decomposition of β-carotene during irradiation with 633 nm laser light. The spectra were taken during the decomposition process at 20 min intervals at (a) 10, (b) 30, (c) 50, and (d) 70 min exposition time. Accumulation time was 10 s, excitation wavelength 633 nm, 10 mW laser power (~1 × 10^6 W/cm^2) on the sample.

defined decomposition of β-carotene, and depletion in carotenoid content was verified by the change in the Raman spectrum excited with 633 nm. Decomposition could be monitored by the decrease of the ν_1 carotenoid signal around 1520 cm^{-1}(compare Figure 1B). Raman spectra used for classification of the pollen were acquired over the range 350−1900 cm^{-1} using an integration time of 10 s per spectrum with 785 nm excitation after irradiation with laser light from a HeNe laser (633 nm).

Spectra from the rye pollen sections were acquired in mapping measurements where the sample stage was moved with respect to the microscope objective with a defined step width in the x and y directions. The diameter of the sampled spots was ~1 μm, step width was 2 μm in each direction, except in one measurement where it was 1.5 μm in x and 3 μm in the y direction. At each position in the predefined grid, a Raman spectrum was measured using an excitation of 785 nm, 18 mW laser power, and an acquisition time of 10 s.

Data Analysis. Prior to further analysis, the Raman shift axes of the spectra were calibrated using the Raman spectrum of 4-acetamidophenol. Pretreatment of the raw spectra, such as vector-normalization and calculation of derivatives, was done using Matlab (The Mathworks, Inc.) or OPUS (Bruker) software. OPUS NT software (Bruker, Ettlingen, Germany) was used to perform the hierarchical cluster analysis (HCA). For HCA, the first derivatives of the spectra were used over the range from 380 to 1700 cm^{-1}. To calculate the distance matrix, Euclidean distances were used, and for clustering, Ward's algorithm was applied.[19] Chemical imaging was done using CytoSpec (CytoSpec Inc., Boston). For the plots of the intensities as a function of spatial coordinate, after baseline correction, the areas under the respective bands were calculated.

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Table 2. Raman Frequencies Observed in Spectra of Pollen and Their Tentative Assignments to the Classes of Molecules and/or Vibrational Modes (Compare Figure 1)

Raman shift (cm^{-1})	tentative assignment [a,b]
1661	U, C, G, C=O stretch
1650	protein backbone, amide I
1608	Phe, Tyr, Trp
1585	proteins: Phe, Tyr
1573	G, A
1522	carotenoid ν_1
1445	lipids, proteins, $\delta(CH_2, CH_3)$
1360	Trp: indole ring
1279	carotenoid ν_5
1268	protein backbone: amide III
1198	Tyr, Phe
1189	carotenoid ν_2
1177	nucleotides: base ν(CN)
1156	carotenoid ν_3, C−H stretch, C−C stretch
1030	proteins, Phe
1010	carotenoid ν_6, C=C (ring breathe)
1012	proteins, Trp
1004	Phe, proteins, C=C (ring breathe)
968	carotenoid
850/830	Tyr, proteins, δ(CCH) aliphatic, Tyr (ring)
780	ν(O−P−O) diester
717	proteins: Met C−S stretch
643	Tyr
620	Phe, proteins (ring)
521	cysteine disulfide bond, S−S-stretch
480	pectin, δ(C−C−C) skeletal vibrations

[a] On the basis of refs 42−45. [b] Abbreviations: ν, stretching; δ, deformation; Tyr, tyrosine; Phe, phenylalanine; Trp, tryptophan; Met, methionine; Cys, cysteine; A, adenine; T, thymine; C, cytosine; G, guanine.

RESULTS AND DISCUSSION

Raman Spectra and Chemical Maps of Pollen Grains. With a microspectroscopic setup, it is possible to collect Raman spectra from individual pollen grains. Examples of Raman microspectra from fresh pollen are shown in Figure 1b,d,e and in Figure 4. The most prominent bands in the spectra are labeled in the examples of Figure 1, their tentative assignments are given in Table 2. As can be seen there, the pollen spectra consist of superimposed signals from a variety of biomolecules, such as nucleic acids, lipids, and proteins. Considering the diameter of the pollen grains as microscopic objects of 15−60 μm and a millimeter penetration depth of the excitation laser in the pollen material, we can assume to probe the outer pollen wall (exine) together with the cellulose-rich intine layer beneath and the cellular pollen interior. In accord with this, we detect the spectral signatures of nucleotide bases, aromatic amino acids in protein side chains, of lipid molecules, the nucleic acid and protein amide backbones, pigments, sugars, and their polymers (compare Table 2).

As described in the Materials and Methods, the spectra (for example, the spectra b, d, and f of Figure 1A) were obtained after irradiation of the pollen samples with 633 nm laser light prior to acquisition of the Raman data with excitation at 785 nm. This approach was chosen because of intense contributions from carotenoids in the Raman spectra of pollen from some species. For an illustration, spectra a, c, and e in Figure 1A were measured before exposure to 633 nm light. In initial experiments that were conducted at 633 nm excitation, we observed that spectral bands ascribed to carotenoid molecules were not stable but diminished steadily (Figure 1B). The irreversible decrease of the carotenoid intensities suggests the photodestruction of these molecules (see arrows in Figure 1B).[20−22] Therefore, 633 nm light was used to deplete the carotenoid molecules before the spectroscopic characterization. With excitation at 785 nm, the spectra of the pollen remain unaltered, independent of exposure time.

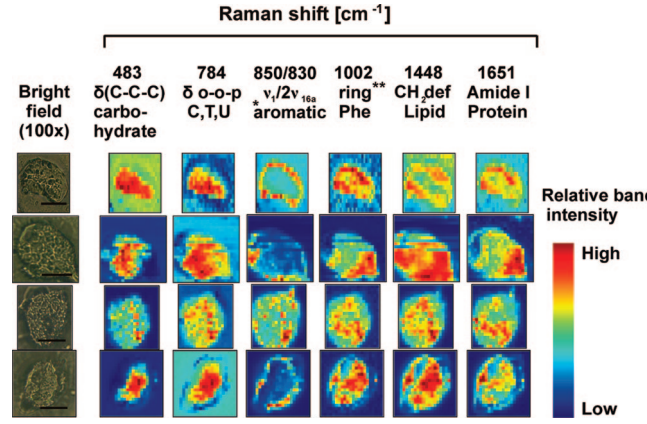

Figure 2. Chemical maps of selected molecular groups in sections of rye pollen grains (four examples). The maps were constructed by plotting the intensities of the respective band as a function of lateral position. For comparison, a photomicrograph (bright field, 100×) is displayed (leftmost column). Scale bars: 20 μm. Abbreviations: ν, stretching; δ, deformation; o-o-p, out-of-plane; Phe, phenylalanine; A, adenine; T, thymine; C, cytosine. *The Fermi doublet at 850/830 cm^{-1} is characteristic for para-disubstituted benzene rings. **The band at 1002 cm^{-1} is due to a breathing vibration of the Phe ring.

To elucidate the contribution of the different histological structures to the average Raman spectral signatures from intact pollen grains, we conducted spatially resolved measurements on sections of rye pollen grains. With the use of the computer-controlled *x*,*y*-stage of the Raman microscope, the sections of the pollen grains were raster-scanned. In this way, a Raman spectrum was obtained in each of the sampling positions of a predefined grid. Figure 2 shows results of four examples of such mapping experiments.

The plots of the intensities of selected characteristic bands as a function of lateral position (so-called chemical maps) provide information on the amount of the respective molecules or molecular groups in the different morphological structures (Figure 2). The band at 784 cm^{-1} can be assigned to out-of-plane deformation vibrational modes of the nucleobases cytosine, thymine, and uracil and serves as indicator for the presence of nucleic acids. At 483 cm^{-1}, a C−C−C deformation of carbohydrate polymers such as starch or pectin is present in some of the spectra. To study the distribution of protein compounds, we analyzed characteristic signals of the amino acid phenylalanine (1002 cm^{-1} ring breathe) as well as of the protein amide I band (1651 cm^{-1}) that is brought about by vibrations of the protein backbones. The maximum of the phenylalanine signal colocalizes with a maximum in protein content (compare examples in Figure

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2). A characteristic vibration, namely, the $\nu_1/2\nu_{16a}$ Fermi doublet at 850/830 cm^{-1},[23] dominates some of the spectra of the pollen sections and identifies para-disubstituted benzene rings. Its maximum signals localize to the pollen capsule, and no overlap is found with the protein signals in the pollen interior. Therefore, it can be inferred that the signal does not stem from the tyrosine side chains of proteins. The signal may be caused by nonprotein tyrosine-containing structures but also by other phenolic compounds, which have been discussed to be building blocks of the exine.[24] Raman signals that can be assigned to a deformation mode of CH_2 groups of aliphatic carbon chains at 1448 cm^{-1} are found in different regions of the pollen grain. Consistent with the fact that such molecules are contained both in the phospholipids of cellular, nuclear, and other membranes but also insofar unidentified aliphatic compounds in the pollen exine, the CH_2 deformation intensity is high in the pollen capsule but also in some cellular regions (Figure 2).

A major advantage of Raman spectroscopy in the chemical characterization of pollen is its applicability in situ. The pollen grains in this study were investigated without prior purification or extraction procedures, which has been the case in most other investigations on pollen composition so far. With the use of Raman microspectroscopy on sections of snap-frozen samples, the chemical composition and very likely also their ultrastructure remain unaltered, and the colocalization of individual components could be studied in the context of pollen micromorphology, simultaneously in very short times (several seconds per spectrum). The distribution of different molecular groups in the various structures of the pollen grains is not homogeneous. As illustrated by the example in Figure 2, the observed localization of molecular groups in the investigated rye pollen sections is in good agreement with cytochemistry. As expected, the area of the sporopollenin capsule can be easily recognized in spatially resolved measurements due to intense signals from, e.g., aromatic and aliphatic compounds. In the cells, the regions of the nuclei (DNA bands) and of carbohydrate reservoirs can be identified. Knowing about the spectral contributions from different parts of the pollen may help to develop classification of pollen fragments in bioaerosols. The ability to obtain molecular information from pollen structures in situ is of significance to pollen research, considering that so far both protoplast and sporopollenin capsules have only been studied after purification by more or less harsh methods.[25,26] The detailed structural interpretation of the in situ Raman spectra of the substructures is extensive and is beyond the scope of this paper but will be reported elsewhere.

Results of Multivariate Analysis. The mapping experiments and separation of predominant spectral contributions from different substructures can help explain the complex spectral pattern of whole pollen grains. To test, whether the full spectral patterns can be used like fingerprints for an overall chemical classification without decomposing the information, we conducted classification studies on whole pollen spectra (corresponding to the examples shown in Figure 1A).

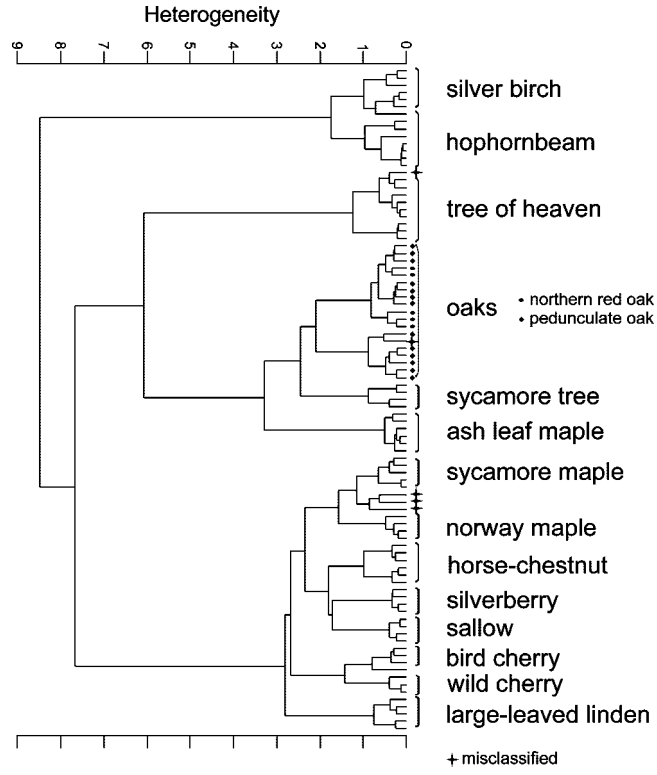

Figure 3. Classification by hierarchical cluster analysis. Dendrogram resulting from cluster analysis containing 91 spectra from 15 tree species (see also Table 1). Cluster analysis was done on first derivatives over the spectral range 380−1700 cm^{-1}. The distance matrix was calculated using Euclidean distance, Ward's algorithm was applied for clustering. Spectra were measured after decomposition of β-carotene with 633 nm irradiation (see Materials and Methods for details). For example, spectra of each species see Figure 4.

To base classification and possible identification upon the whole chemical information contained in the pollen, we used a multivariate method. For an evaluation of overall variance between different pollen and to obtain an unbiased perspective on possible chemical differences, we aim at a classification method that does not require preselection of chemical parameters or weighting of input information. As a consequence, we chose to use hierarchical cluster analysis (HCA) as an unsupervised method. We applied HCA to a spectral data set containing 91 spectra from different samples of 15 tree species using almost the full spectrum (380−1700 cm^{-1}) (Figure 3). The resulting dendrogram reveals formation of three large clusters (see Figure 3). Except for the case of the different oak species and <4% other misclassifications, the spectra of the different samples of each respective species form individual groups within these larger clusters (Figure 3). This result is in accord with the assumption that the variation between different specimens of a particular species is smaller than variation between species and permits us to conclude that most of the pollen samples of the different species studied here can be distinguished based on their Raman spectra. The average spectra of each of the species are shown in Figure 4.

As evidenced by the dendrogram in Figure 3, separation down to the species-level is superimposed by a high similarity within genera. Examples for a high chemical similarity within the same genus are the spectra obtained from two species of *Prunus*, bird

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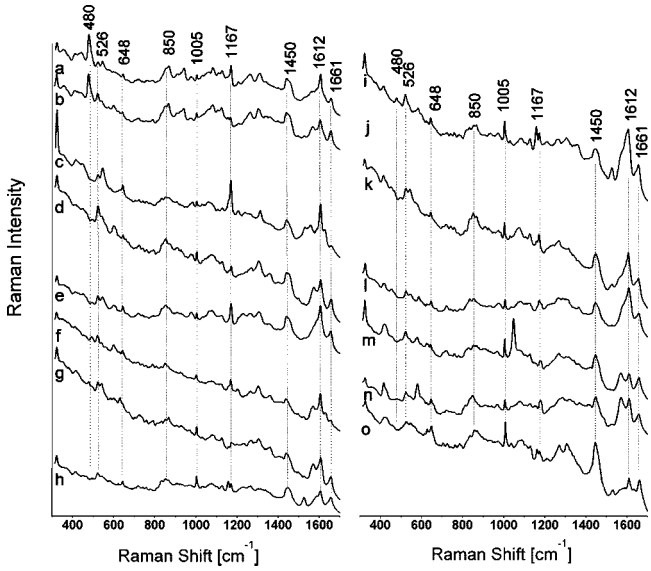

Figure 4. Raman spectra of pollen from the species listed below. The Raman spectra were excited with a wavelength of 785 nm, laser power 18 mW (irradiance 1.8×10^6 W/cm^2), accumulation time was 10 s. For band assignments see Table 2. (a) Silver birch, (b) hophornbeam, (c) tree of heaven, (d) northern red oak, (e) pedunculate oak, (f) sycamore tree, (g) ash leaf maple, (h) sycamore maple, (i) Norway maple, (j) horse-chestnut, (k) silverberry, (l) sallow, (m) bird cherry, (n) wild cherry, (o) large-leaved linden.

cherry and wild cherry. They form a distinct group, as do also two *Acer* species, Norway maple and sycamore maple (see Figure 3). However, the pollen spectra from a third species of *Acer*, the ash-leaf maple (or maple ash), resemble those of oak and sycamore tree pollen rather than the other *Acer* species. The tree is native to North America, different from the other two *Acer* species, which are natives to Europe and Asia.

In good agreement with the order of the systematic taxa, the next level of similarity revealed by HCA is that of plant families, as illustrated by the examples of the Birch and of the Soapberry families (*Betulaceae* and *Sapindaceae*). Silver birch (genus *Betula*) and Hophornbeam (genus *Ostrya*) are members of the Birch family (*Betulaceae*), and their spectra form one of the three main data clusters. Sycamore maple, Norway maple, and horse-chestnut are all members of the Soapberry family (*Sapindaceae*) and are also contained in one group.

Raman Spectra for Pollen Classification. As the reported results indicate (Figure 1), pollen specimens collected in the field exhibit only low levels of fluorescence and enable acquisition of high-quality Raman spectra also at low excitation intensities. In previous studies, Raman spectroscopy on whole pollen was based on feasibility studies on the few freeze-dried pollen species that have been commercially available.[27−29] Some of these spectra in the literature were excited at very high laser powers of several watts and discussed quite high fluorescence backgrounds,[27,28] even at near-infrared (NIR) excitation.[29] The easy utilization of Raman signals from fresh whole pollen is the basis for classification and development of spectral detection methods.

When classifying biological samples by analytical methods, usually the question arises about individual variation vs variation between groups of individuals. To take account such variation between individuals, each of the measurements were performed with 5−10 samples, in different experiments. Variation of spectral signatures is also brought about by structural heterogeneity. Considering that pollen grains are spheroid objects of several (sometimes tens of) micrometers in size with heterogeneous surfaces, the exact position of the excitation laser focus with respect to surface microstucture can influence the background and intensities. Furthermore, the micromorphology of the pollen surface, e.g., different thickness of the exine layer, protrusions, or complete absence at the pollen apertures, represent a certain amount of morphological heterogeneity a spectrum-based pollen classification procedure has to withstand. The cluster analysis results show that such intraspecies differences due to variability and microscale heterogeneity are smaller than interspecies variation (compare Figure 3).

A study that compared infrared spectra of dried pollen from different plants (trees, shrubs, and grasses) demonstrated separation of different species within the *Citrus* genus.[30] The few other vibrational studies of pollen conducted so far investigated the spectra of different species that were also members of different genera, different families, and oftentimes even different orders of plants.[27,29−31] In a study using surface-enhanced Raman scattering (SERS) on four species, which concluded that pollen of the same of two plant families have similar SERS spectra,[32] the pollen did not only belong to different plant families, but also to different orders, classes and even divisions in the plant kingdom. These studies left the question open whether distinction between Raman spectra down to the species level can really be achieved (in the same genus). The results presented here yield an answer to this question: Species that belong to the same genus and of genera that belong to the same families can be distinguished from one another based on a chemical fingerprint.

However, for the oak genus, no distinction can be made between the spectra from the species pedunculate oak (*Quercus robur*) and northern red oak (*Quercus rubra*) in HCA. This indicates that the molecular differences between pollen of these two species may be too small to be detected with HCA as unsupervised method sensu stricto, i.e., using the whole spectral range in a completely unsupervised similarity test. Molecular differences between species may be so small that they only reflect in a few selected spectral alterations localized in short spectral ranges. This observation is in good agreement with other studies that demonstrated spectral classification to species level or even below, e.g., between different strains of one bacterial species.[33−35] In most studies, HCA-based classification is restricted to the use

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of certain spectral ranges or their weighted combination or for supervised classification.[34,35] In this light, the HCA result obtained on full-range pollen spectra is surprising, since the whole spectral range, that is, all classes of molecules are variable to a high extent and can be used for discrimination between most of the species investigated here. The poor spectral discrimination of pollen of the different oak species is in good agreement with the fact that interspecific hybridization is very common among oaks and occurs much more frequently than in other plant species.[36,37] Hybridization has retained oaks a model taxon for species concepts that rely on ecological criteria rather than on reproductive isolation.[38] The high similarity in chemical constitution of different oak pollen, which is verified by a biochemical fingerprint in our Raman experiments, can be interpreted as part of the very weak barriers to being pollinated by another (oak) species.

Raman spectra of the pollen are fingerprints of a particular biochemical composition. Therefore, they reflect part of the phenotype of a plant. To summarize the discussion of the HCA result in the context of phylogenetic groups investigated here, the similarities and dissimilarities observed in the Raman spectra of the pollen are in accord with concepts of systematics and speciation. These are the process of reproductive or mating isolation (which is sensible, considering that pollen are critical parts of mating systems) but also, as was observed here for the case of the Maple genus, ecological and geographical criteria.

Influence of Carotenoid Signals on Classification Results. As illustrated by the spectra displayed in Figure 1, the carotenoid content of the pollen grains from some species is a molecular parameter that can influence the outcome of spectral classification results of pollen. The intense carotenoid signals arise, since the laser used for excitation of the Raman scattering is in resonance or preresonance with electronic transitions in the carotenoid molecules, even at 785 nm excitation, far away from the absorption maximum at 488 nm.[39] Carotenoids have been reported to be commonly involved in pollen pigmentation, mainly in the exine layer, although early hypotheses that carotenoids and carotenoid esters polymerize to form the sporopollenin polymer itself[40] have proven false.[1] The outer pollen wall (exine) is an accumulation site for various pigments, and in a recent report, CARS imaging of an individual cherry pollen grain was based entirely on the ν_1 and ν_3 Raman modes of carotene.[41]

The observed preresonant Raman signals of carotenoids can be orders of magnitude stronger than the normal Raman signals of all other molecules in the pollen. In multivariate analysis, this leads to a classification of the spectra according to carotenoid content. In particular, the spectra of large-leaved linden, sallow, and horse-chestnut are dominated by typical carotenoid features (compare spectra a, c, and e of Figure 1A). Originally, in HCA, the spectra of these species formed one large cluster, obviously due to their high resemblance with respect to the carotenoid spectral features (data not shown). Therefore, the strong carotenoid features have to be taken into account either prior to or post data acquisition. The strong impact the presence of carotenoids can have on pollen classification can be circumvented by a short period of photodestruction using 633 nm light as reported here. Photodecomposition of the carotenoid components is an efficient alternative to postacquisition data treatment. Omitting postacquisition all spectral regions where carotenoid bands may superimpose other features was proposed in studies on microorganisms[34] but does not seem practical here. The width of some of the carotenoid bands would force us to discard numerous important other spectral features hidden beneath the intense carotenoid signals.

CONCLUSIONS

In this paper we have discussed results on the in situ chemical characterization and classification of pollen grains by Raman microspectroscopy. As demonstrated, it is possible to localize different spectral signals to specific morphological microstructures in individual pollen grains. Maps of important molecular constituents in cryo-sections of pollen grains could be constructed simultaneously and without purification or staining. The data permit conclusions about the relative levels and localization of aromatic amino acids along with aliphatic molecules, nucleic acids, protein, and carbohydrate storage in different structures of the pollen. This combined analysis of different classes of molecular constituents in the unaltered material differs from previous purification-based approaches.

By using a Raman-spectroscopic approach, we could prove the working hypothesis that the biochemical makeup of the pollen (as part of a recognition/mating system) is altered during formation of a new biological species and that the species-specific chemical similarities and dissimilarities indeed reflect in the Raman spectral fingerprint. On the basis of the chemical information, unsupervised multivariate analysis consisting of hierarchical clustering revealed in most cases chemical similarities between species that were indicative of both phylogenetic relationship and mating behavior. As illustrated by the data, the spectra of whole pollen can be used for their classification. Such an approach is particularly promising, considering its potential to be employed for automatic pollen detection and warning.

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