# Chemical characterization and identification of Pinaceae pollen by infrared microspectroscopy

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**Main conclusion:**
FTIR microspectroscopy, in combination with spectral averaging procedure, enables precise analysis of pollen grains for chemical characterization and identification studies of fresh and fossilised pollen in botany, ecology and palaeosciences.

**Abstract**
Infrared microspectroscopy (µFTIR) of Pinaceae pollen can provide valuable information on plant phenology, ecophysiology and paleoecology, but measurements are challenging, resulting in unreproducible spectra. The comparative analysis of µFTIR spectra belonging to morphologically different Pinaceae pollen, namely bisaccate *Pinus* and monosaccate *Tsuga* pollen, was conducted. The study shows that the main cause of spectral variability is non-radial symmetry of bisaccate pollen grains, while additional variation is caused by Mie scattering. Averaging over relatively small number of single pollen grain spectra (approx. 5-10) results with reproducible data on pollen chemical composition. The practical applicability of the µFTIR spectral averaging method has been demonstrated by the partial least squares regression-based differentiation of the two closely related *Pinus* species with morphologically indistinguishable pollen: *Pinus mugo* (mountain pine) and *Pinus sylvestris* (Scots pine). The study has demonstrated that the µFTIR approach can be used for identification, differentiation and chemical characterization of pollen with complex morphology. The methodology enables analysis of fresh pollen, as well as fossil pollen from sediment core samples, and can be used in botany, ecology and paleoecology for study of biotic and abiotic effects on plants.

**Keywords:** Fourier transform infrared spectroscopy, Mie scattering, Multivariate analysis, *Pinus mugo*, *Pinus sylvestris*, *Tsuga canadensis*

## Introduction

Pinaceae are one of the most ecologically important plant family with widespread range, in particular considering boreal, costal and mountain forests of the Northern Hemisphere. Moreover, members of the family are widely cultivated for softwood timber and pulpwood, and thus are of greatest economic importance. In general, Pinaceae species have relatively rich fossil record that indicates appearance of Pinaceae ancestors more than 200 million years ago, with diversification of the family during Jurassic and Cretaceous periods (Miller 1999). Fossil pollen grains are often not only the most abundant but also among the best preserved remains of Pinaceae species, thus providing crucial information for the reconstruction of past flora, population sizes and terrestrial communities (Lindbladh et al. 2002). In addition, fresh pollen can provide valuable information on plant phenology, ecophysiology, population dynamics and gene flow (Yazdani et al. 1989; Savolainen et al. 2007).

Pollen analysis is usually based on morphology since pollen can have well preserved form and structure for millions of years, with specific shape, size and texture that are unique for plant taxa (Hesse 2009). Pinaceae are described as saccate pollen due to a large hollow projection (saccus) from the central body of pollen grain. Most of the Pinaceae species, such as *Pinus, Abies, Picea, Cedrus* and *Podocarpus*, have bisaccate pollen, with two laterally-placed sacci (Hesse 2009). In addition to morphometric analysis, chemical analysis of pollen can provide valuable information as well. Pollen wall is generally comprised of an inner layer (intine), an outer layer (exine), and a cover layer (pollen coat). Pollen wall has not only complex surface morphology but also complex and distinct chemical components. Biochemistry of these components depends on a number of metabolic events, involving for example lipid and polysaccharide metabolisms (Pacini and Hesse 2005; Blackmore et al. 2007; Souza et al. 2009; Ariizumi and Toriyama 2011; Jiang et al. 2013; Shi et al. 2015). The most important component of the outer layer of the pollen grain wall (exine) are sporopollenins, an extremely resilient and chemically stable group of compounds that preserves pollen for long periods of time (Bedinger 1992). Sporopollenins are complex biopolymers composed of polyhydroxylated unbranched aliphatic and phenolic constituents (Kim and Douglas 2013), and they are the predominant chemical components of Pinaceae saccus. Sporopollenin phenolic constituents (i.e. phenylpropanoids) serve as protective absorbing screens of solar UV radiation, and, as a result, concentration and composition of phenylpropanoids in pollen grain wall is wavelength-dependent. Therefore, measurement of phenylpropanoids can be used as UV-B proxy, allowing assessment of changes in the flux of UV-B radiation over geological time (Rozema et al. 2001; Watson et al. 2007; Willis et al. 2011; Lomax et al. 2012).

In general, chemical composition of pollen is of importance in order to determine the principal structural, nutritious and metabolic components. For example, triglyceride lipids primarily serve as carbon and long-term energy reserves in a form of lipid bodies, while phospholipids serve as structural components in cell membranes (Piffanelli et al. 1998; Zhang et al. 2016). Carbohydrates, in the form of cytoplasmic saccharides, have a vital function in the resistance to dehydration and temperature stress, as well as serving as cell wall components and energy reserves (Pacini 1996; Speranza et al. 1997). Proteins have both a structural and a functional role, with crucial functions in signalling and interactions of organisms with environment (Roulston et al. 2000; Holmes-Davis et al. 2005). A number of studies have demonstrated the importance of precise measurement of pollen chemical composition (Vanherpen 1981; Vesprini et al. 2002; Lahlali et al. 2014). Unfortunately, measurement of chemical composition of pollen, such as measurements of proteins (Roulston et al. 2000), carbohydrates (Speranza et al. 1997), and lipids (Piffanelli et al. 1998), are rarely conducted since they require complex sample preparation and laborious analysis. During the recent years, a number of techniques, such as laser induced breakdown spectroscopy (LIBS) (Boyain-Goitia et al. 2003), matrix assisted laser desorption ionization mass spectrometry (MALDI-MS) (Liang et al. 2013; Seifert et al. 2015; Joester et al. 2016; Seifert et al. 2016), thermally assisted hydrolysis and methylation pyrolysis gas chromatography/mass spectrometry (THM-py-GC/MS) (Blokker et al. 2005; Watson et al. 2007; Lomax et al. 2008; Willis et al. 2011),

and Raman spectroscopy (Boyain-Goitia et al. 2003; Pummer et al. 2013; Joester et al. 2016; Seifert et al. 2016), have been introduced for the chemical characterization of pollen grains.

In the last decade, analysis of pollen by Fourier transform infrared (FTIR) spectroscopy has seen rapid development in the field of botany and palynology (Pappas et al. 2003; Gottardini et al. 2007; Dell'Anna et al. 2009; Zimmermann 2010; Parodi et al. 2013; Pummer et al. 2013; Lahlali et al. 2014; Zimmermann and Kohler 2014; Bağcıoğlu et al. 2015; Jiang et al. 2015; Zimmermann et al. 2015a; Zimmermann et al. 2015b; Julier et al. 2016; Zimmermann et al. 2016; Bağcıoğlu et al. 2017; Jardine et al. 2017). A major advantage of FTIR spectroscopy is fast and economical measurement of pollen samples which can be conducted without any chemical pre-treatment. In general, FTIR spectra of pollen contain vibrational frequencies of molecular bonds that can be directly related to molecular functional groups of chemical constituents (Pappas et al. 2003; Gottardini et al. 2007; Zimmermann 2010; Zimmermann and Kohler 2014). Given that FTIR spectroscopy is based on the spectral measurement of many different spectral cellular features, the resulting spectrum is a fingerprint of the overall chemical composition of a pollen sample, and not just one type of chemicals. For example, main biochemical constituents of pollen, such as lipids, proteins, and carbohydrates can be easily identified based on their specific infrared signals (Bağcıoğlu et al. 2015; Jiang et al. 2015; Zimmermann et al. 2015b; Jardine et al. 2017). This property enables measurement of structural changes at molecular level, such as phase transition behaviour of lipids (Sowa et al. 1991) and estimation of different protein secondary structures in pollen grains (Sowa et al. 1991; Wolkers and Hoekstra 1995; Lahlali et al. 2014; Depciuch et al. 2017). Furthermore, even chemistry of complex compounds, such as sporopollenins in both pollen and plant spores, has been studied extensively by FTIR (Dominguez et al. 1999; Yule et al. 2000; Watson et al. 2007; Fraser et al. 2012; Lomax et al. 2012; Fraser et al. 2014; Bağcıoğlu et al. 2015; Lomax and Fraser 2015; Zimmermann et al. 2015a; Jardine et al. 2016). Accurate measurement of sporopollenin by FTIR enables, for example, quantification of phenylpropanoids by a non-destructive approach (Watson et al. 2007; Fraser et al. 2011; Jardine et al. 2016; Jardine et al. 2017). A number of studies have demonstrated that FTIR spectra of pollen enable detailed chemical analysis for identification and classification purposes (Pappas et al. 2003; Gottardini et al. 2007; Dell'Anna et al. 2009; Zimmermann 2010; Julier et al. 2016; Zimmermann et al. 2016; Bağcıoğlu et al. 2017). Moreover, FTIR spectroscopy is a valuable method for pollen phenotyping since it provides assessment of environmental effects, such as temperature stress (Lahlali et al. 2014; Zimmermann and Kohler 2014; Jiang et al. 2015) or anthropogenic pollution stress (Depciuch et al. 2016; Depciuch et al. 2017).

Although FTIR microspectroscopy (µFTIR) enables measurement of a single pollen grain, the measurement is quite challenging, resulting in unreproducible spectra, thus severely hindering application of this method in paleoecology (Dell'Anna et al. 2009; Bağcıoğlu et al. 2015; Lukacs et al. 2015; Zimmermann et al. 2015a; Zimmermann et al. 2016). In general, µFTIR of pollen is hindered by strong Mie scattering, since pollen grains, due to size and shape, are highly scattering samples in the infrared (Lukacs et al. 2015; Zimmermann et al. 2015a). The strong light scattering results in anomalous spectral features that can significantly interfere with and distort the signals of chemical absorption. Pollen grains within the range of 5-25 µm show the strongest scattering anomalies, since the sizes are exactly within the magnitude of the mid-IR light (Zimmermann et al. 2015a). For such samples, the conventional measurement on microscope slides is not feasible and different experimental setting needs to be applied, such as embedding in a soft paraffin layer between two sheets of polyethylene foils (Zimmermann et al. 2016). Although grains larger than 40 µm do not show strong scattering and can be measured in conventional way, Pinaceae grains are exception due to complex structure with non-radial symmetry (Bağcıoğlu et al. 2015; Zimmermann et al. 2015a). In addition to the scattering issues, single-grain FTIR spectra of bisaccate Pinaceae pollen have high spectral variability due to differences in chemical absorption bands (Zimmermann et al. 2015a). More precisely, bilateral symmetry of pollen causes distinctive and noninvariant spatial orientations, resulting in high variability of corresponding FTIR spectra (Zimmermann et al. 2015a).

In this study, averaging of µFTIR single-grain pollen spectra was employed in order to obtain reproducible chemical fingerprints for characterisation, identification and differentiation of Pinaceae pollen

species. The practical applicability of the µFTIR spectral averaging method was evaluated by measurement and differentiation of the two pine species: *Pinus mugo* (mountain pine) and *Pinus sylvestris* (Scots pine). Although *P. mugo* and *P. sylvestris* can be differentiated based on quantitative and qualitative characters of tree (sporophyte) phenotypes (Christensen and Dar 1997), there is a considerable overlap in their pollen (gametophyte) morphology, and clear morphological criteria for differentiating pollen of the two species are still lacking (Klaus 1978; Bykowska and Klimko 2015). Therefore, the two pine species represent challenging experimental pollen set for µFTIR-based differentiation study. In addition, the study has included *Tsuga canadensis* (eastern hemlock) pollen samples. *T. canadensis* has monosaccate pollen grains with spherical symmetry, thus it represents good referent sample to morphologically different (Ho and Sziklai 1972; Nakagawa et al. 2000), but chemically quite similar (Zimmermann 2010), bisaccate *P. mugo* and *P. sylvestris*.

## Materials and methods

### Samples

Six pollen samples were collected in 2014 from six different Pinaceae individuals (i.e. parental lineages) growing at campus area of Norwegian University of Life Sciences (Ås, Norway). Two pollen samples per each of the three Pinaceae species were measured: *Pinus sylvestris* L., *Pinus mugo* Turra and *Tsuga canadensis* (L.) Carrière. Each pollen sample was collected from at least ten pollen strobili (male flowers) on one individual Pinaceae tree. Pollen were collected immediately following cone opening and sporangial sac dehiscence. The pollen samples were kept at room temperature for 24 hours, and subsequently stored at -15°C until the µFTIR measurements.

### Measurement

Microscopic transmission measurements of pollen were performed using a Vertex 70 FTIR spectrometer with a Hyperion 3000 IR microscope (Bruker Optik, Ettlingen Germany), equipped with a globar mid-IR source and a liquid nitrogen-cooled mercury cadmium telluride (MCT) detector. The pollen samples were deposited onto 1 mm thick zinc selenide (ZnSe) optical windows without any chemical pretreatment. The spectra were recorded with a total of 64 scans in the 4000-600 $cm^{-1}$ spectral range, with a spectral resolution of 4 $cm^{-1}$. Samples were measured using 15× objective, with 50×50 aperture size. Background (reference) spectra were recorded at the start of measurements (one per sample measurement) by measuring empty areas of ZnSe slides. Visible images of the measured pollen grains were obtained by a charge coupled device (CCD) camera coupled to the microscope. The microscope was equipped with a computer-controlled x/y/z stage. The spectroscopic system was controlled with OPUS 7.5 software (Bruker Optik). Six pollen samples were measured, and 200 spectra per sample were obtained, each corresponding to a different single pollen grains, resulting in 1200 µFTIR spectra of single pollen grains in total.

### Data analysis

For the analysis of spectral set, spectral region of 1900 to 800 $cm^{-1}$ was selected. Prior to analysis, bands belonging to gaseous $H_2O$ and $CO_2$ from ambient air were removed from the spectra by using the OPUS Atmospheric Compensation command in OPUS 7.5. Furthermore, spectra were smoothed and transformed to second derivative form by Savitzky-Golay (SG) algorithm using a polynomial of power 2 with window size 15. After derivation by SG algorithm, spectra were processed using extended multiplicative signal correction (EMSC) with linear, quadratic and cubic component. The SG algorithm was used to enhance spectral features, while the EMSC pre-processing was employed for the normalization and for the separation of chemical and physical variations in vibrational spectra, including the baseline correction (Zimmermann and Kohler 2013). The selection of pre-processing parameters was based on suppressing noise while enhancing intensity of broad amide bands in the 1650−1520 $cm^{-1}$ region, as explained in detail in our previous study (Zimmermann and

Kohler 2013). The Kolmogorov-Smirnov test was applied to assess whether intensity values of pre-processed spectra for each number followed a normal distribution.

Following the spectral pre-processing, three additional sets of spectra were generated by calculating average spectrum of 5, 10 and 20 single pollen grain spectra. Thus calculated average spectra were normalized by using multiplicative signal correction (MSC) for each set of average spectra separately. The spectral set with pre-processed single pollen grain spectra, as well as the three additional spectral sets comprising average spectra, were used in the data analyses.

Biochemical similarities between pollen samples were estimated by using principal component analysis (PCA) and variability test based on Pearson correlation coefficients (PCC). The PCC was calculated for the whole selected spectral region, from 1900 to 800 $cm^{-1}$. The Kruskal-Wallis and Mann-Whitney *U* tests were used to calculate the statistical significance of differences in the PCA principal component scores between species. In addition, partial least-squares regression (PLSR) was used to evaluate classification, either between Pinaceae genera (*Pinus* and *Tsuga*) or between *Pinus* species (*P. sylvestris* and *P. mugo*). The optimal number of components (i.e. PLSR factors) of the calibration models ($A_{Opt}$) was determined using segmented cross-validation using ten segments. The PLSR coefficient of determination ($R^2$) between the taxa was used to evaluate the calibration models. All pre-processing methods and data analyses were performed using The Unscrambler X 10.3 (CAMO Software, Oslo, Norway), as well as functions and in-house developed routines written in MATLAB 2014a. 8.3.0.532 (The MathWorks, Natick, Massachusetts, USA).

## Results

### Morphology of the Pinaceae pollen samples

The optical microscope images of pollen species reveal clear morphological differences between *Pinus* and *Tsuga* pollen grains (Fig. 1). The two laterally-placed sacci of bisaccate *Pinus* pollen are giving this type of pollen two bilateral planes of symmetry, both perpendicular to each other and to the equatorial plane. On the other hand, *T. canadensis* pollen grain is monosaccate, with equatorial saccus (i.e. frill or fringe), giving this type of pollen distinctive biconvex circular symmetry. It should be mentioned that *T. canadensis* pollen has an additional bilateral symmetry perpendicular to the circular axis. However, this symmetry can be disregarded since, due to density and geometric constraints, *T. canadensis* pollen will have overwhelming tendency to be orientated in a polar view on a measuring slide.

### Spectral differences between pollen genera and species

The µFTIR spectra of single pollen grains belonging to the two *Pinus* species have high variability due to the aforementioned variability of spatial orientations on µFTIR slides, caused by bilateral symmetry of the pollen grains (Fig. 2). For instance, the spectrum of the pollen grain that has distal polar orientation has predominant contribution of saccus region, with strong signals of sporopollenin-related phenylpropanoids (Fig. 2a). On the other hand, the spectrum of the pollen grain that has equatorial profile orientation has predominant contribution of corpus region, with strong signals of lipids and proteins (Fig. 2b). In addition, the differences between the two spectra are due to the scattering artefacts, in particular in the low-wavelength spectral region ($< 1000$ $cm^{-1}$).

Contrary to the single grain µFTIR spectra of the two *Pinus* species, spectra of *T. canadensis* pollen are quite invariant (Figs. 2c and 2d). Due to spherical symmetry, with large length difference between polar and equatorial axes, *Tsuga canadensis* pollen grains have consistent spatial orientation on µFTIR slides with almost exclusively polar orientation.

The low reproducibility (i.e. high variability) of the measured µFTIR spectra of *Pinus*, when compared with the spectra of *Tsuga*, can be seen in the PCA score plot in Fig.3. However, it is apparent that the variability is non-random and is significantly species-based. As no wavenumber was found to be normally distributed (according to the Kolmogorov-Smirnov test; Table S1 in the Supplementary material), the Kruskal-Wallis and

Mann-Whitney *U* tests were used to calculate the statistical significance of differences in the PCA principal component scores between pollen species (Table S2 in the Supplementary material). The statistical analyses, based on the PC scores, clearly show that µFTIR spectra of pollen species are significantly different (*P*-values < 0.0001).

The Pearson Correlation Coefficient (PCC), expressed as 1-PCC, was used to estimate the spectral variability of pollen samples (Table 1). The PCC measures correlation between variables, where a value of 1 indicates high positive correlation. Therefore, small variability is indicated by small 1–PCC values. It is apparent that the variability of bisaccate grains of *Pinus* with bilateral symmetry is considerably higher than variability of monosaccate grains of *Tsuga* with radial symmetry.

**Spectral averaging and differentiation of pollen species**

In order to compensate variability due to spatial orientation of the grains, a new set of spectra were created, where each new spectrum was an average of either 5, 10 or 20 single grain spectra. The PCA and PLSR results (Fig. 4 and Table 2), as well as the statistical analyses (Tables S2 and S3 in the Supplementary material), show that the average spectra have high reproducibility and enable characterization, identification and differentiation of pollen samples.

The PLSR model based on discrimination of single pollen grains has relatively high discrimination power, with $R^2 = 0.88$ (Table 2). However, the PLSR model has relatively high optimal number of components ($A_{Opt}$=11) with probable over-fitting result, since the regression coefficient shows strong contribution of noise and artefacts, such as white noise, water vapour, and Mie scattering (Fig. 5). In general, PLSR models are greatly improved by spectral averaging, with $R^2 > 0.95$, and considerably lower optimal number of components ($A_{Opt} \approx$ 5), even when averaging is based on only five grains. In order to avoid an overly-optimistic estimate of discrimination power, PLSR models with fewer factors were preferred. For example, the PLSR model based on 10 grain average ($R^2$=0.96) has considerably higher discrimination power than the model based on discrimination of single pollen grains ($R^2$=0.83), provided that the number of components is the same ($A$ = 4; Table 2). The separation between the two *Pinus* species is based predominantly on signals belonging to lipids (1740 $cm^{-1}$, C=O stretch; 1475 $cm^{-1}$, $CH_2$ bending), proteins (1650, amide I: C=O stretch; 1550, amide II: NH deformation and C–N stretch), and sporopollenins (1622, 1517 and 833 $cm^{-1}$, all bands related to phenylpropanoids building blocks) (Fig. 5) (Zimmermann 2010; Bağcıoğlu et al. 2015).

## Discussion

Automated and objective analysis of samples is a persistent problem in palynology (Holt and Bennett 2014). In this study, a highly challenging pollen samples, covering two closely related pine species of high economic and ecological importance, have been chosen for µFTIR analysis. *P. mugo* and *P. sylvestris*, are highly variable species showing wide range of morphological and physiological characteristics as well as adaptive traits for specific environments. *P. sylvestris* has the largest geographical distribution of all pines, covering the whole Europe, from the Mediterranean to well within the Arctic Circle, while *P.mugo* is a predominantly high-altitude species with restricted spread across south and central European mountain ranges (Wachowiak et al. 2011; Wachowiak et al. 2015). Genetic studies have shown that *P.mugo* and *P. sylvestris* share similar genetic background, indicating divergence in recent evolutionary past (Wachowiak et al. 2011; Wachowiak et al. 2015). Moreover, hybrids of the two species (*P. mugo* × *sylvestris*) are relatively widespread in natural populations since the parent plants readily undergo spontaneous hybridization in the autochthonous populations (Staszkie.J and Tyszkiew.M 1969; Christensen 1987; Christensen and Dar 1997; Wachowiak et al. 2011). Interspecific gene flow and high morphological polymorphism causes serious taxonomic problems, in particular regarding *P. mugo* (Christensen 1987; Businsky and Kirschner 2006; Wachowiak et al. 2011; Boratynska et al. 2015). In this study, pollen samples were obtained from *P. mugo* subsp. *mughus* (*P. mugo* sensu stricto), a polycormic shrub subspecies with prostrate growth.

In general, morphometric analysis enables taxonomic determination of *Pinus* pollen grains at the level of the *Strobus* and *Pinus* subgenera, due to the difference in outline of the sacci in polar view (Bykowska and Klimko 2015). However, differentiation at species level is extremely challenging and requires detailed analysis by scanning electron microscopy (Nakagawa et al. 2000). Concerning interspecific gene flow between *P. sylvestris* and *P. mugo*, with widespread hybridization, it is reasonable that the two species lack morphological features for pollen differentiation (Klaus 1978; Bykowska and Klimko 2015).

**Spectral differences between pollen genera and species**

The µFTIR spectra of single pollen grains belonging to *Pinus mugo* and *Pinus sylvestris* have relatively high variability, compared to *Tsuga canadensis* spectra, due to bilateral symmetry of bisaccate pollen grains of *Pinus* (Fig. 2). The high variability of the µFTIR spectra of the two *Pinus* pollen species is consistent with the results of our previous studies on bisaccate pollen of *Pinus*, *Picea*, *Cedrus*, *Abies* and *Podocarpus* (Bağcıoğlu et al. 2015; Zimmermann et al. 2015a). Due to spherical symmetry of monosaccate pollen grains, *T. canadensis* spectra are relatively invariant. It should be noted that all three studied species have relatively similar chemical composition of pollen (Zimmermann 2010). Therefore, the comparative study between *Pinus* and *Tsuga* samples shows that the majority of spectral variation originates due to either spatial orientation on microscope slides or scattering on substructures of bisaccate pollen grains.

The previous study has demonstrated that differentiation of congeneric species of pollen with radial symmetry can be obtained by µFTIR spectroscopy (Zimmermann et al. 2016). Here, it has been demonstrated that congeneric species of pollen with bilateral symmetry can be differentiated as well. In this respect, the presented averaging µFTIR methodology is more similar to multigrain µFTIR measurements (Dell'Anna et al. 2009; Bağcıoğlu et al. 2015; Julier et al. 2016), than to single grain µFTIR measurements (Zimmermann et al. 2016).

**Application of µFTIR approach**

It should be noted that certain variation in chemical composition between pollen grains of the same parental (sporophytic) lineage is always present, due to genetic differences between the pollen grains. Chemical composition of pollen depends both on sporophytic (usually diploid) genome which controls development of exine and pollen coat, as well as gametophytic (usually haploid) genome which controls development of intracellular materials and inner layer of pollen grain wall (intine) (Piffanelli et al. 1998; Jiang et al. 2013). The vast majority of µFTIR spectral signals are related to the intracellular structures, such as lipid and carbohydrate nutrients synthesized in the vegetative cell of the pollen grain under the control of the gametophytic genome. Unfortunately, spectral averaging reduces these chemical differences between individual pollen grains, resulting in an average µFTIR spectra of a pollen population.

Although µFTIR approach is limited regarding precise chemical characterization of a single grain bisaccate pollen, it can still have broad application. For example, the spectral averaging methodology enables measurement of chemical variation between pollen samples originating from different strobili or branches of an individual sporophyte, thus local biotic and abiotic effects on pollen can be studied, such as influence of pathogens, pollution or partial shading. Likewise, the presented FTIR methodology enables analysis of pollen grains from sediment core samples, thus providing chemical characterization of a stratigraphic sequence of pollen.

Moreover, chemical information of pollen obtained by this methodology can be combined with the results of other multigrain-based methods, such as THM-py-GC/MS (Blokker et al. 2005; Watson et al. 2007; Lomax et al. 2008; Willis et al. 2011) and MALDI-MS (Liang et al. 2013; Seifert et al. 2015; Seifert et al. 2016), in order to obtain comprehensive chemical fingerprints of pollen species and ecotypes. Regarding pollen grain wall chemistry, FTIR analysis of pollen cannot yet provide the same level of quantitative and qualitative chemical analysis of sporopollenins as THM-py-GC/MS. However, it is reasonable to assume that methodology will significantly improve in future by combining FTIR spectroscopy with chemometrics of multivariate

regression, as was the case for other complex biological samples (Zimmermann and Kohler 2013). This has already been indicated in a UV irradiance study where phenylpropanoid estimates were based on FTIR measurements of *Lycopodium* spores from a natural shading gradient (Jardine et al. 2017). It should be taken into account that a typical THM-py-GC/MS measurements of phenylpropanoids in *Pinus* pollen is conducted on approx. 50 pollen grains (Willis et al. 2011), while this study has demonstrated that reproducible chemical fingerprint of pollen can be obtained from even smaller number of pollen grains (5-20). Furthermore, FTIR measurements are non-destructive and thus can provide additional level of chemical information, such as lipid and protein secondary structure (Sowa et al. 1991; Wolkers and Hoekstra 1995; Lahlali et al. 2014; Depciuch et al. 2017), which cannot be obtained by destructive mass spectrometry approaches. Finally, chemical analysis of pollen samples by µFTIR, combined with DNA sequencing from single pollen grain (Parducci et al. 2005; Nakazawa et al. 2013), could provide valuable information in paleoecology for reconstruction of past communities, environments, and plant-environment interactions.

## Conclusions

Comparative analysis of µFTIR spectra belonging to morphologically different Pinaceae pollen, namely bisaccate *Pinus* and monosaccate *Tsuga* pollen, proves that the main cause of spectral variability is non-radial symmetry of bisaccate pollen grains. The spectral effects due to pollen grain spatial orientation on microscope slide, as well as scattering effects, can be considerably reduced by spectral averaging approach. Moreover, the value of the µFTIR methodology has been demonstrated on the two closely related *Pinus* species with morphologically indistinguishable pollen. The spectral averaging over relatively small number of single pollen grain spectra results in high spectral reproducibility as shown by the PCC. The reliable PLSR models, with high discrimination power ($R^2 > 0.95$) and clear chemical fingerprints, were obtained for differentiation of *P. sylvestris* and *P. mugo* pollen. Therefore, the study has demonstrated that the µFTIR approach can be used for identification, differentiation and chemical characterization of Pinaceae pollen and other pollen species with non-radial symmetry.

## Supplementary material

Additional Supplementary material may be found in the online version of this article at the publisher's website.


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**Tables**

**Table 1** Variability of spectral data, with values for single grain spectra and spectra based on different averaging factor, and with designated number of spectra used in the variability tests.

| Type of variability | Infrared region 800-1900 $cm^{-1}$ (1-*PCC**) * $10^{-4}$ | | | |
|---|---|---|---|---|
| | Single grain (200 spectra) | 5 grain average (40 spectra) | 10 grain average (20 spectra) | 20 grain average (10 spectra) |
| **Tsuga canadensis #1** | 113 | 24 | 13 | 7 |
| **Tsuga canadensis #2** | 98 | 23 | 14 | 8 |
| **Pinus sylvestris #1** | 838 | 251 | 137 | 67 |
| **Pinus sylvestris #2** | 895 | 296 | 194 | 120 |
| **Pinus mugo #1** | 662 | 190 | 101 | 43 |
| **Pinus mugo #2** | 546 | 165 | 84 | 47 |

**Table 2** The PLSR coefficient of determination ($R^2$) for differentiation between the taxa, either Pinaceae genera (*Pinus* vs *Tsuga*) or *Pinus* species (*P. sylvestris* and *P. mugo*), with the number of components in parenthesis ($A_{opt}$ - optimal number: $A_4$ - four components); results are stated for different averaging factor (AF).

| AF | Genera | | Species | |
|---|---|---|---|---|
| | $R^2$ ($A_{opt}$) | $R^2$ ($A_4$) | $R^2$ ($A_{opt}$) | $R^2$ ($A_4$) |
| 1 | 0.942 (10) | 0.897 | 0.878 (11) | 0.830 |
| 5 | 0.980 (9) | 0.966 | 0.961 (6) | 0.944 |
| 10 | 0.986 (7) | 0.982 | 0.968 (5) | 0.963 |
| 20 | 0.996 (6) | 0.991 | 0.970 (4) | 0.970 |

**Figures**

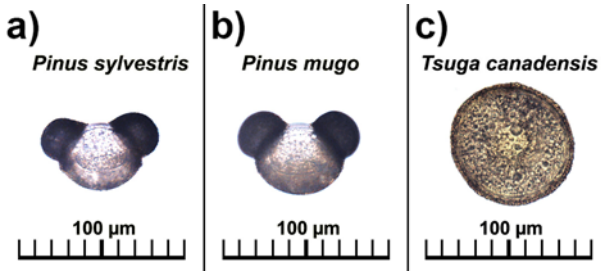


**Fig. 1** Microscope image of pollens. **a** *Pinus sylvestris*, equatorial view. **b** *Pinus mugo*, equatorial view. **c** *Tsuga canadensis*, polar view

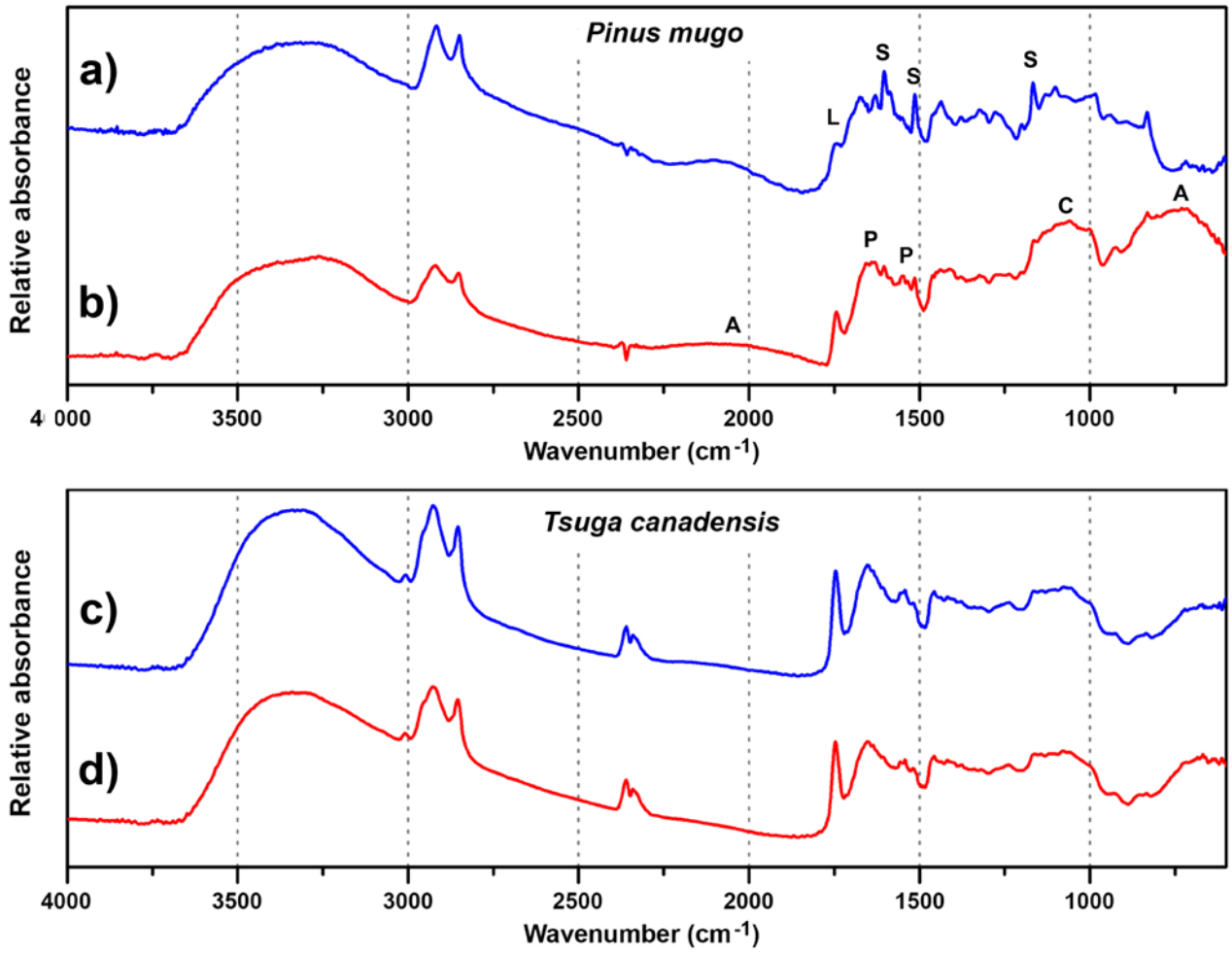


**Fig. 2** FTIR microspectroscopy spectra of single pollen grains. µFTIR spectra belonging to two *Pinus mugo* single pollen grains, one with distal polar orientation (**a**) and another with equatorial profile orientation (**b**). µFTIR spectra belonging to two *Tsuga canadensis* single pollen grains with polar orientation (**c**, **d**). For better viewing the spectra are offset; the marked vibrational bands are associated with lipids (L), proteins (P), carbohydrates (C) and sporopollenins (S); the scattering artefacts (A) are present in **a** and **b**

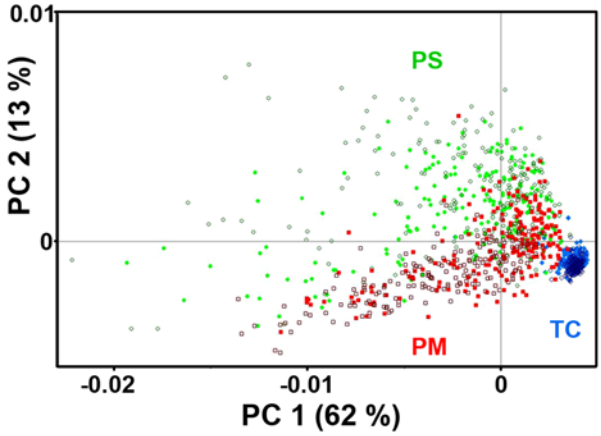


**Fig. 3** PCA score plot of FTIR microspectroscopy spectral set. The set includes six samples with 200 single grain measurements per sample: *Pinus sylvestris* (PS: green, dark green), *Pinus mugo* (PM: red, dark red) and *Tsuga canadensis* (TC: blue, dark blue)

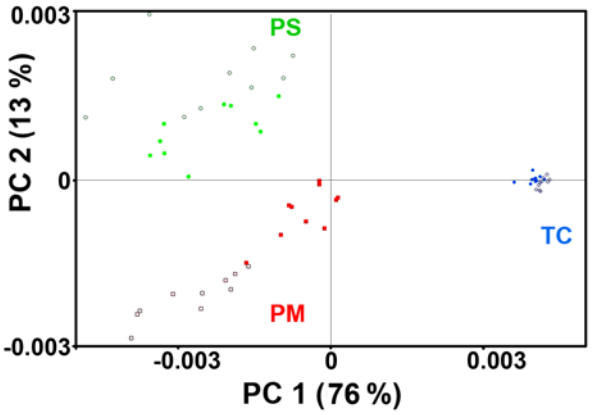


**Fig. 4** PCA score plot of FTIR microspectroscopy spectral set with averaging. The set includes six samples with 10 average spectra per sample; each average spectrum is based on 20 spectra of single grains; *Pinus sylvestris* (PS: green, dark green), *Pinus mugo* (PM: red, dark red) and *Tsuga canadensis* (TC: blue, dark blue)

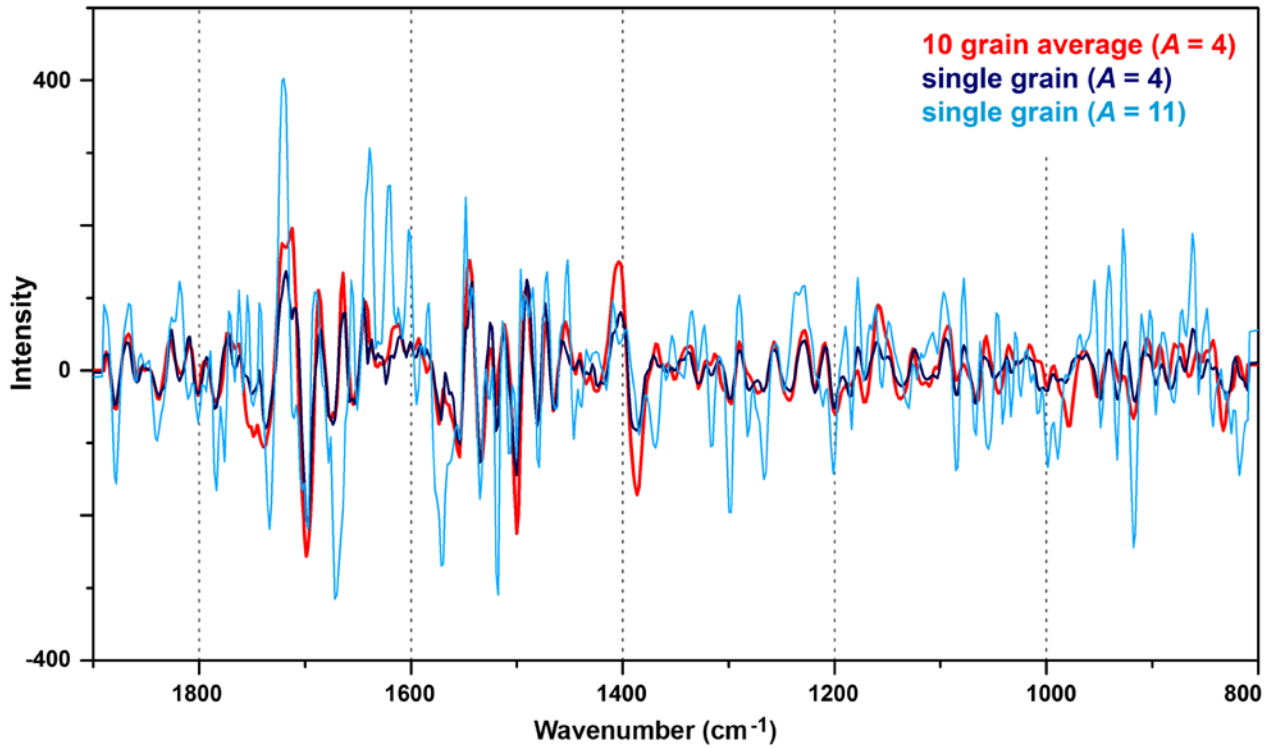


**Fig. 5** Plot of PLSR regression coefficients for differentiation between *Pinus* species based on single grain and average spectra; number of components of the calibration models are designated in parenthesis