Monitoring of plant-environment interactions by high throughput FTIR spectroscopy of pollen

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Running title: High throughput infrared spectroscopy of pollen

Key words: infrared spectroscopy, pollen, Betulaceae, Fagaceae, Ecology and environmental sciences, *Betula*, *Corylus*, *Quercus*, *Fagus*, *Alnus*

Word count: Total – 6743; Introduction - 944; Materials and Methods – 883; Results and Discussion - 2726; Conclusions - 241; Acknowledgements - 85; Supporting Information - 17; References - 1444; Table legends - 40; Figure legends - 363

SUMMARY

1. Fourier transform infrared (FTIR) spectroscopy enables chemical analysis of pollen samples for plant phenotyping to study plant-environment interactions, such as influence of climate change or pathogens. However, current approach, such as microspectroscopy and Attenuated Total Reflection spectroscopy, does not allow for high-throughput protocols. The paper at hand suggests a new spectroscopic method for high-throughput characterization of pollen.

2. Samples were measured as thin films of pollen fragments using a Bruker FTIR spectrometer with a high-throughput eXTension (HTS-XT) unit employing 384-well plates. In total, 146 pollen samples belong to 31 different Fagales pollen species, collected during three different pollination seasons (2012-2014) at locations in Croatia, Germany and Norway were analyzed. Critical steps in the sample preparation and measurement, such as variabilities between technical replicates, between microplates and between spectrometers, were studied.

3. Measurement variations due to sample preparation, microplate holders and instrumentation were low, and thus allowed differentiation of samples with respect to phylogeny and biogeography. The spectral variability for a range of Fagales species (*Fagus*, *Quercus*, *Betula*, *Corylus*, *Alnus* and *Ostrya*) showed high species-specific differences in pollen's chemical composition due to either location or pollination season. Significant inter-annual, intra-seasonal and locational differences in the pollen spectra indicate that pollen chemical composition has high phenotypic plasticity and is influenced by local climate conditions. The variations in composition are connected to lipids, proteins, carbohydrates and sporopollenins

that play crucial roles in cold and desiccation tolerance, protection against UV radiation, and as material and energy reserves.

4. The results of the study demonstrate the value of high throughput FTIR approach for the systematic collection of data on ecosystems. The novel FTIR approach offers fast, reliable and economical screening of large number of samples by semi-automated methodology. The high-throughput approach could provide crucial understanding on plant-climate interactions with respect to biochemical variation within genera, species and populations.

INTRODUCTION

The vast majority of land plants are sporophyte-dominant seed plants that have male gametophytes with microscopic sizes and short lifespans. However, pollen is crucial in reproduction and survival of seed plants since it enables production of genetically diverse offspring, and thus it is exposed to a diversity of selection pressures (Delph, Johannsson & Stephenson 1997; Williams & Mazer 2016). Climate effects, including global climate change, can have a strong detrimental effect on reproductive structures of plants, including pollen. For example, temperature stress can have a damaging effect on development, transport and germination of pollen (Endo *et al.* 2009; Chakrabarti *et al.* 2011; Bokszczanin, Fragkostefanakis & Thermotolerance 2013). Pollen development has been pointed to as the most heat-sensitive process in plant sexual reproduction (Bokszczanin, Fragkostefanakis & Thermotolerance 2013). An increase in sterility of pollen results in lower seed yield, and thus significantly reduced population resilience as well as higher economic damages to agricultural crops.

It has been hypothesized that phenotypic plasticity will play an important role in the persistence of plants in increasingly variable climatic conditions (Nicotra *et al.* 2010; Anderson *et al.* 2012). Regarding pollen, it is critical to estimate plasticity of the most important traits such as longevity, viability, hydration and germination speed, as well as pollen tube growth rate and length. The majority of these traits should have correlation with chemical composition of pollen grains, for example with nutrient reserves in form of triglycerides and polysaccharides that play crucial role in pollen tube growth (Speranza, Calzoni & Pacini 1997; Rodriguez-Garcia, M'rani-Alaoui & Fernandez 2003). Therefore, to monitor climate-effects on plant communities it is imperative to improve phenotyping of pollen. Standard pollen analysis by optical microscopy can provide only characterization based on morphological analysis, while chemical information and information on pollen quality cannot be obtained. Infrared spectroscopy is one of the most widely used methods for chemical characterization of samples, and in the last decade, rapid progression in infrared studies of plant material was conducted.

Development and implementation of Fourier transform infrared (FTIR) spectroscopy of pollen is a novel approach with the potential to greatly expand palynological and botanical research. Infrared spectra of pollen are precise fingerprints of the overall biochemical composition of a pollen grain, and contain specific signals of lipids, proteins, carbohydrates, cell wall biopolymers, and water. The spectra can be obtained by using diverse techniques, such as single- (Gottardini *et al.* 2007; Zimmermann 2010; Lahlali *et al.* 2014; Zimmermann & Kohler 2014; Bagcioglu, Zimmermann & Kohler 2015; Jiang *et al.* 2015; Zimmermann *et al.* 2015b) and multi-reflection (Mularczyk-Oliwa *et al.* 2012) attenuated total reflection

(ATR) FTIR, diffuse reflectance FTIR (DRIFT) (Pappas *et al.* 2003; Mularczyk-Oliwa *et al.* 2012), transmission FTIR measurements between windows (Crowe, Hoekstra & Crowe 1989; Sowa, Connor & Towill 1991) and in pellets (Pappas *et al.* 2003; Zimmermann 2010; Mularczyk-Oliwa *et al.* 2012; Zimmermann & Kohler 2014; Bagcioglu, Zimmermann & Kohler 2015), FTIR microspectroscopy measurements of single pollen grains (Pappas *et al.* 2003; Gottardini *et al.* 2007; Pummer *et al.* 2013; Zimmermann & Kohler 2014; Bağcıoğlu, Zimmermann & Kohler 2015; Zimmermann *et al.* 2015a; Zimmermann *et al.* 2016) as well as multi grains (Wolkers & Hoekstra 1995; Wolkers & Hoekstra 1997; Dell'Anna *et al.* 2009; Bagcioglu, Zimmermann & Kohler 2015), and photoacoustic FTIR spectroscopy (Parodi, Dickerson & Cloud 2013). Recent studies have shown that FTIR spectroscopy achieves simple, economical, and rapid identification and classification of pollen by spectral fingerprinting, as well as characterization and biochemical interpretation with respect to environmental stress (Lahlali *et al.* 2014; Zimmermann & Kohler 2014; Jiang *et al.* 2015). The identification and classification of samples is mostly based on infrared spectral databases, thus offering an unbiased and operator-independent approach with huge potential for automatization (Pappas *et al.* 2003; Dell'Anna *et al.* 2009; Zimmermann 2010; Zimmermann *et al.* 2016). Our recent study on aeroallergen pollen by FTIR microspectroscopy has shown that the FTIR has great potential for an automated pollen analysis (Zimmermann *et al.* 2016). Moreover, since the method is compatible with standard air-samplers, it can be employed in the assessment and control of bioaerosols, including allergy forecasts.

Standard methodologies, including microscopies and sequencing, are overly complex, expensive and time-consuming. Therefore, they are slow in providing the massive data needed for determining and monitoring effects of abiotic and biotic stress on plant communities. Since FTIR spectroscopy supports high-throughput measurements, the technique is ideal for rapid screening of plant populations under different conditions. A high throughput screening (HTS) FTIR system has been successfully applied in a variety of studies in natural and biomedical sciences for the investigation of microorganisms and tissues (Dean *et al.* 2010; Sirikwanpong *et al.* 2010; Ollesch *et al.* 2013; Kohler *et al.* 2015; Mignolet & Goormaghtigh 2015). An extension of this approach on studies of pollen would have a great potential, particularly for comprehensive monitoring of terrestrial ecosystems. An HTS FTIR methodology has never been used in pollen analysis. Our previous study, on comparative analysis of FTIR techniques, has shown that spectra of ground pollens, obtained by either ATR or in pellets, is optimal for gathering information on their chemical composition (Bagcioglu, Zimmermann & Kohler 2015). Thus, a high-throughput FTIR approach where samples are prepared by pulverization of pollen into water suspension and by subsequent drying as films, should offer an analogous result. In the paper at hand, we explore the usage and development of a new high-throughput FTIR-based protocol for the biochemical characterization of pollen. The FTIR study was conducted on a substantial set of samples, consisting of six different genera of Fagales (*Fagus*, *Quercus*, *Betula*, *Corylus*, *Alnus* and *Ostrya*), collected during the three different pollination seasons (2012-2014) at three different locations (Germany, Croatia and Norway). The potential of high-throughput FTIR

spectroscopy of pollen as a tool for studying plant communities and plant-environment interactions is elucidated.

MATERIALS AND METHODS

Samples

The studied pollen belong to 31 different Fagales pollen species, covering 146 pollen samples in total (Table 1). Each sample comprised pollen belonging to approximately 10 male flowers (catkins) from the same individual plant. The pollen samples were collected directly from plants at flowering time during 2012, 2013 and 2014 pollination seasons at locations in Croatia, Germany and Norway. Pollen samples from Croatia were collected in Zagreb at the Botanical Garden of the Faculty of Science, the University of Zagreb. Pollen samples from Germany were collected in Berlin at the Berlin-Dahlem Botanical Garden , the Free University of Berlin. Pollen samples from Norway were collected in Ås at campus area of Norwegian University of Life Sciences. After the sampling, the samples were kept at room temperature for 24 hours, and subsequently stored at −15 °C until measurements.

Sample preparation

In order to produce homogeneous suspensions of pollen samples that could be transferred to high-throughput FTIR plates and measured in transmission, the pollen samples were sonicated. Approximately 1 mg of a pollen sample was transferred into a 1.5 ml microcentrifuge tube containing 500µl of distilled H_2O. The sample was sonicated in an ice

bath, by a 2mm probe coupled to a Q55 Sonicator ultrasonic processor (QSonica, LLC, USA) under 100 % power. The sonication period was two minutes in total, with 30s intermission after the first minute of sonication. Following the sonication, the sample was centrifuged with 13000 rpm for 10 min, and the suspension was concentrated by removing 400 µl of the supernatant. Out of the remaining suspension, three aliquots, each containing 8 µl, were transferred onto an IR-transparent silicon 384-well microtiter plate (Bruker Optik GmbH, Germany). The microtiter plate was dried for 1 hour at r.t. to create adequate films for FTIR measurement.

From each pollen sample, only one sample solution was prepared. In order to test variability between different sonications and dry film preparations, *Alnus incana* (Norway, 2014) was measured on different microtiter plates using different instruments. For the sonication variability test, ten independent pollen suspensions were prepared by using the described procedure with the following modifications: microcentrifuge tubes were filled with the double amount of pollen and water, and, following centrifugation, 800 µl of supernatant was removed. Ten aliquots (each containing 8 µl) from each of the ten prepared suspensions were transferred onto one silicon 384-well microtiter plate, designated as *sonication variability test plate*, thus creating 100 sample films. For technical replicates variability, ten spectra for each suspension were used. For sonication variability, average spectra for each suspension were used. Following the preparation of the *sonication variability test plate*, the remaining suspensions were combined into one stock suspension. For microplate variability test, five different microtiter well plates were prepared by using ten aliquots (each containing 8 µl) of the stock suspension. The five plates, designated as *microplate variability test plates*,

contained 50 sample films in total. For microplate variability, average spectra for each microtiter plate were used. For instrument variability test, five *microplate variability test plates* and the *sonication variability test plate* were measured using two different HTS-XT FTIR spectrometer systems. For instrument variability, average spectra for each suspension from the *sonication variability test plate* were used. For variability between different pollen samples (taxa, pollination seasons, and locations) average spectra of three technical replicates were used. Schematic diagram of variability test study is in the Supporting Information (Fig. S3).

Measurement

Infrared spectroscopy measurements were obtained using a HTS-XT extension unit coupled to a TENSOR 27 spectrometer (both Bruker Optik GmbH, Germany) (referred to as instrument #1). For instrument variability test, an additional spectrophotometer system, comprising HTS-XT extension unit coupled to a VERTEX 70 spectrometer (both Bruker Optik GmbH, Germany), was used (referred to as instrument #2). Both systems are equipped with a globar mid-IR source and a DTGS detector. The spectra were recorded in transmission mode, with a spectral resolution of 4 cm^{-1} and digital spacing of 0.964 cm^{-1}. Background (reference) spectra of an empty well on a microtiter plate were recorded before each sample well measurement. The spectra were measured in the 4000−500 cm^{-1} spectral range, with 32 scans for both background and sample spectra, and using an aperture of 5.0 mm.

Data acquisition and instrument control was carried out using the OPUS/LAB software (Bruker Optik GmbH, Germany).

Spectral preprocessing and data analysis

The whole spectral range was used in the variability study, while the spectral region of 1900 to 700 cm^{-1} was selected for PCA since this spectral region contains bands distinctive for pollen grains (Zimmermann & Kohler 2014; Bağcıoğlu, Zimmermann & Kohler 2015). All spectra were transformed to second derivative form by the Savitzky-Golay algorithm using a polynomial of degree two and a window size of 21 points in total. The window size was chosen based on noise level and digital spacing (Zimmermann & Kohler 2013). Following the transformation to derivative form, the spectra were processed by extended multiplicative signal correction (EMSC), an MSC model extended by a linear, quadratic and cubic component (Kohler *et al.* 2005; Kohler *et al.* 2008; Zimmermann & Kohler 2013). Biochemical similarities between pollen samples were estimated by using principal component analysis (PCA) and variability test based on Pearson correlation coefficients. All pre-processing methods and data analyses were performed using in-house developed routines written in MATLAB 2014a. 8.3.0.532 (The MathWorks, Natick, USA).

RESULTS AND DISCUSSION

Effects of sample preparation

The high throughput FTIR unit employs silicon microtiter plates as sample holders for thin films of pollen samples, created by drying of pollen suspensions. Following the preparation of films, the fully automated transmission measurement of the microplate was performed. The microtiter plate has 384 positions (wells) for sample suspensions, thus 127

samples can be measured in a single run, assuming triplicate measurements per sample and an empty position for background measurement.

Recording of an optimal spectrum critically depends on the preparation of the dry sample film. Our previous study has demonstrated that pulverization and homogenization of pollen by grinding obtains more comprehensive information on grain chemistry than measurement of intact pollen.(Bagcioglu, Zimmermann & Kohler 2015) Moreover, intact pollen grains can create saturation effects when measured in transmission mode (Zimmermann *et al.* 2015a). The saturation effect happens when the central part of the grain is opaque for a large part of the infrared spectrum of the light, and the small fraction of light that reaches the detector has passed through the periphery areas of pollen grain (i.e. grain wall). The resulting spectrum has high baseline contribution and oversaturation of signals, while the spectral information is predominantly obtained from the grain wall. Lastly, intact pollen grains can cause wavelength-dependent scattering effects since the sizes of pollen grains are at the same order as the wavelength range employed in the infrared spectroscopy measurements.

The covered Fagales pollen have grain sizes within 25-50 µm range, and the first step of sample preparation was grain breakdown by sonication treatment. Pollen grains have a double-layered grain wall composed predominantly of natural polymers: cellulose and sporopollenin. The grain wall has exceptional mechanical and chemical resilience and thus offers excellent protection from ambient effects. In particular, the outer layer of pollen grain wall (exine) offers vital protection to physical stress due to remarkable properties of sporopollenins, the highly resistant and complex biopolymers. Therefore, relatively strong

disruptive and prolonged force needs to be applied in order to obtain complete breakdown of pollen samples. As can be seen in Figures 1 and 2, incomplete fragmentation of pollen is obtained after short sonication times, resulting in low quality spectra. On the other hand, prolonged sonication treatment results in extensive fragmentation of sporopollenous exine and homogenization of intracellular material (Figure 1B).

Figure 2 shows the effects of the sonication treatment on the quality of the FTIR spectra. In general, a high quality FTIR spectrum has a flat baseline positioned around zero absorbance and high signal-to-noise ratio (Figure 2). Since a sample film is acquired from 5-10 µL of a liquid sample, the exact quantity and concentration of pollen suspension should be adjusted for obtaining high quality spectra. Ideally, the pollen IR spectra should display an absorbance signal of the amide I band within 0.4-1.0 absorbance range (Kohler *et al.* 2015). Amide I is used as a reference signal since it is usually the strongest absorption band in the FTIR spectra of biological cells. In the principal study, the pollen suspension was prepared by mixing approx. 1 mg of pollen sample with 500 µL of water, which is the minimal liquid amount needed for sonication. Following the sonication, the pollen suspension was centrifuged, concentrated by removing 400 µL of supernatant, and using 8 µL of the remaining suspension for the preparation of sample film, resulting in high quality FTIR spectra (Figure 2). The red spectrum in Figure 2 shows characteristic signals for pollen chemical constituents: carbohydrate at 1200-900 cm^{-1} (C-O-C, C-C and C-O stretching vibrations), sporopollenin at 1605, 1515, 1170 and 835 cm^{-1} (all vibrations are related to phenyl ring vibrations), lipid signals at 1745 (C=O stretch in esters), 1705 (C=O stretch in carboxylic acids), 1470 (CH_2 deformation), and 1170 cm^{-1} (C-O-C stretch), and protein

signals at 1660-1630 (amide I:C = O stretch) and 1555-1530 (amide II: NH deformation and C–N stretch) (Gottardini *et al.* 2007; Zimmermann 2010; Zimmermann *et al.* 2015b). In addition, the spectrum shows signals at 3050 cm^{-1} (=C-H stretch) and 2960-2830 cm^{-1} (C-H stretch in $–CH_3$ and $–CH_2–$) that are predominantly associated with lipids, and in a smaller degree with carbohydrates and proteins as well.

Measurement variability in high throughput FTIR

Dry films can have variations in morphology, including thickness and texture. This leads to noticeable baseline and absorbance variations in their FTIR spectra. Although these variations can be significant, they are predominantly additive and multiplicative effects, and thus can be readily suppressed by spectral preprocessing. In this study, we have converted spectra into second derivatives followed by EMSC, resulting in relatively small variation between replicate measurements. Conversion into second derivatives enhances chemical spectral features and suppresses baseline variations, while EMSC enables correction of wavelength-dependent scattering effects and thus provides better separation of physical (i.e. scattering) and chemical information (Zimmermann & Kohler 2013).

In order to investigate remaining measurement variability (after preprocessing) and to compare it to chemical variability between the samples, a measurement variability test was performed. It was expected that sonication pre-treatment and dry film preparation are the critical steps in high throughput measurement of pollen. Substantial variations in any of these two steps could considerably influence spectral reproducibility. Therefore, the measurement variability was examined regarding variability between sonication preparations and between

technical replicates. The variability between sonication preparations refers to independently prepared suspensions of the same pollen sample by using the same sonication procedure. Technical replicates refer to preparation of dry films, i.e. to repeated FTIR measurements using the same pollen suspension that was applied to different sample positions on the silicon microplate. In addition, variability between microplates and between spectrometers was estimated as well. The variability between microplates was estimated by measurement of the same sample suspension on different silicon microtiter well plates, while the instrument variability was estimated by measuring the same sample plates with two FTIR spectrometers.

The Pearson Correlation Coefficient (PCC), expressed as 1-PCC, was used to estimate the spectral variability of pollen samples (Table 2). 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. The PCC was calculated for two infrared region: 3200-2800 and 1800-1500 cm^{-1}.

As can be seen in Table 2 and Figure 3, the measurement variability is relatively small. The biggest source of variation are technical replicates, i.e. imperfections in preparation of dry films on a microplate.

The variations between measurement microplates, as well as between sample preparations by sonication, were smaller then for the variation caused by technical replicates. The difference between instruments was small, with slightly better precision for newer and more advanced instrument #2 (VERTEX 70 spectrometer). In general, the measurements reproducibility for a given pollen sample is high compared to variability between different

pollen samples. Therefore, the new high throughput FTIR approach can be considered as robust.

Biochemical fingerprints of pollen samples related to taxonomy

The studied set of pollen samples covers related species of Fagales. The set includes six genera of Betulaceae (birch family) and Fagaceae (beech family), with 31 species in total. Moreover, the set contains samples from three distinct pollination seasons from 2012 to 2014, as well as from three distinct locations in the Northern hemisphere: Croatia, Germany and Norway. Therefore, the set offers a broad phylogenetic diversity with good representation at different taxa levels, as well as temporal and spatial diversity with good representation of climate conditions (Table 1).

The data show an expected increase in spectral variability regarding taxonomy, going from species and genera to families and order (Table 2). In addition to the calculation of PCC values, principal component analysis (PCA) was performed to examine main variation patterns in the FTIR spectra (Figure 4). The variability within the different phylogenetic levels is approximately 1 to 2 orders of magnitude higher than on the technical replicates level, and even variability within species can be 20-30 times higher than the technical replicates. One exception is the high consistency of *Fagus sylvatica* pollen composition. *Fagus sylvatica* pollen samples were collected in Norway and Croatia, and they show extremely small intra- and inter-locational variability (Table 2 and Figure 4).

Spectral variability for a range of Fagales species shows high differences in pollen's chemical composition across taxa, in accordance with the published studies (Pappas *et al.*

2003; Gottardini *et al.* 2007; Zimmermann & Kohler 2014; Zimmermann *et al.* 2016). It is important to notice that FTIR spectra present relative chemical composition of pollen grains, and the difference between *Fagus* and *Quercus* pollen composition is a good example. *Fagus* pollen shows higher relative amount of lipids and lower relative amount of proteins than *Quercus* pollen, as indicated by lipid related signals at 1745, 1463 and 1180 cm^{-1} and protein related signals at 1655 and 1545 cm^{-1}. This result is consistent with standard measurements of pollen composition, that have shown pollen protein content of 30.4% and 17.4 % for *Quercus robur* and *Fagus sylvatica* respectively (Roulston, Cane & Buchmann 2000). On the other hand, one needs to take into consideration that a typical pollen grain of *F. sylvatica* has approx. double the size of *Q. robur* grain (radii of approx. 22 and 17 μm respectively). Therefore, regarding absolute chemical content, both species have similar amount of proteins per individual grain, while *F. sylvatica* has significantly higher amount of lipids per grain compared to *Q. robur*. However, our previous FTIR studies have shown that pollen lipids between various species of *Quercus* can vary tenfold, with some, such as *Q. cerris*, *Q. libani* and *Q. frainetto*, having approx. three times higher amounts of lipids that *Q. robur* (Zimmermann & Kohler 2014).

Chemical composition of pollen and phenotypic plasticity

The variability shows high differences in pollen's chemical composition due to either location or pollination season. For example, pollen samples of *Quercus robur*, collected during the two consecutive seasons at the same location in Norway, show big variations in stored triglyceride nutrients (Figure 4 and Figure S1 in the Supporting Information). Higher

triglyceride relative content (compared to sporopollenin) is present in the pollen grains from 2014 pollination season, as indicated by changes in lipid associated signals at 1745, 1470 and 1170 cm^{-1}. Regarding location, *Quercus robur* pollen samples from Germany show even lower amounts of triglycerides. In addition, German samples show slight differences in the sporopollenin composition, as indicated by changes in signals at 1602, 1515, 1171 and 833 cm^{-1}, as well as differences in lipids as indicated by signals at 1744 and 1706 cm^{-1} associated with triglycerides and free fatty acids respectively (Figure S1 in the Supporting Information).

The differences in sporopollenin composition are of interest due to intricate correlation between sporopollenin chemistry and environmental conditions. Sporopollenin is the most complex and robust plant extracellular matrix, and is composed of polyhydroxylated unbranched aliphatic and phenolic constituents (Kim & Douglas 2013). The composition of sporopollenin is not uniform, but rather a group of related polymers with significant taxon-specific variations in chemical composition (Dominguez *et al.* 1999). The main components of sporopollenin are oxygenated phenylpropanoid building blocks, particularly p-coumaric and ferulic acids. Production of these phenolic acids in plants is induced by solar ultraviolet (UV-B) via the phenylpropanoid pathway, the same pathway responsible for synthesis of lignin and suberin biopolymers. Studies have shown that concentrations and ratios of phenylpropanoids in sporopollenin depend on the magnitude of UV-B radiation to which plants had been exposed, and could serve as a proxy for estimating variations in UV-B radiation in palaeoecology (Rozema *et al.* 2009; Fraser *et al.* 2011; Willis *et al.* 2011). Moreover, pollen grains are covered with pollenkit, a sticky pollen coat composed mainly of lipids and plant pigments, and, alike sporopollenin, composition of pollenkit is taxon-specific

(Pacini & Hesse 2005). Both sporopollenin and pollenkit are developed under control of sporophytic genome, as opposed to development of intracellular materials and intine that are under control of gametophytic genome (Blackmore *et al.* 2007). Alongside sporopollenin, pollenkit is also responsible for protection against UV radiation. Therefore, slight differences in the sporopollenin and lipid composition for *Quercus robur* samples collected in Germany and Norway could be result of variations in exposure to UV-B. In general, detailed FTIR measurements of sporopollenin and pollenkit compositions could offer valuable information on plant-environment interaction and photoprotection mechanisms in plants.

Results for spectral variability for *Betula pendula* pollen samples are similar to the results obtained for *Quercus robur* samples, in regards that separations based on seasons and locations are present as well (Figure 5 and Figure S2 in the Supporting Information). In general, the spectra from German samples indicate a lower amount of carbohydrates compared to those of the Norwegian samples. Carbohydrates have several important roles in pollen, including energy reserves and cold tolerance, and all of them would be beneficiary in relatively cold Norwegian environment (Speranza, Calzoni & Pacini 1997). Moreover, inter-annual differences in the Norwegian pollen samples are based on higher lipid and protein relative content (compared to sporopollenin) for the 2014 pollination season. The climate data for Ås (Table S1 in the Supporting Information) shows big difference between the seasons, with 2-4 °C higher average temperature and 50% more precipitation for February-May period in 2014 compared to 2013. Study on pollen of Petunia hybrida, has shown that protein composition was dependent on the day temperature during the plant growth (Herpen 1986). Similar to our result, the total amount of protein was higher for the plant grown at

high temperature. In addition, in case of Petunia hybrid, pollen tube length was positively correlated with growth temperature (Herpen 1986). It has been stipulated that pollen developed at the high temperature has more reserve material and thus makes longer pollen tubes than pollen developed at the low temperature. However, the increase of lipids in pollen with the increase of temperature is opposite to the results of study on Pinaceae pollen. The study on several *Abies*, *Picea* and *Pinus* species has shown negative correlation between pollen lipid content and ambient temperature (Zimmermann & Kohler 2014). Considering that Fagaceae and Pinaceae are far related taxa, such difference in plant-environment interaction is not entirely unexpected. In general, pollen triglycerides are valuable energy and material reserve, and higher amounts of lipids in pollen grain should be advantageous for the grain germination. Nevertheless, triglyceride lipids are not the only nutrient reserve in pollen. For instance, pollen starch reserves can be interconverted to lower molecular weight saccharides as well as lipids, thus creating a complex balance of pollen nutrients (Pacini & Juniper 1984).

The relatively large intra-seasonal variations for *Alnus glutinosa*, *Betula pendula* and *Quercus robur* indicate that differences in pollen composition can be substantial, even for the same species living at the same location. In particular, this is prominent for *Corylus avellana* pollen samples collected in Germany during winter 2014. The samples were collected from six plants, with large differences in pollination phenology spanning more than five weeks. As can be seen in Figure 6, the composition of German *Corylus avellana* pollen samples changes dramatically during the course of the season. The early pollinating German plants (mid January – early February) have the lowest lipid-to-protein ratio, followed by huge

increase in the ratio in mid-pollinating plants (mid February). Late pollinating German plants (late February) have lower lipid-to-protein ratio, and their pollen composition is quite similar to the Norwegian plants.

The high variation in pollen composition could be explained by *Corylus avellana* pollination strategy. *Corylus avellana* starts production of pollen during summer, and in late fall enters a dormancy period (Frenguelli *et al.* 1994). Dormant male catkins have high cold tolerance and can survive severe freezing and chilling conditions. Dormancy is finished with the rise of mean daily temperature, and the beginning of pollination is usually in late winter or early spring. The warming periods can vary substantially from year to year, and thus *Corylus avellana*, as well as other early pollinating species such as *Alnus incana*, often have large inter-annual variations in pollination phenology. Due to different adaptations to cold and warm weather, it can be expected that early pollinating species have larger variations in pollen composition between populations than late pollinators such as *Fagus sylvatica* and *Quercus robur*. Germany has several wild populations of *Corylus avellana* (Wanjiku & Bohne 2015), and it is highly probable that the plants growing in the Berlin botanical garden originate from different wild populations or cultivars. The Norwegian plants belong to the same cultivar, *Corylus avellana* 'Aurea', thus explaining more uniform phenology and pollen chemical composition.

CONCLUSIONS

The high throughput FTIR approach discussed here enables rapid screening of large numbers of pollen samples. High quality spectra can be obtained, provided that sonication

achieves sufficient fragmentation of pollen grains. Measurement variations due to sample preparation, microplate holders and instrumentation are low, and allow differentiation of samples with respect to phylogeny and biogeography. Specifically, the high quality of the data enables an observation of significant inter-annual, intra-seasonal and locational differences in the pollen spectra, which provide evidence that pollen chemical composition has high phenotypic plasticity and is influenced by local climate conditions. The changes are predominantly connected to changes in lipid triglyceride nutrients that play a crucial role in pollen germination and pollen tube growth.(Rodriguez-Garcia, M'rani-Alaoui & Fernandez 2003; Zhang *et al.* 2009) Regarding inter-annual variations, both *Betula pendula* and *Quercus robur* pollen samples from Norway had a higher relative content of triglycerides in 2014 samples compared to the samples from 2013 pollination season. Large inter-seasonal variations in *Corylus avellana* pollen collected in Germany in 2014 are probably associated with population-specific adaptations to quick and early pollination.

The results of the study, conducted on 146 Fagales samples, demonstrate the value of high throughput FTIR approach for the systematic collection of data on ecosystems. The novel FTIR approach offers fast, reliable and economical screening of large number of samples by semi-automated methodology. The high-throughput approach could provide crucial understanding on plant-climate interactions with respect to biochemical variation within genera, species and populations.

Acknowledgements

The research was supported by the Norwegian Research Council (IS-DAAD project No. 233941, DAAD No. 57068987), and by the European Commission through the Seventh Framework Programme (FP7-PEOPLE-2012-IEF project No. 328289 and FP7-IDEAS-ERC-2010-StG project No. 259432). The authors have declared that no conflict of interests exist. **Author Contributions:** Conceived the research idea: AK, BZ, JK. Designed the experiments: AK, BZ, MB. Performed the experiments: BZ, MB, SS. Analyzed the data: AK, BZ, MB. Wrote the paper: BZ, MB. Discussed and revised the paper: AK, JK, SS.

Data accessibility

Spectral data will be stored in the Dryad repository (http://datadryad.org/) after acceptance of the manuscript.

Supporting Information

Figure S1. PCA of IR spectral data set of Fagaceae pollen

Figure S2. PCA of IR spectral data set of *Betula pendula* pollen

Figure S3.Schematic of samples for the variability study

Table S1. Climate data for Ås, Norway, 2013-14

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Table 1. List of the pollen species. Sampling locations: **N** – Norway, **G** – Germany, **C** – Croatia.

Family	Genus	Species	N		G	C			Total number
			2013	2014	2014	2012	2013	2014	
Fagaceae	***Quercus***	*Q. cerris*		1		1			2
		Q. cocciena		1					1
		Q. shumardii				1			1
		Q. palutris		1					1
		Q. robur	4	7	2		1		14
		Q. libani				1	1	1	3
		Q. ilex					1		1
		Q. petreae		1		1	1		3
		Q. faginea					1		1
		Q. frainetto					1		1
	Fagus	*F. sylvatica*		6		1	6		13
Betulaceae	***Betula***	*B. pendula*	3	6	4	1	4	1	19
		B. alleghaniensis	1	2					3
		B. divaricata		1					1
		B. ermanii	2	2			1		5
		B. lenta		2					2
		B. papyrifera	1	2					3
		B. pubescens		2					2
		B. raddeana	1						1
		B. utilis	1	4					5

	B. fruticosa	1	1					2
Alnus	*A. glutinosa*	1	10	3	1			15
	A. hirsuta	1	2					3
	A. incana	4	9	1	1			15
	A. viridis	1	3					4
Corylus	*C. americana*	1	2					3
	C. avellana	4	1	6	1			12
	C. chinensis				1			1
	C. maxima	2		3				5
	C. cornuta	1	1	1				3
Ostrya	*O. virginiana*		1					1

Table 2. Variability for different technical replicates, sonications, microtiter plates, instruments, taxa, and pollination seasons. # designates number of spectra used in the variability tests.

Type of variability	3200-2800 cm^{-1} (1-*PCC**)x10^{-4}	1800-1500 cm^{-1} (1-*PCC**)x10^{-4}	Number of spectra
Technical replicates; instrument #1 [1]	0.70±0.40	3.09±2.47	100
Sonications; instrument #1	0.37	1.23	10
Microplates; instrument #1	0.31	1.75	5
Technical replicates; instrument #2 [1]	0.43±0.25	2.28±1.63	100
Sonications; instrument #2	0.31	1.09	10
Microplates; instrument #2	0.08	0.58	5
Instruments [1]	0.63±0.10	0.40±0.04	100
Within Fagales	20.47	82.72	146
Within Fagaceae	9.59	62.21	41
Within Betulaceae	19.09	66.04	106
Within *Alnus*	16.88	44.40	37
Within *Corylus*	16.42	62.18	23
Within *Betula*	18.13	54.45	43
Within *Quercus*	10.55	44.20	28
Within *Alnus hirsuta*	8.05	19.54	3
Within *Alnus viridis*	9.87	30.14	4
Within *Alnus incana*	12.63	30.25	15
Intraseasonal of *Alnus incana*, Norway 2014	12.78	16.75	9
Within *Alnus glutinosa*	16.73	44.17	15
Intraseasonal of *Alnus glutinosa*, Norway 2014	8.40	35.68	10

Within *Corylus avellana*	15.64	69.08	12
Intraseasonal of *Corylus avellana*, Germany 2014	8.89	61.62	6
Intraseasonal of *Corylus avellana*, Norway 2013	3.93	10.02	4
Within *Betula pendula*	18.69	44.85	19
Interannual of *Betula pendula*, Norway	18.29	44.46	9
Intraseasonal of *Betula pendula*, Norway 2014 [4]	15.59	19.50	4
Intraseasonal of *Betula pendula*, Norway 2013 [5]	11.14	25.09	3
Within *Quercus robur*	10.50	29.29	28
Interannual of *Quercus robur*, Norway	8.90	25.04	11
Intraseasonal of *Quercus robur*, Norway 2013	7.14	6.56	4
Intraseasonal of *Quercus robur*, Norway 2014	6.03	22.67	7
Within *Fagus sylvatica*	0.99	3.91	13
Intraseasonal of *Fagus sylvatica*, Norway 2014	0.84	4.39	6
Intraseasonal of *Fagus sylvatica*, Croatia 2013	1.05	2.91	6

[1]Based on 10 samples with 10 technical replicates each (± one standard deviation)

[4] Based on 4 samples from the same population, collected in Norway 2014

[5] Based on 3 samples from the same population, collected in Norway 2013

Figure legends

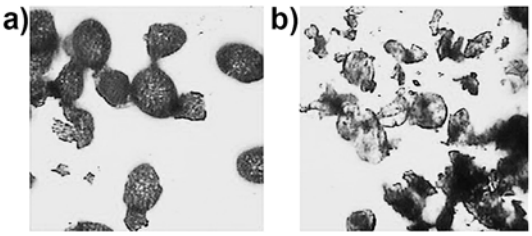

Figure 1. Optical microscopic images of *Quercus robur* (Pedunculate oak) pollen grains with respect to different sonication treatment: A) 1 min, and B) 2 min.

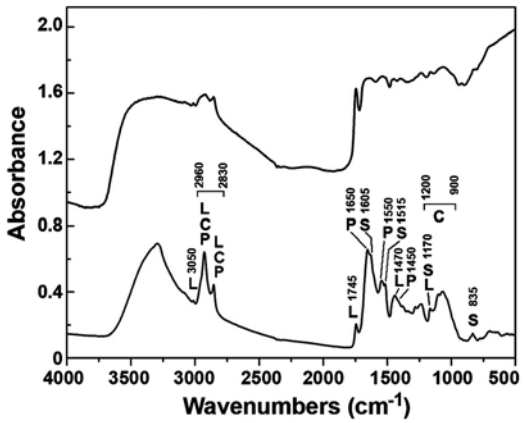

Figure 2. FTIR spectra of *Alnus glutinosa* (Black alder) pollen showing effect of sample preparation. Spectra obtained after: 1 min of sonication (top) and 2 min of sonication (down). The marked bands are associated with molecular vibrations of (P) proteins, (L) lipids, (C) carbohydrates and (S) sporopollenins.

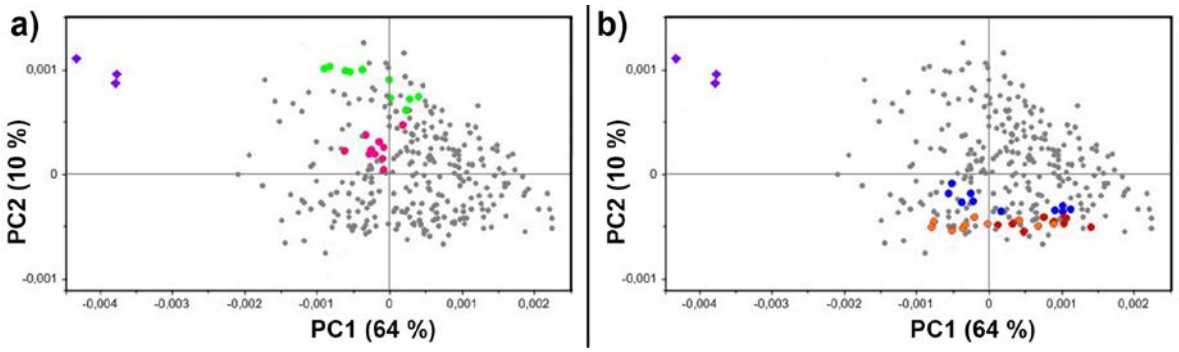

Figure 3. (A) Variability between the two independently prepared pollen suspensions of the same sample (green and magenta); each sample includes 10 technical replicates. (B) Variability between the two microplates (orange and dark red) and between the two instruments (dark red and blue); each measurement includes 10 technical replicates. In addition, the PCA score plots include 300 measurements of a *sonication variability test plate* and a *microplate variability test plate* in two instruments (dots). The control sample (purple diamonds) is pollen sample from different *Alnus incana* tree, belonging to the same population and collected on the same day as the sample for the variability study.

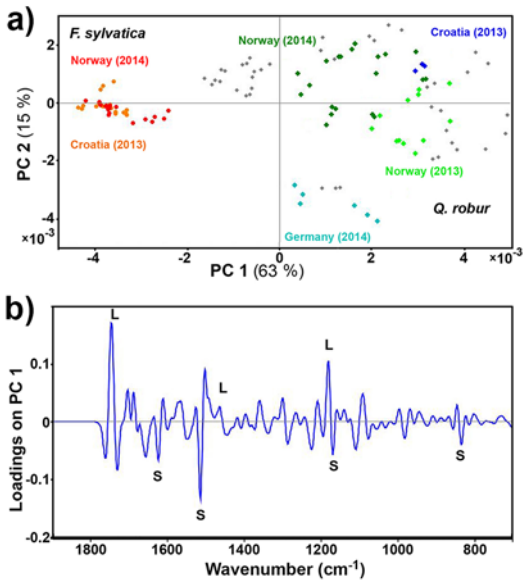

Figure 4. PCA plot of IR spectral data set of Fagaceae pollen (three spectra per sample), with depiction of plant genera (dot=*Fagus*, diamond=*Quercus*), locations and seasons. The percent variances for the first five PCs are 63%, 15%, 9%, 4% and 2%. (B) Loadings plot on the first principal component of the PCA; the marked signals are associated with vibrational bands of lipids (L) and sporopollenins (S).

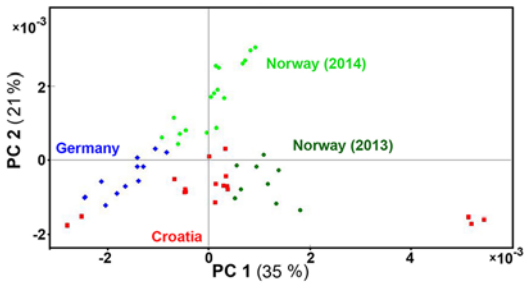

Figure 5. PCA plot of IR spectral data set of *Betula pendula* pollen (three spectra per sample), with depiction of plant locations and seasons. The percent variances for the first five PCs are 35%, 21%, 14%, 9% and 9%.

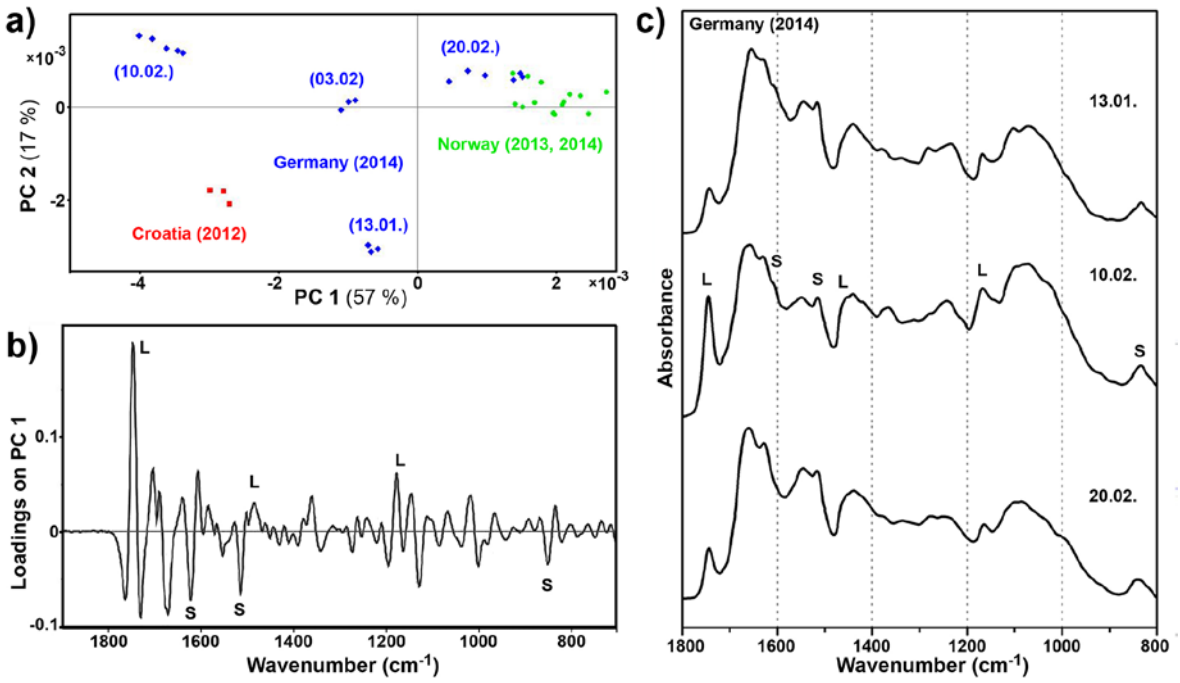

Figure 6. (A) PCA plot of IR spectral data set of *Corylus avellana* pollen (three spectra per sample), with depiction of plant locations and seasons. The percent variances for the first five PCs are 57%, 17%, 13%, 5% and 3%. (B) Loadings plot on the first principal component of the PCA. (C) *Corylus avellana* pollen spectra of samples collected at different dates in Germany 2014; the marked signals are associated with vibrational bands of lipids (L) and sporopollenins (S).