New methods in Palaeopalynology: Classification of pollen through pollen chemistry

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Thesis for the degree of Philosophiae Doctor (PhD)
University of Bergen, Norway
2021

UNIVERSITY OF BERGEN

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Date of defense: 04.11.2021

Year: 2021

Title: New methods in Palaeopalynology: Classification of pollen through pollen chemistry

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Print: Skipnes Kommunikasjon / University of Bergen

Scientific environment

This thesis was written at the Department of Biological Sciences of the University of Bergen. The candidate, Florian Muthreich, is part of the PalaeoChem lab within the Ecological and Environmental Change Research Group (EECRG). This work is part of the PollChem project funded with a FRIPRO Grant from the Research Council of Norway (PollChem 249844). The extensive field work was possible thanks to funding from the L. Meltzer Høyskolefond (2017/05/LMH) and the Olaf Grolle Olsen Legat (2017/52/FOL).

Acknowledgements

This thesis would not be possible without the collaboration of many friends and colleagues, whose input and discussions I greatly appreciate and value. Even though I often will write in the first person, many of the decisions were informed after discussions with colleagues, friends and supervisors.

Many thanks to Alistair Seddon for his trust and mentorship. The pollen sampling and collection of reference material was possible due to help from Sally Dawson, who showed me the *Eucalyptus* collection at the herbarium in Kew Gardens, London and from Nigel England from the Australian tree seed centre at CSIRO and Matthew Parker from the National Arboretum Canberra, who helped me to identify and collect *Eucalyptus* pollen in and around Canberra. Janelle Stevenson and Simon Haberle from ANU graciously hosted me in Canberra, where we visited Bega Swamp. I hope to return to Bega, to explore finally the *Eucalyptus* record in the future. Many thanks to Carlos Vila-Vicosa, without him the *Quercus* data-set would not have been possible. Thanks to Simon Honey from the Royal Botanic Garden Edinburgh, the staff at royal botanical gardens Victoria and Donatella Magri, who provided *Quercus* pollen from their collections

Thanks to Achim Brauer, Francesco Muschitiello and Daniela Festi, who provided sediment samples for analysis of fossil pollen. Unfortunately, I was not able to analyse them all, but hope to return to those samples in the future. Thanks to Linn Krüger, Silje Östman and Arild Breistøl for assistance in the lab and introduction to the pollen laboratory. Also, to Anne Bjune, who answered my organisational question or told me who I should ask instead. Thanks to Boris Zimmermann for explaining me the inner workings of FTIR and Raman and answering my questions.

Lastly, thanks to my family at home in Germany for the support and to Ragnhild Holtet for giving me time in the last weeks of writing this thesis. I am eternally grateful to Mari for being there and taking this journey with me and thanks to Leonora and Luna for being the light in my heart to distract me when I needed it most.

Abstract

Pollen grains are one of the primary tools of palaeoecologists to reconstruct vegetation changes in the past. The description, counting and analysis of pollen grains (palynology) has contributed to our understanding of establishment and dynamics of past and present plant communities. Advances in identification accuracy, precision and increased taxonomic resolution have greatly improved our understanding of biogeography and plant community interactions. Nevertheless, the techniques by which palynological studies are performed have not fundamentally changed. Taxonomic resolution and automation have been identified as some of the key challenges for palynology and palaeoecology. Chemical methods have been proposed as a potential alternative to morphological approaches and have demonstrated promising results in the classification of modern pollen grains and in the analysis of pollen chemical responses to UV-B radiation. The application of chemical methods for palynological needs have not been thoroughly explored, with analysis of (sub-)fossil pollen lagging behind their modern counterpart. Especially the application of infrared methods have gained popularity as an alternative to traditional morphological approaches.

In this thesis, I explore the use of infrared methods for palynological applications, by exploring the chemical variation in modern pollen grains and in the analysis of fossil pollen grains with IR microscope approaches. The objectives of this thesis are formulated into three research objectives:

- Collect modern pollen and explore the variation in chemical composition
- Apply chemical methods to fossil material
- Explore microscopy chemical methods on modern pollen

The thesis is structured into four studies to study these objectives. Papers I and II explore variation and classification based on the chemical composition of modern *Quercus* pollen using two IR approaches, Fourier transform infrared spectroscopy (FTIR) and Fourier transform Raman spectroscopy (FT-Raman). After exploring modern chemical composition of pollen, paper III investigates FTIR methods for the analysis of fossil pollen, in spectra of Holocene *Pinus* pollen. Additionally, the effects of acetolysis and

density separation on *Pinus* pollen is described. Paper IV addresses the challenge of scattering signals when measuring small pollen grains of four *Quercus* species with FTIR microscopy and ways to surpress or weaken the scattering signals.

The results from paper I and II show classification success, surpassing traditional morphological approaches, at the *Quercus* section level and ~90% recall on species level with both IR approaches. Chemical bands most useful for classification are lipids, sporopollenin and proteins for both FT-Raman and FTIR. We observe differences in the importance of chemical functional groups for the classification. FT-Raman relies more on sporopollenin chemistry, while FTIR utilizes more variation in lipid bands. After finding considerable variation in sporopollenin chemistry in modern pollen samples, FTIR methods were applied to pollen from sediment cores spanning the Holocene. Paper III examines the differences between modern and sub-fossil pollen and reported large differences between them, mainly the removal of labile components, such as lipids and protein peaks from the sub-fossil spectra during diagenesis. Additionally, paper III finds changes to pollen chemistry caused by acetolysis in the 1200 - 1000 cm^{-1} region of the spectra, when comparing acetolysed spectra to non-acetolysed spectra. The paper concludes with findings of unwanted inorganic signals (BSi) and contamination from density separation media in the sediment pollen spectra. Paper IV demonstrates two successful methods of removing scattering signals from pollen spectra. Two approaches were examined, embedding and processing with signal correction algorithms. Spectra from embedded pollen have no scattering anomalies, but part of the spectra is unusable, because of absorbance of the embedding matrix (paraffin). The signal processing algorithm removes most of the scatter components and allows the scatter components to be extracted. Classification of the different data-sets (spectra without correction, embedded spectra, processed spectra, scatter parameters) reveals that scatter correction methods reduce classification success and that scatter parameters contain taxonomic information. This suggests that scatter corrections may not be the best approach for applications mainly focused on classification or identification, while reconstructions of, for example, UV-B radiation may benefit from scatter correction methods, when measuring single grain spectra.

This thesis shows that the performance of IR methods surpasses traditional morphological methods for pollen classification and that a considerable amount of taxonomic infor-

mation is stored in functional groups associated with sporopollenin (phenylpropanoids). In a study on fossil pollen, this thesis demonstrates that conventional chemical extraction methods, such as acetolysis, alter the chemical composition of pollen and may not be ideal for palaeochemical purposes. Additionally, the scatter correction methods show that IR can provide non-chemical information in the form of scatter parameters, which contain taxonomic information. These results are useful additions to the growing knowledge on chemical methods for palaeoecological and palynological analyses.

List of Publications

Paper I

Muthreich, F., Zimmermann, B., Birks, H.J.B., Vila-Vicosa, C.M., Seddon, A.W.R. 2020. Chemical variations in *Quercus* pollen as a tool for taxonomic identification: Implications for long-term ecological and biogeographical research. *Journal of Biogeography*, 47:1298-1309. https://doi.org/10.1111/jbi.13817

Paper II

Muthreich, F., Tafintseva, V., Zimmermann, B., Kohler, A., Vila-Vicosa, C.M., Seddon, A.W.R. 2021. Evaluating the use of FT-Raman spectroscopy for pollen chemical characterization (*Manuscript*)

Paper III

Muthreich, F., Zimmermann, B., Seddon, A.W.R. 2021. Assessing variations in the chemistry of subfossil and modern *Pinus* pollen (*Manuscript*)

Paper IV

Muthreich, F., Heitmann Solheim, J., Almklov Magnussen, E., Kohler, A., Tafintseva, V., Seddon, A.W.R., Zimmermann, B. Analytical and experimental solutions for pollen measurements by Fourier transform infrared microspectroscopy. (*Manuscript*)

Contributions to other publications during the PhD period

Heitmann Solheim, J., Borondics, F., Zimmermann, B., Sandt, C., **Muthreich, F.**, Kohler, A. An automated approach for fringe frequency estimation and removal in infrared spectroscopy and hyperspectral imaging of biological samples. (*Submitted to Journal of Biophotonics*)

Author Contributions

Author contribution roles following CRediT.

Table 1: Author contributions following CRediT. Authors listed alphabetically. Initials used in the list: FM, Florian Muthreich; BZ, Boris Zimmermann; AWRS, Alistair Seddon; HJBB, John Birks; CVV, Carlos Vila-Vicosa; AK, Achim Kohler; VT, Valeria Tafintseva; JHS, Johanna Heitman Solheim; EAM, Eirik Almklov Magnussen

Task	**Paper I**	**Paper II**	**Paper III**	**Paper IV**
Conceptualization	AWRS, BZ, **FM**, HJBB	AWRS, BZ, **FM**	AWRS, **FM**	BZ, **FM**,
Data curation	CVV, **FM**	CVV, **FM**, VT	AWRS, **FM**	**FM**
Formal Analysis	AWRS, BZ, **FM**	AWRS, **FM**, VT	**FM**	EAM, **FM**, JHS, VT
Funding Aquisition	AWRS, **FM**	AWRS, **FM**	AWRS	**FM**, BZ, AWRS
Investigation	AWRS, BZ, **FM**	CVV, **FM**	**FM**	BZ, EAM, JHS
Methodology	AWRS; BZ, CVV, **FM**	BZ, VT	AWRS, BZ, **FM**	AK, BZ, EAM,**FM**, JHS, VT
Resources	AWRS, BZ	AK, BZ	AWRS, BZ	AK, BZ
Software	**FM**	**FM**, VT	**FM**	BZ, EAM, JHS, VT
Validation	BZ, **FM**	**FM**, VT	AWRS, BZ, **FM**	AK, BZ
Visualization	**FM**	**FM**	**FM**	BZ, **FM**
Writing original Draft	AWRS, BZ, **FM**	AWRS, BZ, **FM**, VT	AWRS, BZ, **FM**	BZ, **FM**
Writing review and editing	AWRS, BZ, CVV, **FM**, HJBB	AWRS, BZ, CVV, **FM**, VT	AWRS, BZ, **FM**	AK, AWRS, BZ, EAM, **FM**, JHS, VT

Contents

1 Background

Sub-fossilised remains of plants and animals are used by palaeoecologists to reconstruct vegetation changes in the past. The pollen produced by plants is both abundant and easily preserved (e.g. in bogs and lakes), which makes it a useful tool to investigate vegetation changes over thousands of years. The study of pollen grains (palynology) has a long tradition of reconstructing past vegetation since the presentation and publication of the first pollen diagram by Lennart von Post (von Post 1916, 1918). Von Post counted arboreal pollen from peat samples in southern Sweden and showed changes in relative abundance (Birks and Berglund 2018). Although the fundamentals and theory behind pollen analysis have changed remarkably little since Von Post, newer methods for the extraction, description, radiometric dating and analysis of pollen and their sediment samples (Erdtman and Praglowski 1959, Fægri and Deuse 1960, Fægri and Iversen 1989) have allowed insights into the past to become more detailed and complex: from descriptive studies of past vegetation, to gaining new information of long-term successional processes, the establishment of and dynamics of plant communities (Delcourt et al. 1982, Ritchie 1995, Mitchell and Cole 1998) and temporal and spatial changes in plants communities as linked to climate and anthropogenic change (Webb 1986, Odgaard 1999, Haskell 2001, Davis and Shaw 2001, Birks 2019). Reliable and detailed identification of pollen or other sub-fossil remains is an integral part of detailed environmental, archaeological and ecological reconstructions, which can give answers to complex questions. Advances in the quality of plant fossil identification have played a key role in improving our understanding of historical plant geography (Godwin 1975, Lang 1994, Magri et al. 2006, Birks 2008, 2014, Birks and Berglund 2018).

Nevertheless, taxonomic resolution remains a challenge for palynology and palaeoecology as a whole, because pollen data are often constrained by low taxonomic resolution (Rull 2012). Current taxonomic resolution of pollen analysis is limited by a number of factors, including: i. technical limitations to the optical resolution of light microscopes; ii. limitations of modern reference collections resulting in insufficient sample sizes; iii. variety in reference collections based on regional differences; iv. the abilities or confidence of an expert palynologist to identify pollen at the lowest taxonomic level.

Difficult to identify taxa are often classified to the level the analyst feels comfortable, which can be sub-genus, genus or even family in some cases (Prentice 1988). In the age of big data (Brewer et al. 2012), this poses a challenge when combining several datasets originating from different analysts and from different locations. To counteract differences in taxonomic resolution, often the broader classification is chosen to opt for more data instead of more detail. The reliable identification of pollen is a requirement for palaeoecological reconstructions of past environments (Mitchell et al. 2014) and has implications for the modelling of past and future vegetation/climate responses (Finkelstein et al. 2006).

Pollen data with too low taxonomic resolution may not provide the necessary detail for reconstructions, e.g. related to assessing human impact (Deza-Araujo et al. 2021). Anthropogenic pollen indicators have been established based on their modern occurence as agriculturual plants or weeds, yet only a few are identified to species, while a number of domesticated species is grouped in a few families (e.g. Brassicaceae). An increase in taxonomic precision would help to separate the influence of anthropogenic land-use on vegetation change from other drivers, such as climate. Furthermore, improved precision of taxonomic identification would improve fossil pollen as a record of past biodiversity (Odgaard 1999). Environmental and ecological preferences are more detailed and defined for species than they are for higher taxonomic groupings. Pollen-based biome reconstructions, for example, would be improved by higher taxonomic resolution, because certain taxon associations have persisted for long time periods, but changes in species associations may be masked by the taxonomic resolution of the pollen data. Williams et al. (2004) report, for example, an association between *Carya* and *Quercus* for over 21,000 yrs, but both taxa represent a variety of species with specific preferences and associations that occupy distinct niches and biomes. Another example is the distribution and abundance of oaks, which are usually identified to two sub-genus morphotypes, but a recent attempt to model future responses of *Quercus* in Europe using fossil pollen were based on *Quercus* pollen resolved to genus (Nogués-Bravo et al. 2016). *Quercus* species in Europe often have distinct geographical distributions and species-specific responses to climate (Acácio et al. 2017). Limitations on taxonomic resolution (e.g. *Quercus* or similar taxa) have implications for the reconstruction and interpretation of past environments using modern analogues and the modelling of past and future vegetation/climate

responses, and hinders the ability to develop accurate predictions.

1.1 Technical limitations

Light microscopy (LM) is most commonly used to identify pollen, because of its ease of use and long established standardized protocol for pollen extraction from sediment samples (Erdtman and Praglowski 1959, Fægri and Deuse 1960, Fægri and Iversen 1989). There have been innovations, such as extraction of pollen from sediments avoiding strong acids (e.g. density separation using sodium polytungstate, see Regnéll and Everitt 1996, Nakagawa et al. 1998) and technical improvements to LM, but the actual description and counting of pollen has largely stayed the same since Erdtman and Praglowski (1959), because of its efficiency in allowing the counting of a large number of pollen grains (e.g. 300) extracted from the same sample. Scanning electron microscopy (SEM) is a method with much higher resolution and detail, which is also more expensive, requires special expertise, additional sample preparation and remains a tool for detailed morphological description of pollen for taxonomic purposes (Solomon 1983a, 1983b, Denk and Grimm 2009, Denk and Tekleva 2014) instead of vegetation-assemblage reconstruction.

1.2 Limitations of expert evaluation

Pollen counting is a subjective process according to Stillman and Flenley (1996), who called it the 'personal equation' that influences identification. Other decisions, such as deciding which grains are within the counting window or access to and training with reference collections are part of the 'personal equation'. Palynologists do their best to ensure consistency between analysts and between labs through reference databases (Martin and Harvey 2017) or counting protocols (Regal and Cushing 1979, Fægri and Iversen 1989), which standardise decisions on which grains are counted. Nevertheless, there are differences in the specifics of how pollen is counted between individual palynologists, laboratories and communities.

One aspect of pollen counting is the total number of pollen grains counted for each sample. Pollen counting using LM is time consuming and rarely are all pollen grains

counted that are found within a given sample slide. The number of samples and pollen grains counted are often limited by the time or budget allocated to palynological analysis. The recommendation is that at least 500-1000 grains are counted per sample to estimate rare taxa sufficiently (Birks and Birks 1980, Moore et al. 1991, Bennett and Willis 2002, Weng et al. 2006), but this threshold is not always met and other studies found lower pollen counts sufficient, e.g. 200-250 (Barkley 1934, Hill 1996) or as low as 150 (Lytle and Wahl 2005). Generally, pollen counts of ~300 grains are widely used (Birks and Birks 1980, Keen et al. 2014). Pollen assemblages are biased representations of their parent vegetation, and the variance in richness or evenness of this vegetation. Pollen-count sums that are too low may therefore under- or over-estimate the importance of some taxa compared to others, based on the abundance/ rarity of specific pollen types. Furthermore, pollen assemblages are biased towards species that have a high pollen productivity or disperse pollen well, e.g. wind-pollinated species. Tree and grass taxa are overrepresented in pollen assemblages, as opposed to other flowering plants.

In addition, there may be human biases to expert evaluation, such as declining precision due to fatigue, over familiarity with certain samples or other identification biases due to reference-library access or study area familiarity (MacLeod et al. 2010). Studies that evaluate expert accuracy and reproducibility are not very common (Gobalet 2001, Kelly 2001, Culverhouse et al. 2003), but have shown in some cases inconsistencies between experts and in some cases call into question the accuracy of reported data (Gobalet 2001). These examples are from other proxies (e.g. dinoflagelates), but the underlying problem is the same for pollen.

Studies that evaluate automated approaches' consistency compared to palynologists' give some insight into palynologists' performance in counting pollen grains, even though they are the benchmark standard in this example. Holt et al. (2011) shows that pollen-count standard deviations are higher for human experts compared to their automated pollen counting system (Autostage). Unfortunately accuracy was not evaluated. Tcheng et al. (2016) develop a system to differentiate two types of spruce and evaluate the performance of human experts to identify the *Picea* variants on slides with different ratios of the two pollen variants. Their results show that human experts generally overcounted the variant that was less represented on the slide, while their automated system counted closer to the

true ratios. These findings show that there is considerable variability, even though these tests were very specific examples aimed at evalutating the performance of automated approaches. Studies on the accuracy of palynologists ability to identify pollen would be a vital resource to evaluate the performance of automated approaches and evaluate uncertainty in pollen reconstruction. Some taxa are very hard to identify, even for experts of those genera, which implies a certain degree of imprecision and/ or inaccuracy to palynology, that is not well studied, but accepted as the 'personal equation'.

1.3 New approaches to an old problem

One possible development in palynology which may help resolve issues related to observer bias and data collection speed is automation. For the past 25 years several studies have outlined the prospect and challenges of automation in palynology (Stillman and Flenley 1996, Li et al. 2004, Hodgson et al. 2005, Holt and Bennett 2014), with promising results for partly- or fully-automated systems (Holt et al. 2011, Punyasena et al. 2012, Holt and Bebbington 2014, Riley et al. 2015, Tcheng et al. 2016, Sevillano et al. 2020). Alternatively, chemical methods have been developed (with focus on using infrared (IR) radiation, e.g. Laucks et al. 2000, Dell'Anna et al. 2009, Zimmermann 2010) to provide more objective techniques for identification with potential for automation. The potential of these approaches was of interest at the start of my studies, and below, I outline some fundamentals of these approaches.

1.3.1 Computer Vision approaches

Automation of pollen counting has been recognized as a solution to address a number of the disadvantages in palynology (Stillman and Flenley 1996). Some of these I have described above: limits to taxonomic resolution, objectivity of human palynologists, pollen counting takes a long time. Additionally, they identify the need for finer resolution and larger counts, to discover more detailed vegetation responses to e.g. climate change or human impact from pollen records.

New methods and techniques have consistently been developed parallel to the refinement

of palynology that aim to reduce the time spent counting pollen. They range from improved databases to aid identification (Walker et al. 1968, Guppy et al. 1973) to texture analysis based on digitised images of the exine surfaces (Langford et al. 1990). With advances in computing capacity and performance, analysis of digital pollen images assisted by machine-learning techniques have resulted in a number of studies. These approaches are generally using morphological parameters extracted from images (Kaya et al. 2013, Mander et al. 2013, Tcheng et al. 2016) or image-stacks (Punyasena et al. 2012), which are used for further identification using custom algorithms (Tcheng et al. 2016) or machine-learning methods (e.g. k-nearest-neighbour, Mander et al. 2013, or artificial neural networks Holt et al. 2011). In parallel, the challenge of pollen detection and segmentation on images has been investigated by analytical methods (Landsmeer et al. 2009, Tcheng et al. 2016) and an automated microscope which captures images of pollen directy from sample slides (Allen 2006, Holt et al. 2011). These approaches mostly rely on the extraction of morphological and surface texture parameters (size, tectum thickness, shape, etc.) instead of using the images directly. With advances in computer vision, neural networks have became more and more sophisticated and capable of difficult identification and segmentation tasks (He et al. 2015, Ren et al. 2015, Szegedy et al. 2015b, Krizhevsky et al. 2017, Yu et al. 2019). These developments have found application in palynological studies (Sevillano et al. 2020) and have great potential in the automation of pollen counting.

1.3.2 Infrared Spectroscopy

Chemical analysis of pollen grains is an alternative approach to automation using computer-vision and machine-learning approaches. Infrared spectroscopy is a fast, inexpensive and reproducible method to collect information about the chemical composition of a sample (Zimmermann et al. 2017, Mondol et al. 2019). It gained popularity in the ecological sciences and later palaeoecology after infrared spectrometers became more accessible and affordable. In IR-spectroscopy, the sample is irradiated with infrared light and specific chemical-functional groups absorb the IR radiation at specific wavelengths. Different types of bonds absorb IR radiation, which causes characteristic types of vibrations or stretching of particular bonds. The difference in IR intensity

(absorbance) is recorded and called a chemical spectra. One type of IR spectroscopy is Fourier transform infrared spectroscopy (FTIR). FTIR is more sensitive to vibrations of polar bonds, found in carbonyl-functional groups (alcohols, ethers, esters, etc.). The spectra provided by FTIR spectrometers provide balanced information on a variety of types of chemical compounds, lipids, carbohydrates, proteins and more complex compounds, such as sporopollenin.

A second type of IR spectroscopy, Raman spectroscopy operates in much the same way but with important differences. Raman causes stronger vibrations in different types of functional groups than FTIR, namely carbon-to-carbon bonds and hydrocarbons (e.g. aliphatic bonds). Raman spectroscopy is based on inelastic scattering of photons, when the photons interact with matter they transfer energy (matter gains vibrational energy) and the photon changes direction (Raman scattering). The energy of the photons is shifted, which gives information about chemical composition. This information is complementary to FTIR spectroscopy, where Raman preferentially induces vibrational modes to non-polar based chemical functional groups (e.g. carotenoids, sporopollenin, etc.). This sensitivity to aliphatic functional groups potentially makes Raman a better choice, when samples are rich in complex compounds such as lignin, carotenoids and sporopollenin. There are, however, a number of undesired effects that occur when analysing pollen samples with Raman, especially fossil pollen. First, unequal absorption of radiation can reduce Raman-band intensities, which is caused by near-IR absorption by the sample, and which can weaken the incident laser. Second, a common problem for pollen is intense heating and in some cases thermal decomposition, caused by absorption at the laser wavelength. Adjustments to Raman spectroscopy, such as different lasers are used to address these problem with varying results (Chase 1986, Baranski et al. 2005, Kairyte et al. 2012).

1.3.3 Application of IR in Ecology and Palaeoecology

The study of pollen grains using IR methods is relatively young, even though the chemical composition of pollen has been studied before, (e.g. Todd and Bretherick 1942, Baker and Baker 1979, Hemsley et al. 1996, Moore et al. 2006). However, since the beginning of my PhD in 2016, the application of IR spectroscopy to palaeoecological

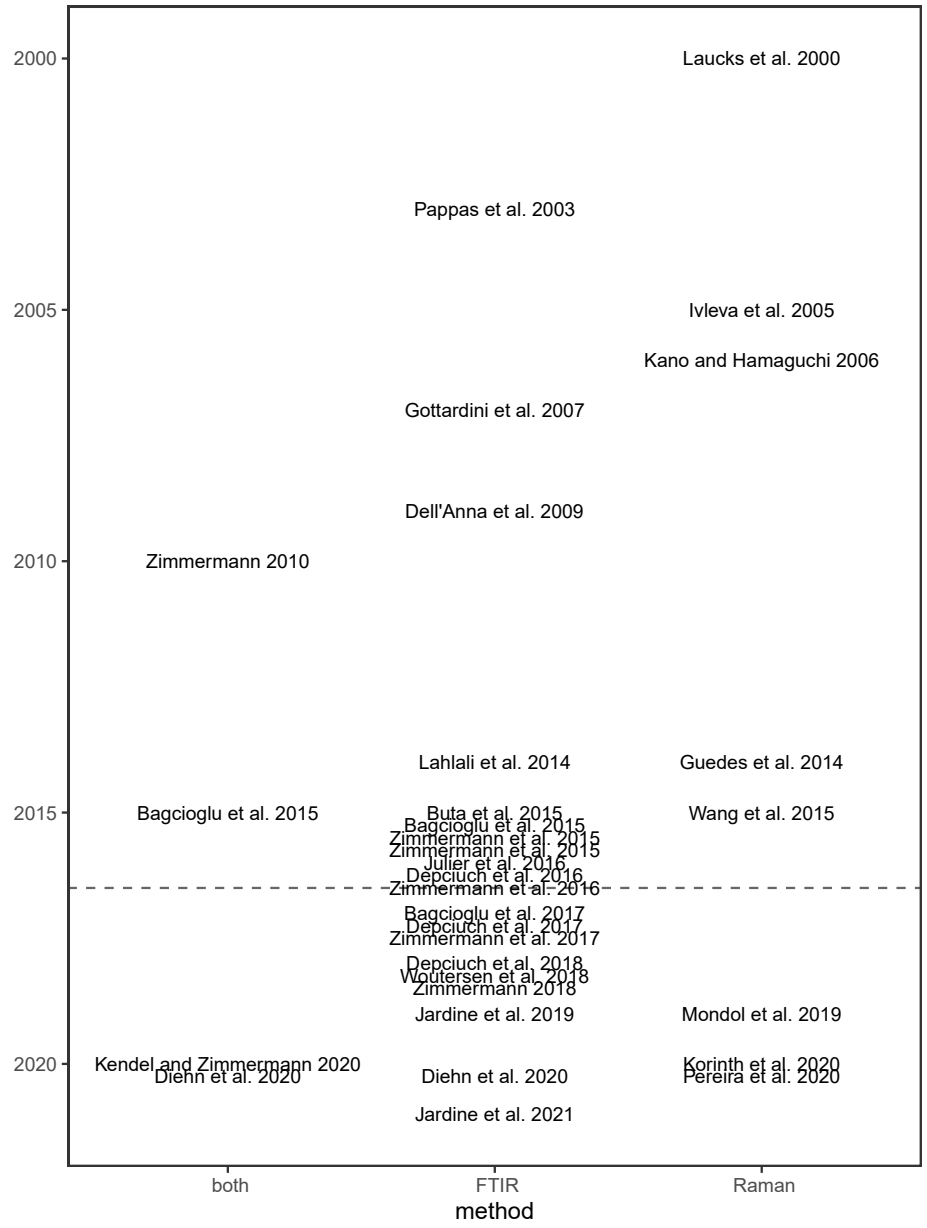

Figure 1: Studies published between 2000 and 2021 using IR spectroscopy to identify or classify pollen grains. Studies are grouped if methods used were FTIR, Raman or both approaches. Not included are studies focused purely on effects of UV-B radiation on pollen or other chemical methods (e.g. MALDI-TOF). Dashed line represents the start of my PhD.

research, in addition to other related pollen-chemistry methods (including pyrolysis-Gas Chromatography Mass Spectrometry, Bell et al. 2018, Seddon et al. 2021) has seen rapid progress and development. Figure 1 shows the progression in the field since 2000. Since a large number of the studies that have a palaeoecological application in mind (in terms of classification of pollen grains) were published during or shortly before I started my PhD in 2016, I will discuss these more recent studies together with my findings in the discussion. In this section I will describe the overall state of the knowledge that was available at the time and how that informed objectives for this thesis.

Most studies present classifications of tree and grass pollen types that are common allergens (e.g. Poaceae, *Alnus*, *Tilia*, etc) and outline the potential of IR for the automization of allergenic monitoring and identification of bioaerosols (Laucks et al. 2000, Pappas et al. 2003, Ivleva et al. 2005, Gottardini et al. 2007, Dell'Anna et al. 2009, 2009, Zimmermann 2010, Guedes et al. 2014, Wang et al. 2015, Zimmermann et al. 2015b, 2016, 2017). These studies generally have high accuracy (>90% recall) and a high number of different taxa. A variety of IR methods are employed in these studies, some use bulk FTIR (e.g. Zimmermann 2010), some use IR microscopy (Raman and FTIR) to analyse single grains (e.g. Ivleva et al. 2005, Dell'Anna et al. 2009), whilst others use bulk Raman measurements (e.g. Laucks et al. 2000). What all of these studies have in common is that they use modern material collected from public parks, botanic gardens or purchased from commercial suppliers. The results from these studies show that a variety of IR methods (FTIR, Raman, bulk, microscopy) are capable of identifying pollen based on their chemistry.

Based on these findings, building a system that could reliably identify pollen using their chemical composition seems feasible, considering the promising performances of Pappas et al. (2003), Dell'Anna et al. (2009), Zimmermann (2010) and Zimmermann et al. (2015b), which carried out analyses across a diverse range of taxa. From my perspective, these previous studies show the powerful capabilities of IR methods and show promising results for application of chemical identification using FTIR or Raman microscopy. At the same as FTIR methods were developed for pollen classification, there were parallel developments happening in UV-B research where IR and other chemical methods were being applied to fossil pollen grains to recover a UV-B signal (Blokker et al. 2005, 2006,

Willis et al. 2011, Jardine et al. 2016).

Nevertheless, if chemical methods are to be applied to palaeoecological research, it was clear that there were challenges that had to be overcome. The first was that the amount of variation in pollen chemical composition was not clear, and whether it showed regional patterns similar to morphological variations. Most of the classification studies mentioned above had quite low replication for each of their sampled species, often only one specimen per species. Low replication is a potential challenge, because low replication cannot capture potential regional variation in pollen chemistry. Some studies up until that point had used IR spectroscopy to show the plasticity of pollen chemistry. For example, Lahlali et al. (2014) demonstrated responses in pollen protein and lipid content to heat stress and Buta et al. (2015) showed a possible link between biochemical composition, viability, and germination capacity, but important work that demonstrated the variability of pollen chemistry across large sample sets was relatively limited (Bağcıoğlu et al. 2017, Zimmermann et al. 2017). Understanding the natural variability of pollen in the modern setting is a necessary step before applying chemical methods to fossil pollen. In particular, understanding species and even subspecies differences, as well as geographic differences in chemical composition in modern material, is something that is necessary to achieve before application to fossil pollen grains. The spectra obtained from pollen would then be an important reference with which to compare fossil pollen to. This is analogous to reference collection for morphological identification, which can show geographic variation in certain morphological characteristics. Sampling trees with regional variety is one way to capture some of this variability of pollen chemistry.

The second challenge was the technical limitations of FTIR microscopes, where scattering interference would be maximised while measuring small particles (<30 µm) (Lukacs et al. 2015, Zimmermann et al. 2015a, Blümel et al. 2018). Analytical solutions to this type of scattering have been demonstrated on biological particles and some pollen types (Lukacs et al. 2015) by filtering scattering anomalies. Zimmermann et al. (2016) and Zimmermann (2018) demonstrated scatter correction methods on more pollen types and show promising results. The ability to capture single grain spectra of pollen is very important for palaeoecological purposes and poses one of the biggest challenges for this method.

Whilst one side of the challenge to measure FTIR spectra of single pollen grains is technical (e.g. issues of scattering, reproducability of spectra, mounting medium, etc.), the other side concerns the pollen samples and the possible effects of common palaeoecological sample processing methods (e.g. acetolysis). At that point, FTIR studies on fossil pollen or spores are rare (Fraser et al. 2012, 2014) and the effects of acetolysis are tested on commercial *Lycopodium* spores using FTIR (Jardine et al. 2015). Acetolysis is one of the most common techniques used to extract and isolate pollen from sediments (Erdtman and Praglowski 1959, Fægri and Iversen 1989) for Quaternary pollen analysis. It successfully hydrolyses most undesired organic debris during sample preparation and leaves pollen and spores largely intact morphologically if used with care. It is unclear how it may impact pollen chemically. Jardine et al. (2015) find limited impact of acetolysis under normal processing parameters on sporopollenin chemistry. These are promising results that acetolysis would be a suitable method to extract fossil pollen from sediment samples for chemical analysis. Given the large variations in both sediment types and in the FTIR spectra of different pollen grains (Zimmermann 2010), it was clear that it would be important to understand the impacts of processing procedures on additional taxa. For chemical methods to work on fossil pollen, it is necessary to understand the chemical differences between sub-fossil, untreated pollen and modern pollen. This requires comparisons between processing types and what effect they have on pollen chemical composition, both modern and fossil.

In summary, the state of knowledge with regards to the application of IR methods to palaeoecology in 2016 can be described as follows:

- i. chemical methods show promising classification performances, even with high species numbers.
- ii. there are technical limitations for IR microscopes that have potential solutions in need of testing (e.g. embedding to deal with scattering)
- iii. it is unclear how variable pollen chemical composition is based on regional or other factors (e.g. climate)

1.4 Objectives of the Thesis

The objective of the thesis is to investigate the application of chemical methods for palaeoecology as a new method for classification of pollen. Note that I also initially explore the possibilities of computer vision approaches in the beginning of my PhD. The results of these preliminary investigations can be found in Appendix A. The rest of this thesis will focus on chemical methods and their application for palaeoecological investigations. For this purpose I formulate the following research objecctives:

- i. *Collect modern pollen and explore variation of chemical composition.* The first aim was to study modern pollen chemistry to gain a better understanding of the variability in chemical composition and estimate the sources of variation.
- ii. *Explore microscopy chemical methods (e.g. FTIR microscopy) on modern pollen.* Microscope IR methods have their own challenges (see above) and to explore solutions to, e.g., scattering of small pollen, tested on modern material
- iii. *Apply chemical methods on fossil material.* Proof of concept of IR methods on fossil pollen to explore fossil pollen chemistry.

In the following chapters I introduce the studies I performed to reach these goals and present the results of my research.

2 Design of Thesis

This thesis investigates the application of different IR methods with the goal to improve taxonomic classification, it consists of four main studies: The first paper is designed to investigate the variability of modern pollen chemistry and examine any differences between pollen chemical components. The second paper is a closer investigation of sporopollenin chemistry comparing the results of two IR methods, FTIR and FT-Raman. The third paper is an application of a new type of FTIR microscope detector on fossil pollen and examines two pollen extraction methods. The fourth and last paper utilizes analytical and technical approaches to investigate scatter effects in FTIR spectra that typically occur measuring small pollen or other particles. Here, I introduce each paper individually to explain the research goals, methods and the data-sets used in the studies.

2.1 Paper I - Chemical variation in *Quercus* pollen

This paper addresses two of the major challenges faced in the application of pollen chemistry techniques to palaeoecological research (see introduction), that is understanding the amount of chemical variation across species from different populations and regions, and the ability of FTIR techniques to discern closely related (congeneric species). There are several studies that report influences of temperature and UV-B radiation on pollen chemistry, such as protein, lipid content and building blocks of sporopollenin (Rozema et al. 2001, Bağcıoğlu et al. 2015, Jardine et al. 2017, Bell et al. 2018, Kendel and Zimmermann 2020, Diehn et al. 2020). The aims of this paper are threefold:

- i. investigate the capabilities of FTIR to discern closely related (congeneric) species
- ii. capture as much chemical variation as possible and determine which parts of the pollen chemistry are more variable.
- iii. determine if this variation is present in the parts of the pollen grain that are thought to be preserved in fossil pollen.

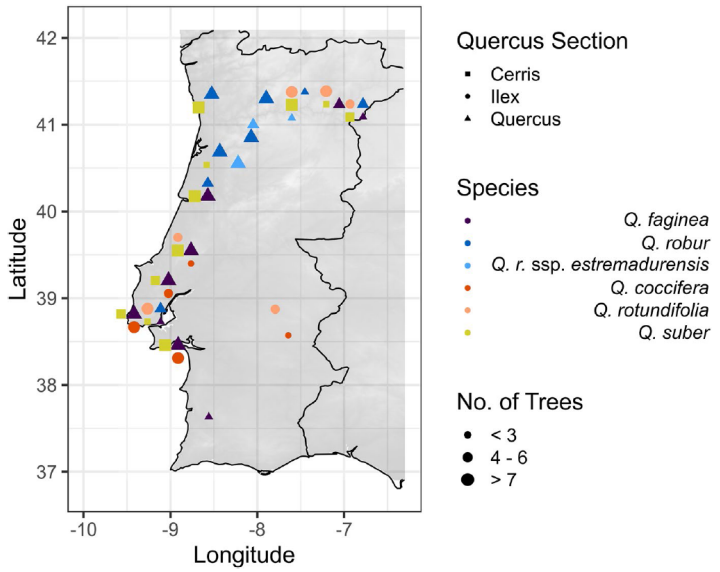

Figure 2: Map of Portugal showing the locations where the *Quercus* trees where sampled. Colour indicates species, shape indicates *Quercus* section following Denk et al. (2017) and size of the symbol indicates number of trees sampled per location

2.1.1 Data-set

To achieve this, I collected pollen from 297 trees in Portugal. Compared to other studies published at the time, this data-set greatly increased the number of replicate pollen samples, both for each species and at each location. I collected pollen from a variety of geographic and climatic conditions between Porto and Lisbon and towards the Spanish border along the River Douro. The trees were either from wild populations, public parks or a few botanic garden trees. The Iberian Peninsula, and especially Portugal, is characterised by a bioclimatic range, from dry Mediterranean in the SE, to wet temperate Atlantic in the North. I sampled trees ranging from evergreen sclerophyllous forests with dry and hot summers to temperate areas, with cold winters and mild rainy summers (Neophytou et al. 2010). Each of these environments is diverse in its species composition and thus, the presence of different oak species are likely indicative of changes in ecological and edaphic conditions (García-Mijangos et al. 2015, Rivas-Martinez et al. 2017). *Quercus* contains 22 native species in two subgenera and three sections in Europe (Denk et al. 2017), which often have distinct geographical distributions, e.g. clear tran-

sitions of evergreen to decidous ecosystems are marked with striking changes in *Quercus* presence. For this data-set I collected pollen from three *Quercus* sections, 5 species and 1 sub-species (*Quercus suber*, *Quercus coccifera*, *Quercus rotundifolia*, *Quercus faginea*, *Quercus robur* and *Quercus robur estremadurensis*).

2.1.2 Spectroscopy and statistical analysis

I used a FTIR approach that measures bulk samples, based on previous studies examining the chemical composition of pollen from multiple species (Gottardini et al. 2007, Zimmermann 2010). Bulk measurements allow for the rapid analysis of a larger number of samples at higher spectral quality than FTIR microscopy approaches. It was important for me to record high quality spectra to investigate fully the chemical variability. I used chemometric techniques that allow for multivariate classification from the partial least squares (PLS) family of methods. To evaluate the classification, *n*-fold cross validation was performed, where multiple training- and test-versions of the data-set were created to assess the classification. Performance metrics were evaluated across folds.

2.1.3 Environmental variable reconstruction

To investigate the influence of environmental variables on pollen chemistry, I reconstructed temperature, precipitation and solar radiation from the *Q. suber* samples of the data-set. *Q. suber* was present in most of the locations sampled and represents the largest variation in environmental conditions in the data-set. I used weather data from monitoring stations in Portugal, which were between 2 and 30 km from the sample locations to extract the last 14 days of weather data before sampling of each tree. The predictions were done using a PLS model with the environmental variables as responses and spectra of *Q. suber* as dependant variables. This reconstruction was part of an earlier version of the manuscript, but was removed in a later version in order to focus the manuscript more on classification and differences between the sections.

2.2 Paper II - Sporopollenin chemistry of *Quercus* pollen

While the first paper explores the variability of modern pollen and identifies that some of the variation is stored in sporopollenin, this second paper explores the sporopollenin chemistry in more detail by using FT-Raman spectroscopy. Specifically, FTIR can be heavily influenced by non-sporopollenin components for classification. This may not be ideal for the purposes of palynology where most of these components are assumed absent. In order to focus more on the sporopollenin chemistry, the Portugal *Quercus* data-set was analysed with FT-Raman. FT-Raman is more sensitive to sporopollenin building blocks and can give more information on the variability that is more relevant for fossil applications. The goals for this paper are as follows:

- i. compare the classification performance of FT-Raman compared to FTIR and when both sets are combined,
- ii. explore sporopollenin chemistry in more detail on *Quercus* section/species level. Phenylpropanoids are the main bands used to study sporopollenin chemistry with IR, but with Raman, additional information is obtained, such as more sensitivity to carotenoids.

In order to build on the results of paper I, paper II uses the same data-set as the first paper.

2.2.1 Spectroscopy and statistical analysis

FT-Raman analysis were performed as bulk samples, similar to paper I, and the results from the FT-Raman analysis were combined with the results from FTIR in paper I. The specific settings are outlined in the detailed methods section in the manuscript. Raman spectroscopy of pollen can cause autoflouresence and even thermal decomposition. I observed some amount of both phenomena, but only in a few samples, which were measurable without such intereference after adjusting laser power. FT-Raman in general is less prone to autoflorescence with pollen than other Raman approaches, due

to differences in the wavelength of the laser. Generally, high wavelength near infrared lasers are less prone to autofluorescence than low wavelength visible lasers.

Classifications were performed using a hierarchical-classification tree, which identified *Quercus* sections and then the species. This was done to simplify the task for the PLS model as it improves classification performance (Tafintseva et al. 2018). A novel multivariate tool was used, multiblock (Westerhuis et al. 1998), to compare directly the importance of chemical components from FT-Raman and FTIR for the classifications. Normally, the regression coefficients of two PLS models are not directly comparable, which was possible using multiblock. With multiblock, the different datablocks, in our case pollen spectra from two methods (FT-Raman and FTIR), corresponding sample measurements are established side by side. This integrated approach allows for the maximization of common variation in the data blocks, whereby variable variation patterns can be visualized and compared between data blocks.

2.3 Paper III - Fossil Pollen chemistry

After exploring the variation in modern pollen, and finding strong signals in sporopollenin components of modern pollen, the next step was to apply IR methods to fossil material. The main goals for this article are threefold:

- i. to investigate the chemistry of fossil pollen compared to modern pollen,
- ii. to investigate the effect of acetolysis on fossil pollen chemistry and
- iii. to assess the performance of a new type of FTIR microscope detector that allows simultaneous capture of spectra of multiple single grains.

An important motivation for this paper was to compare fossil pollen to modern material to investigate how diagenesis and extraction methods can affect the chemical signal. There are a variety of chemical extractions that are used to extract/isolate pollen grains from lake or bogs. One of the most common used procedures is acetolysis, which uses a combination of strong acids to hydrolyse organic material that are not pollen and spores. Acetolysis may alter or remove taxonomic or other desirable information that is present in

untreated fossil pollen. Some studies have investigated the effects of acetolysis on pollen and found that it does alter parts of the chemistry under certain conditions (Jardine et al. 2015, 2017, 2021). Therefore, if we want to apply FTIR and other chemical analytical methods on fossil pollen, we need to understand the differences between modern and fossil material and how extraction methods can impact pollen chemistry. In this paper, I also use an alternative extraction method, density separation, to extract pollen without the use of acids and compare their chemistry to acetolysed fossil pollen and modern material.

An additional motivation was to use a new detector in combination with a FTIR microscope, which allows the capture of multiple single grains, by capturing multiple spectra over a sample area (e.g. 500 x 500 µm) as opposed to single point spectra. This allows extraction of multiple single-grain spectra from the same image and opens the door to potential automization methods using deep learning approaches (Großerueschkamp et al. 2017).

To achieve these goals, three cores from different locations were subsampled. Two varved cores from Germany and a core from Greenland. The cores are Dalmuttladdo (DAL), Tiefer See (TSK) and Meerfelder Maar (MFM). The samples have different ages, but all from within the last 13,000 years. Samples from the DAL core span the past 9,800 years, while TSK samples are from the past 80 years and MFM samples were depositied between 13,000 and 11,000 BP.

The chemical spectra were captured using a FTIR microscope to collect single grain spectra. To answer the questions, I focused on *Pinus sylvestris* pollen, which is very common in European sediment cores, easily identifiable and therefore a good candidate for this study. Another more practical reason for chosing *Pinus sylvestris* was that the FTIR microscope used for this study does not produce images of good enough quality to identify pollen smaller than 50 µm, e.g. *Betula*, *Alnus* or *Corylus*. The pollen was extracted from the sediment using density separation, using sodium polytungstate (SPT) as extraction medium, to avoid any acid treatment (e.g. acetolysis). Density separation has been used in other studies where chemically unaltered pollen is desirable, such as pollen carbon-isotope studies (Regnéll and Everitt 1996).

2.3.1 Adjustments to the study as a result of Covid-19

For this paper, I originally planned to analyse spectra of fossil *Quercus* grains from several cores in Europe in addition to other common tree pollen taxa (e.g. *Alnus*, *Pinus*). For this purpose, we had access to several cores from Italy, Germany and Iberian Peninsula through colleagues that provided me with some of their residues, or even re-sampled the cores. Unfortunately, the Covid-19 lockdown and other delays required changing the design of the paper. Travel- and lab-restrictions greatly limited the available lab time for me and I had to adjust the number of samples in each core and the number of pollen types I was able to target.

Therefore, I decided to focus on *Pinus* pollen and three cores. *Pinus* pollen is much larger than *Quercus* and other tree pollen, more abundant and therefore easier to find in sediment samples. Focusing on *Pinus* pollen saved a lot of time in the lab, which meant I could capture more grains from more samples as opposed to using a lot of time to look for *Quercus* pollen. Despite the change in the studied genus, *Pinus* is still a fitting choice for this type of research because *Pinus* pollen is a potential candidate for a UV-B proxy (Willis et al. 2011, Jardine et al. 2017, Seddon et al. 2019) and *Pinus* pollen is hard to identify to species using traditional LM. An obvious next step would be to apply these methods to *Quercus*.

2.4 Paper IV - Scatter-correction approaches

In addition to the challenges of assessing chemical changes related to the extraction fossil pollen grains, vibrational spectroscopy approaches such as FTIR on small particles (i.e. $>$ 30 µm), introduces anomalies in the spectra caused by scattering of the IR beam (Lukacs et al. 2015, Zimmermann et al. 2015a, Blümel et al. 2018). These scattering interferences are present in larger objects, but much more pronounced in smaller objects. This is problematic for the analysis of pollen, because a lot of pollen taxa are smaller than 30 µm and interesting for palaeoecologists, such as *Quercus* or Poaceae. For palaeoecological applications, the effects of scattering and any potential methods used to correct for them should be explored. In other disciplines scattering

causes similar problems, e.g. analysis and/or identification of micro-plastics (Hufnagl et al. 2019), diatoms (Alipour et al. 2016) and dinoflagellates (Versteegh et al. 2012).

Here, we undertook an exploratory study of a number of potential methods to correct for scattering caused by small particles. We also aimed to investigate if there was any taxonomic information stored in scattering parameters. Scattering effects can either be addressed during measurement, or analytically during spectral processing. For example, one solution to remove the effect of scattering is to embed the pollen in paraffin between two layers of polyethylene (PEP Zimmermann et al. 2016). This method prevents any scattering anomalies, but at the cost of losing part of the spectra (1520 - 1290 cm^{-1}), which are dominated by peaks of the polyethylene and paraffin matrix. Alternatively, pollen grains can be measured on standard IR microscope slides (such as zinc selenide slides), and analytical corrections can be made to identify scattering anomalies and correct them. During scattering part of the radiation is absorbed by the particle and partly scattered, therefore lost to the detector. The main type of scattering observed with pollen grains is Mie-type scattering, which can be corrected by spectra processing algorithms such as Mie-extinction extended multiplicative signal correction (ME-EMSC) or averaging of single grain spectra until scattering effects are reduced (Zimmermann 2018). Mie-type scattering can be described by Mie theory of electromagnetic radiation scattering on spherical objects. Pollen grains can be approximated as spheres and typical Mie scatter correction algorithms calculate scattering spectra for spheres of different radii and match them to the spectra that need to be corrected. We use Mie-extinction extended multiplicative signal correction (ME-EMSC) in this paper (Bassan et al. 2012, Konevskikh et al. 2018, Solheim et al. 2019) in addition to pollen embedding with PEP.

Using a subset of the *Quercus* pollen samples collected for previous studies, we selected a number of representative samples to measure pollen both conventionally on ZnSe slides

Table 2: Species in the 'Scatter correction' data-set. Taxonomy follows Denk et al 2017.

Subgenus	**Section**	**Species**	**Location**
Quercus	Lobatae	*Q. palustris*	Australia
Quercus	Quercus	*Q. robur*	Portugal
Cerris	Cerris	*Q. suber*	Portugal
Cerris	Ilex	*Q. rotundifolia*	Portugal

and embedded in paraffin/polyethylene matrix. This data-set was curated from the Portugal data-set and supplemented with one species from a sampling field trip to Australia in order to add an additional *Quercus* section. The data-set comprises four *Quercus* species (Table. 2) The pollen of *Q. palustris* was collected in Melbourne, October 2017, while pollen of *Q. robur*, *Q. rotundifolia* and *Q. suber* were collected in Portugal in April 2018.

Each species is from a different section of the *Quercus* genus and has morphological differences that are used for light-microscope identification to Section level (Beug 2004), such as grain size and ornamentation.

Microscopic transmission measurements of pollen were performed using a Vertex 70 FTIR spectrometer with a Hyperion 3000 IR microscope (Bruker Optik, Ettlingen, Germany). All pollen samples were measured, without any chemical pretreatment, under two different experimental settings: (1) on zinc selenide (ZnSe) optical windows, and (2) embedded in a paraffin-polyethylene (PP) matrix. For the ZnSe measurements, the pollen samples were deposited onto 1 mm thick zinc selenide (ZnSe) optical windows. For each pollen sample, 50 spectra of different individual single pollen grains were obtained, corresponding to 200 spectra per species. Thus, each experimental set (ZnSe and PP) contained 800 µFTIR spectra of single pollen grains.

3 Results

3.1 Paper I

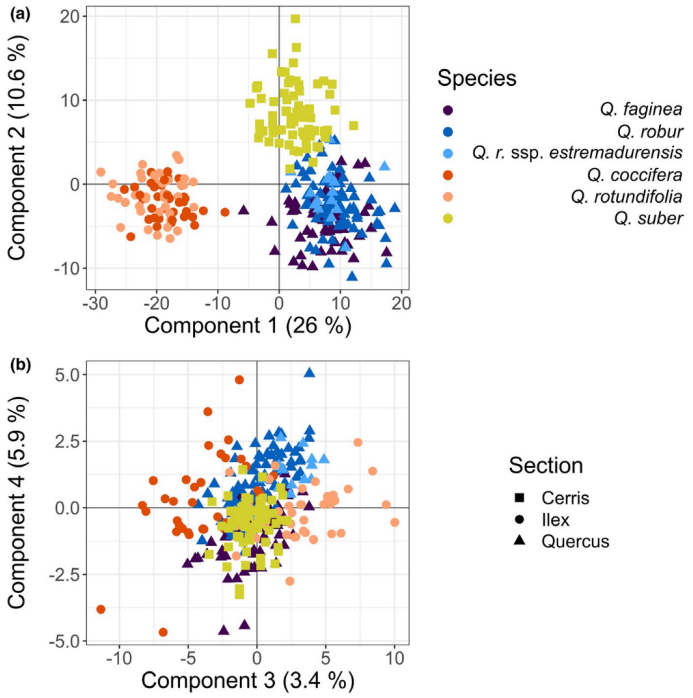

Figure 3: Ordination of components 1–4 of canonical powered partial least squares (CP-PLS) models (100-fold cross-validation). Each point represents a sampled *Quercus* tree. The mean training scores for each sample over the 100-folds were calculated. Proportion of variance explained by each component in parentheses. Colour indicates species, while symbol indicates *Quercus* section

The first study used FTIR spectroscopy to investigate chemical differences across *Quercus* sections and allowed us to separate *Quercus* pollen at the section level using major differences in chemical composition. For example, our PLS model was able to clearly differentiate the three *Quercus* sections using the first two components (Fig. 3). and achieved some success on species level utilizing the third and fourth component.

The reconstruction of environmental variables shows a positive correlation for all three variables (temperature, precipitation and solar radiation), but the predictions are quite noisy. For example, predictions at precipitation 0 range from -1 to 1, while the entire ob-

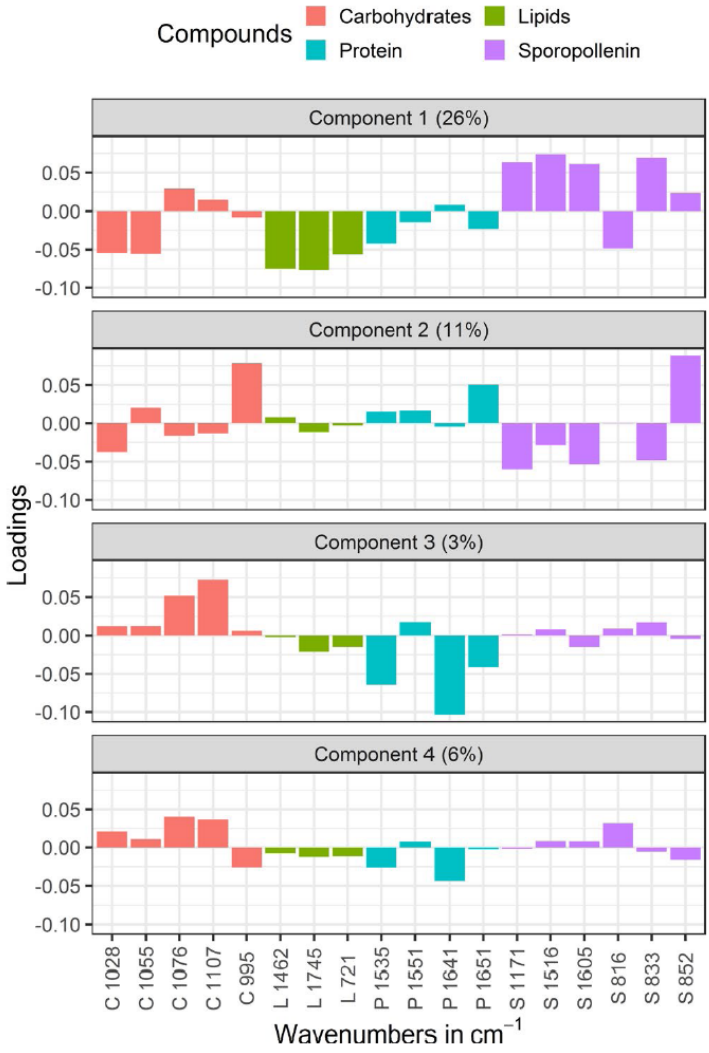

Figure 4: Loadings plot of classification of canonical powered partial least squares (CP-PLS) model. Lipids (L), protein (P), sporopollenin (S), and carbohydrates (C). High absolute loading indicates a high importance of a given wavenumber for the corresponding component. Loadings are chosen in such a way as to describe as much as possible of the covariance between the variables (wavenumbers) and the response (species). Proportion of variance explained by each component in parentheses

Table 3: Confusion matrix of linear discriminant analysis on the test sets using four components of the fitted canonical powered partial least squares (CPPLS) model

Pred/Ref	*Q. faginea*	*Q. robur*	*Q. r. estr.*	*Q. coccifera*	*Q. rotund.*	*Q. suber*
Q. faginea	64 ± 12	18 ± 8	11 ± 13	0	0	3 ± 4
Q. robur	30 ± 12	76 ± 10	81 ± 18	0	0	1 ± 3
Q. r. estr.	1 ± 2	5 ± 6	6 ± 13	0	0	2 ± 6
Q. coccifera	1 ± 3	0	0	75 ± 14	25 ± 11	0
Q. rotund.	1 ± 2	0	0	25 ± 14	75 ± 11	0
Q. suber	3 ± 4	1 ± 3	2 ± 6	0	0	98 ± 2

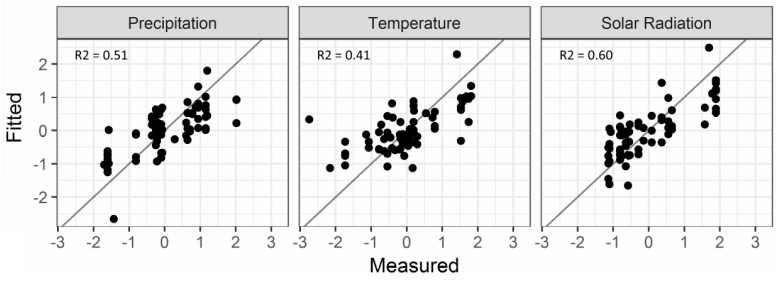

Figure 5: Reconstruction of environmental variables using *Q. suber* spectra from Portugal data set. A CPPLS model (four components) was fitted using 100-fold cross-validation of environmental data as response and second derivative FTIR spectra as predictor.

served precipitation range is from -2 to 2. The environmental variables were normalised, because of the vastly different scales in measurement units for each environmental variable.

We observed quite large variation between species of the same section, e.g. *Q. rotundifolia* and *Q. coccifera* (Fig. 3 a). The model was able to use some of the variation on components 3 and 4 to differentiate between section Ilex species (*Q. rotundifolia* and *Q. coccifera*) and section Quercus species (*Q. robur* and *Q. faginea*) (Fig. 3 b). The model also identified the chemical functional groups that could best explain the variance between sections, high loadings (greater importance) of lipids, carbohydrates and sporopollenins on component 1 and 2 (section level) and proteins and carbohydrates on component 3 and 4 (species level) (Fig. 4). Our classification success is on par with SEM and more detailed than most reported *Quercus* pollen counts (i.e. at section level as opposed to *Quercus* decidous type vs *Quercus* evergreen type). Given that sporopollenin was identified as one of the key chemical discriminators across the data-set, we then decided to examine the sporopollenin chemistry of *Quercus* with the FT-Raman approach.

3.2 Paper II

Our results show that FT-Raman analyses of Quercus pollen grains provided similar overall results to those obtained using FTIR. For example, at the section level both FTIR and FT-Raman were able to correctly classify *Quercus* sections (Table 4), while there were differences within sections.

We fitted PLS models that use the vibrational bands to differentiate the pollen by section first and then by species. On each level we fitted three models using only FTIR, only FT-Raman and using both (multiblock). For section Ilex, FTIR performed better (Table 5), while FT-Raman performed better for section Quercus (Table 6). Overall, the classification performance from paper I was exceeded and reached 90% to 95% accuracy on species level. The regression coefficients that indicate which chemical functional groups were the most important showed a high importance of carbon hydrogen bonds for FT-Raman, such as spropollenin peaks, e.g. the 1600 cm^{-1} region (Fig. 6 b). For FTIR, lipids, proteins and sporopollenins had high regression coefficients (Fig. 6 a). The importance of sporopollenin peaks was quite different between sections, which can be seen in the regression coefficients of the 1600 cm^{-1} peaks and 1225 cm^{-1} peak (Fig. 6 b). Section Cerris pollen has the highest positive regression coefficients (1600 and 1225 cm^{-1}), while section Quercus and Ilex have negative regression coefficients for the same coefficients.

We compared the classification performance and important wavebands that had high regression coefficients (high importance) for the classification model between FTIR and FT-Raman. We observed vibrational bands belonging to the same functional groups in both spectra, but with differences in intensity, e.g. the 1745 cm^{-1} peak is much stronger in FTIR and weaker in FT-Raman, while the 1600 cm^{-1} peak is much wider and stronger in FT-Raman compared to FTIR, which indicates phenylpropanoid groups. In general, phenylpropanoid functional groups (eg. 1225 cm^{-1}) are stronger in FT-Raman spectra than the corresponding bands in FTIR spectra.

These results give an overview of modern *Quercus* pollen chemistry and show the differences between FTIR and FT-Raman methods.

Table 4: Model performance for 1st Node differentiating spectra into the three *Quercus* sections (Quercus, Ilex and Cerris). Comparison of model performance between methods: Multiblock and single block models. Models were fitted using short spectra region of interest (700-1900 cm^{-1}) and window size 29 in Savitzky Golay step during preprocessing.

	Recall in %			
Method	Cerris	Ilex	Quercus	Acc.
FTIR	100.0	100	99.2	99.6
FT-Raman	100.0	100	100.0	100.0
Multiblock	98.5	100	100.0	99.6

Table 5: Model performance for 2nd Node differentiating section Ilex pollen. Comparison of model performance between methods: Multiblock and single block models. Models were fitted using short spectra region of interest (700-1900 cm^{-1}) and window size 29 in Savitzky Golay step during preprocessing.

	Recall in %		
Method	*Q. coc*	*Q. rot*	Acc.
FTIR	91.2	88.2	89.7
FT-Raman	88.2	85.3	86.8
Multiblock	94.1	85.3	89.7

Table 6: Model performance for 3rd Node differentiating section Quercus pollen. Comparison of model performance between methods: Multiblock and single block models. Models were fitted using short spectra region of interest (700-1900 cm^{-1}) and window size 29 in Savitzky Golay step during preprocessing.

	Recall in %		
Method	*Q. fag*	*Q. rob*	Acc.
FTIR	88.7	93.5	91.5
FT-Raman	96.2	93.5	94.6
Multiblock	92.5	92.2	92.3

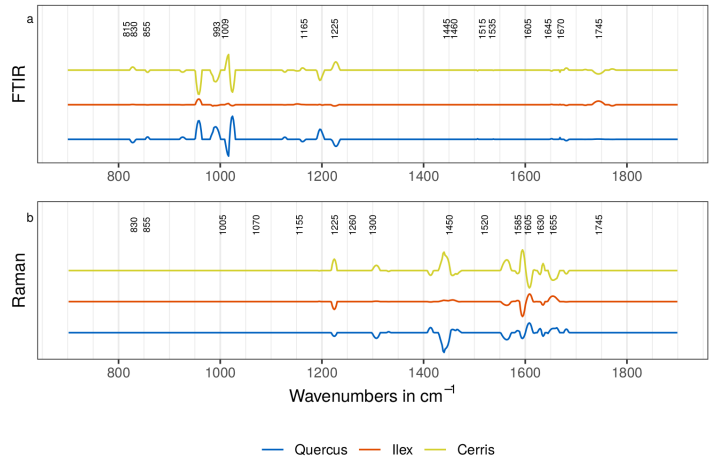

Figure 6: Model regression coefficients for the first node: Classifying *Quercus* sections. Multiblock model regression coefficients for FTIR data block (a) and FT-Raman data block (b). Regression coefficients are off-set in figure a and b. Higher regression coefficients indicates higher importance for the model. Preprocessing parameters for this model were short SROI (1900 - 700 cm^{-1}) and windowsize 29 for both data blocks.

3.3 Paper III

A FTIR microscope equipped with a FPA detector successfully recorded spectra from both modern and fossil pollen grains. Furthermore, the results from this paper showed differences between modern and fossil pollen grains (Fig. 7), e.g. the lipid peak at 1745 cm^{-1} and protein peak at 1550 cm^{-1}, were not visible in the fossil pollen grains. Prominent and characteristic sporopollenin peaks (1600, 1510 and 1170 cm^{-1}), on the other hand, were visible in the fossil pollen and modern pollen. There were also several differences in the fingerprint region (1500 to 1800 cm^{-1}) and carbohydrate region (1000 to 1250 cm^{-1}) between modern and fossil pollen grains. The differences between fresh and fossil grains were very strong, as seen in the ordination (Fig. 8), where fossil and modern spectra strongly separated on the first component axis.

When examining only the fossil grains, there were some differences, mainly in the carbohydrate region (1000 to 1250 cm^{-1}). Acetolysed pollen grain spectra were dominated by two peaks in this region, at 1040 and 1170 - 1190 cm^{-1}, while non-acetolyzed pollen was missing the 1040 cm^{-1} peak and showed the sporopollenin peak at 1170 cm^{-1} and a wide peak at 1090 cm^{-1}. We also observed two possible contaminations in the spectra.

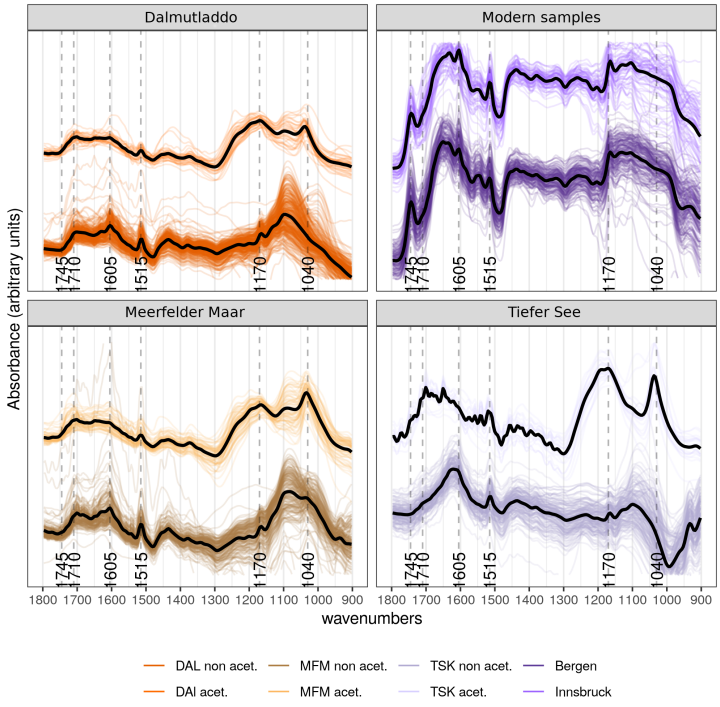

Figure 7: Mean spectra of fossil *Pinus* pollen from each sediment core and modern samples. For each core the mean of the acetolysed and non-acetolysed spectra are shown.

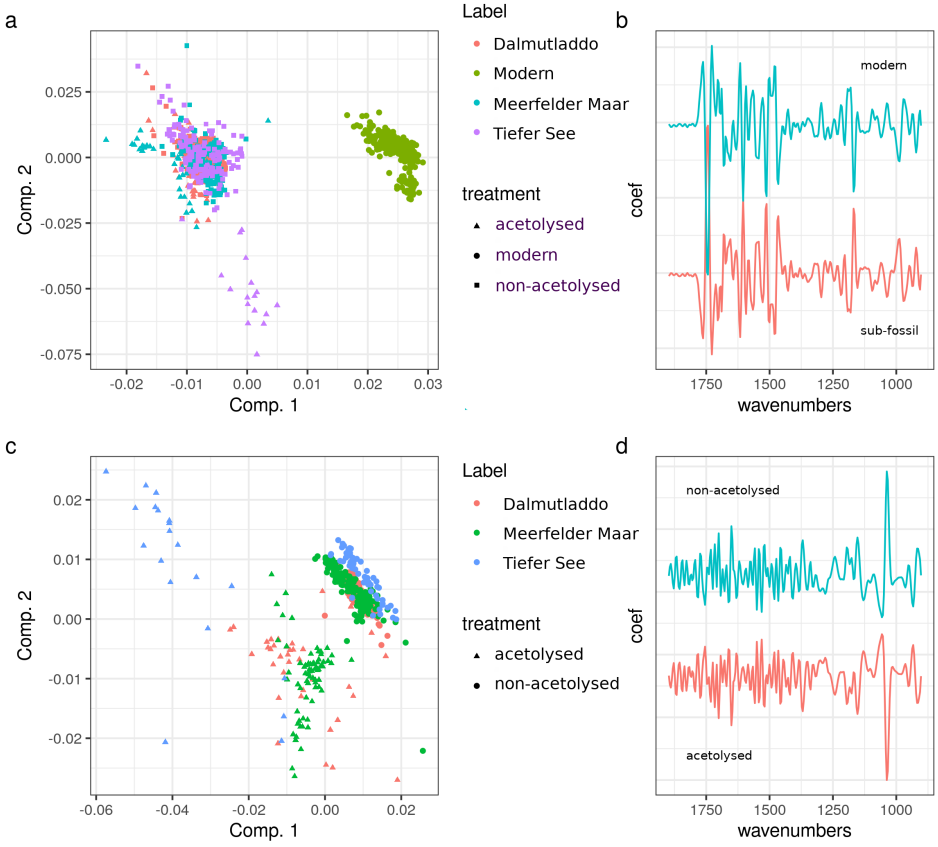

Figure 8: a. PLSR ordination scores of all samples showing the difference between fresh vs fossil pollen b. regression coefficient of component 1 c. regression coefficient of component 2; d PLSR ordination scores using only samples from sediment cores. Difference between non-acetolysed and acetolyses; e. regression coefficient component 1; f. regression coefficient component 2

A peak at 1090 cm^{-1} is found in all non-acetolyzed samples and an introduced peak at 1630 cm^{-1} in all non acetolyzed samples of Tiefer See. These two peaks were relatively wide and not present in any of the other fresh or fossil pollen samples. The 1090 cm^{-1} was most similar to spectra of dissolved silica and the 1630 cm^{-1} peak was identified as part of a typical sodium polytungstate spectra, the density separation medium.

3.4 Paper IV

An analytical and a experimental approach successfully removed scattering signals in spectra of single grain pollen. The scattering signals were visible in the 2500-1800 cm^{-1} region of the spectra (Fig. 9 b), where there are no chemical absorbance signals in *Quercus*. Scattering signals were successfully removed with PEP embedding, but with a trade-off, since the absorbance bands of the paraffin-polyethylene matrix hid any signals of the pollen in the region 1520 - 1290 cm^{-1}. The analytical method (ME-EMSC) substantially removed the scatter signals, but did not completely suppress them (not pictured here; see Fig. 2 in the paper).

The type of scattering observed in pollen grains is Mie-scattering, which depends on the radius of the scattering particle. The pollen grains of the four species (Table 2) studied in this paper showed differences in size. Although there is a significant overlap in pollen size ranges, *Q. rotundifolia* pollen was generally smaller (polar d.: ~26 µm; eq. d.: ~19.9 µm) than pollen of the other three species (polar d.: ~34 µm; eq. d.: ~29.5 µm) (Table 7) (Beug 2004).

From the classification results we see that both scatter correction methods reduced the classification accuracy (81.0%) compared to the uncorrected ZnSe data-set (91.5%) (Table 8). In addition to the corrected spectra, the filtered scatter parameters from the ME-EMSC method could also be used for classification and had a classification accuracy of 66%, which shows that taxonomic information is stored in the scatter parameters. These results showcase the successful suppression of scatter signals during measurement (PEP embedding) and analytically during spectra processing (ME-EMSC).

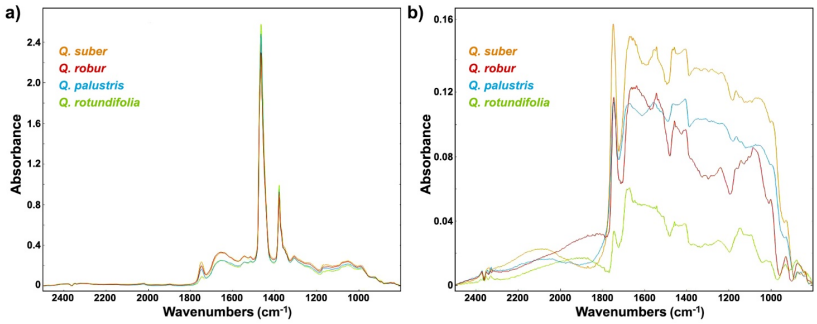

Figure 9: Pollen spectra measured: (a) in paraffin-polyethylene (PP) matrix, and (b) on ZnSe optical windows. Spectra were baseline corrected and averaged (average of 200 spectra of individual pollen grains).

Table 7: Pollen grain sizes of species used in this study according to Beug 2004. Mean diameter and ranges are based on 100 grains for each species.

Species	Polar diameter in μm	Equatorial diameter in μm
Q. robur	36 (31-34)	28.9 (26.3-32)
Q. suber	33.1 (27.4-41.4)	30.4 (25.3-35)
Q. ilex (close relative to *Q. rotundifolia*)	26.4 (21.2-30.2)	19.9 (15.2-24.7)
Q. palustris	34.6 (26-39.2)	

Table 8: Results of classification data analyses: classification accuracy (CA) for artificial neural networks (ANN) and random forest (RF) classifiers.

Dataset	CA_{ANN} (%)	CA_{RF} (%)
PP	81.0	80.0
ZnSe	76.0	91.5
ZnSe ME-EMSC spectra	75.0	81.0
ZnSe ME-EMSC parameters	66.5	58.0

4 Discussion

4.1 Species classification using IR methods

One of the main goals of this thesis was to investigate the application of IR methods for taxonomic purposes, for example, to improve classification of cryptic species such as *Quercus* based on pollen-grain chemistry. In order to do this with fossil pollen, it was first important to understand the variation in modern pollen as much as possible. I showed, using different vibrational spectroscopy approaches (FTIR, FT-Raman, FTIR microscopy), that there is taxonomic chemical information stored in modern grains, which matches or exceeds the performance of morphological approaches (e.g. SEM and LM). The approaches I used were bulk sample FTIR (Papers I and II), bulk sample FT-Raman (Paper II) and single grain FTIR microscopy (Paper IV) on *Quercus* pollen. The taxonomic information reconstructed from the pollen spectra is stored in both the chemically inert components of the grain wall (sporopollenin), and in the more labile intracellular components (lipids, proteins, carbohydrates). In addition, we also found some taxonomic information in the scatter anomalies (Paper IV). As expected, the relative importance of the chemical components varied depending on the IR method used. For FTIR, lipids and other carbon-oxygen bond-based components were more informative, while sporopollenin and carbon-carbon and carbon-hydrogen based components were more informative in FT-Raman. The addition of FT-Raman and a different variation of PLS model improved the classification performance on species level substantially, from ~73% in paper I, to ~90% in paper II.

This work builds on the results of other studies examining pollen chemistry (Zimmermann 2010, Bağcıoğlu et al. 2015, Jardine et al. 2019). Zimmermann (2010) showed the variability of pollen chemical composition between ~30 different species of pollen, with a high number of congeneric species, which inspired me to focus this thesis on cryptic taxa to explore the potential of FTIR for increasing taxonomic resolution in palaeoecology. A more recent study, Jardine et al. (2019) reported classification accuracy for congeneric Poaceae species ranging from 25% to 87% recall. Further work by Bağcıoğlu et al. (2015) utilised Raman and FTIR approaches to characterise pollen chemistry,

which showed the potential of Raman for this thesis. The results from papers I and II expand on these studies: paper I shows the between species variability, while paper II utilizes Raman to explore spropollenin chemical composition in modern pollen samples and improve classification to species level substantially.

Studying the performance of IR methods for con-generic species is especially important, because taxa such as *Quercus*, *Eucalyptus* or *Pinus* are ecologically diverse and improving the taxonomic resolution of these taxa would greatly improve our understanding of past vegetation and dynamics. For example, studies forecasting the effect of climate change on Mediterranean forest dynamics (Nogués-Bravo et al. 2016) use *Quercus* records on subgenus level and may miss the species specific responses of trees to climate (Acácio et al. 2017). The same is true for other taxa, such as *Eucalyptus*, *Pinus* and others. This thesis shows that it is possible to identify *Quercus* at a species level using FTIR, FT-Raman and FTIR microspectroscopy.

Despite our successes in classifying *Quercus* pollen, a majority of the variation was unexplained or not utilized by the models used for classification. We attributed the variation to be of largely environmental origin and due to population differences.

4.2 Influences on modern pollen chemistry

One of the most important contributions of papers I and II is that it studies pollen chemistry using a smaller set of congeneric species with very high replication within species (i.e. 50 $\pm$ 23 tree replicates per species) and locations (15), compared to other studies with fewer replicates (Zimmermann 2010, Julier et al. 2016, Woutersen et al. 2018, Jardine et al. 2019) or fewer congeneric species (Julier et al. 2016, Zimmermann et al. 2017). Other studies that focused on application of IR methods for palaeoecology were influencial for this thesis (Julier et al. 2016, Woutersen et al. 2018, Jardine et al. 2019) and I was able to demonstrate the importance of including larger number of sample replicates for classification of cryptic taxa. The variability within *Quercus* was quite high and overlaped considerably between species, and it is this which makes it important to sample enough replicates for each species. In Zimmermann (2010) we see considerable variability between *Pinus* species and Bağcıoğlu et al. (2017) showed year

to year and geographic variation. Low replication would underestimate this chemical variation and inflate classification success, as suggested by Woutersen et al. (2018). The design of the Portugal data-set was designed to build on these findings and capture as much variability as possible, by increasing replication at each location and sample from a variety of locations.

My studies on the *Quercus* data show a substantial amount of variation in pollen chemistry, especially at subgenus and species level, thanks to the high number of replicates and sampling locations. The approach I took was to sample a high number of trees from wild populations, botanical gardens and parks in different parts of Portugal. This approach was similar in some aspects compared to other studies that were published with environmental impacts on pollen chemistry at the time. For example, Depciuch et al. (2016) and Depciuch et al. (2017) studied impacts of pollution on pollen chemistry using pollen from a national park and wild populations. Fraser et al. (2011) also used wild populations studying the impacts of UV-B on spore chemistry. Bell et al. (2018) and Seddon et al. (2021) used a combination of botanic garden and naturally occuring *Cedrus* and *Pinus* populations, respectively, to study the impact of UV-B radiation. Our approach was unique in this regard, because it utilized wild populations and high replication (>50 specimen per species). Zimmermann et al. (2017) used similarly high replication (>60 specimens) to study the effects of growing conditions and population.

However, one of the disadvantages of sampling this large number of replicates over a large spatial area is the difficulties in characterising the effects of environmental variability on pollen chemistry. For example, I reconstructed temperature, precipitation and solar radiation for the Portugal set by using climate data from weather stations close to the sampled trees. My reconstructions showed a positive correlation, but were quite noisy and the variation they explained was relatively low (Fig. 5). Another challenge is the fact that there is a strong correlation between both the climatic and geographic variables, which makes it difficult to separate population-level from other environmental effects.

One approach that tries to untangle environmental influence on pollen chemistry are fine-scale studies and common garden experiments, which have become more popular with pollen chemical studies in the last 3-4 years. Here, some of the strongest results, which show the plasticity of pollen chemistry, have been achieved with specimens grown

in controlled environments (e.g. green houses), such as Zimmermann et al. (2017) and Benca et al. (2018). Here, for example, temperature, growing conditions and UV-B exposure are controlled and their impact on chemical variability are studied. Alternatively, field experiments could be used, in combination with careful monitoring of local climate conditions (Seddon et al. 2021).

In addition, a number of similar common garden studies have now shown that pollen-chemistry response time to environmental stimuli may be as short as 14-21 days (Zimmermann and Kohler 2014, Jokerud 2017, Seddon et al. 2021) and may, in part, be driven by population and genetic origin (Zimmermann et al. 2017, Bell et al. 2018). These results demonstrate that green-house and field experiments are better suited to untangle environmental and population differences. At the time of conception of the Portugal data-set, the scale of environmental influence on pollen chemistry was not fully known and my results show that a large amount of information is environmental, in addition to taxonomy. The challenge going forward is to try and further resolve environmental, population and geographic differences using fine-scale studies.

4.3 Applying modern knowledge to fossil pollen

In papers I and II the goal was to investigate the potential for chemical variations to be used to identify modern *Quercus* pollen. The next step was to apply our understanding of modern pollen chemistry to fossil pollen. For this, we would ideally apply our knowledge from modern pollen chemistry to fossil pollen, e.g. compare unknown pollen spectra (e.g. of a *Quercus* pollen or any other taxa) to multiple modern spectra and find the closest match, or measure the part of the spectra most responsive to, e.g., UV-B exposure and apply a transfer function created from modern pollen calibrations. The results from papers I and II show that FTIR and FT-Raman are both capable of differentiating *Quercus* pollen at species level. Others have shown similar results for other taxa (Zimmermann 2010, Bağcıoğlu et al. 2015, Kendel and Zimmermann 2020), which, taken together, suggest that we could compare fossil pollen to modern samples to identify to species. Unfortunately for us, this is not as straightforward, because, as expected, the results from paper III show that fossil pollen are chemically different com-

pared to their modern counterparts. The main differences between fossil and modern material in our results and from Jardine et al. (2021) show that labile components, such as lipids and proteins are completely or partly removed from the pollen grain and not detectable in the fossil pollen. These changes occur during diagenesis and it is not clear what kind of chemical processes are causing them. Furthermore, deposition and burial in lakes and bogs may differ depending on the type of sedimentation environment and chemical conditions during burial and fossilisation. We found some differences between cores measured in paper III, which suggest differences based on diagenesis, a finding also made by Jardine et al. (2021). Understanding what occurs during diagenesis is critical for any study using chemical methods on fossil material, be it identification of fossil pollen or reconstruction of UV-B radiation.

We do not currently have a way to mimic the diagenetic processes present in lakes and bogs, so that modern pollen grains might chemically resemble fossil pollen grains. Our approach in paper III was to understand fossil pollen chemistry better, by extracting non-acetolysed pollen from the sediment sample and compare it to acetolysed replicates. We showed that acetolysis, a commonly used method to extract fossil pollen, alters the chemical composition of the pollen. Comparing untreated fossil *Pinus* pollen with acetolysed *Pinus* pollen revealed differences in the 1000 - 1200 cm^{-1} region. We observe two large peaks at 1040 and 1190 cm^{-1}, which are new C-O bonds that are created through acetolysis and are the results of acetylation of hydroxyl groups according to (Moore et al. 1991) and which were also observed in acetolysed *Pinus pinaster* pollen by Dominguez et al. (1998). There have been other IR studies that report spectra of *sub-fossil* pollen (Jardine et al. 2017, 2020, 2021), but these used multiple chemical extractions to isolate the fossil pollen, such as acetolysis, HF, KOH, etc., which makes it challenging to attribute the chemical alterations observed. Jardine et al. (2020) extracted fossil *Pinus* pollen with HF and acetolysis from a varved core. Their spectra are similar in the 1800 - 1500 cm^{-1} region, but show larger dissimilarities in the 1200 - 1000 cm^{-1} region, where we observe two large peaks at 1040 and 1190 cm^{-1}, while their spectra show multiple sharp peaks in the same region.

In another study, acetolysed spectra that are similar to ours in the 1200 - 1000 cm^{-1} region are acetolysed *modern* samples from herbaria of Nitrariaceae and Poaceae pollen

(Woutersen et al. 2018, Jardine et al. 2021). Jardine et al. (2021) also report spectra from chemically treated fossil Poaceae pollen, which are not similar to our *Pinus* spectra nor the acetolysed modern Poaceae set from the same study. Not all the fossil Poaceae samples were treated with acetolysis, but with a combination of lignite, HCl and $Na_4P_2O_7$. The two acetylation peaks we and others (Dominguez et al. 1998, Woutersen et al. 2018, Jardine et al. 2021) observed in acetolysed pollen are not visible in other studies that use acetolysis in addition to other chemical treatments (Jardine et al. 2020, 2021). Dominguez et al. (1998) write further on the nature of these acetylation peaks, that the bands are removed by treatment with methanol under reflux conditions. It seems that these peaks are only reported in pollen that is treated with acetolysis only, which are often modern samples that are treated in order to mimic subfossil or fossil pollen. It is very important to investigate the effect of sediment extraction methods and other chemical treatments carefully going forward. Studies, like paper III, reporting untreated or minimally treated fossil pollen and acetolysed replicates (or other treatments) are important, to assess the changes due to chemical extraction methods, acetolysis among them.

One of the difficulties with FTIR and IR methods in general is that spectra are converted to relative absorbance, which makes it difficult to identify which part of the spectra has changed. ABsolute absorbance is difficult to use, because e.g. scattering issues prevent the use of Beer-Lamberts law. Modern studies can assume that changes in the sporopollenin absorbance bands are relatively stable compared to changes in lipid peaks, but this is not the case for fossil applications or reconstructions of UV-B. In our results, we can identify the main sporopollenin peaks (phenylpropanoid) in the fossil spectra (1600, 1515, 1170 cm^{-1}), but the relative peak height of these wavebands does change between modern, acetolysed and non-acetolysed pollen (Fig. 7). Here, any changes to the relative absorbance of sporopollenin peaks may as well be caused by changes to everything else. Our results show that acetolysis alters the chemical spectra quite drastically compared to untreated fossil and modern samples. It is therefore also important to examine the effects of other chemical extractions on untreated fossil material to identify potential changes.

There is a variety of methods being used to extract pollen or purify modern pollen,

that progressively remove "labile" components from sporopollenin. In addition there are multiple methods that are commonly used for sediment sample preparation. Studies examing the chemical composition of sporopollenin over the past decade have revealed a variety of possible sporopollenin chemical compositions (Wehling et al. 1989, de Leeuw et al. 2006, Jardine et al. 2017, Li et al. 2019). It may be argued that these differences may be based in differences of methods and species used. Nierop et al. (2019) demonstrate that different species produce structurally very different sporopollenins by examing the composition of early plant spores sporopollenin and noticing differences in the amount of phenolic acids bound to sporopollenin. Sporopollenin seems to be more of a collection of chemically similar complex biopolymers that has evolved in parallel with the development of plants. Indeed, we have observed differences in *Quercus* sporopollenin chemistry analogous to morphological differences, which indicates that the different surface ornamentations of the *Quercus* sections are also chemically distinct. The suggestion from Nierop et al. (2019) is that the ornaments may have differences in the relative amount of different polyphenolic compounds (e.g. para-coumaric, ferulic or caffeic acid). Nevertheless, due to the relative nature of IR spectra and overlapping absorbance bands of, e.g., lipids with sporopollenin components, makes it difficult to attribute the chemical differences observed in the spectra purely to sporopollenin. Additional chemical methods, such as NMR spectroscopy or pyrolysis GC-MS in addition to IR methods, may supplement the chemical information from FTIR and FT-Raman with insights on chemistry of sporopollenin components.

Other applications interested in pollen chemistry avoid acetolysis because it can contaminate the pollen with modern carbon isotopes, and use alternate extractions without carbon-based heavy acids (e.g. only sulphuric acid see Loader and Hemming 2000). Alternate extraction methods, such as heavy density separation may be a suitable replacement, because it offers a way to extract pollen without the use of strong acids. Paper III demonstrated the advantages and challenges for chemical palaeoecology of density separation. It allowed us to analyse untreated fossil pollen, but it also outlined challenges: i. pollen density varies for certain sediment types and requires testing and adjustment of extraction density. ii. density separation requires careful washing to remove any residue of the separation medium. I found contamination of SPT in samples of one of the cores, despite no difference between cores in washing procedure after density separation. In

summary, careful evaluation of the effects of chemical extractions on untreated fossil pollen and modern material may provide a solution that identifies a method to fossilize modern grains to resemble fossil grains.

4.4 Discussion of FTIR vs Raman.

The results of paper I and II have shown that FTIR spectra of modern pollen are most variable in the labile components (lipids, carbohydrates, proteins). The sporopollenin peaks visible in FTIR are connected to phenylpropanoids and are less variable. From analysis of fossil *Pinus* pollen I showed that the phenylpropanoid peaks are also characteristic for fossil pollen, in both acetolysed and non-acetolysed pollen. Raman is an alternative vibrational spectroscopy method, that is less used than FTIR, and which provided more detail on *Quercus* sporopollenin chemistry at the cost of less variation in other areas (e.g. the lipid peak at 1745 cm^{-1} is almost not visible in Raman). For pollen studies, Raman may be the a more suitable methods for identification and proxy development. The results from paper II show that it provides complementary information, which is mainly more detail on sporopollenin chemistry that improved the models ability to differentiate *Quercus* pollen. Using FT-Raman we were able to utilise increased detail on phenylpropanoid groups to increase the classification of *Quercus* pollen.

There are important draw-backs to Raman, however, compared to FTIR. FTIR as a method is more transferable from bulk analyses to microscopy FTIR, which the result of paper III and IV demonstrate. Our FTIR spectra of modern *Pinus* pollen is very close to published spectra of bulk analysis FTIR techniques, such as ATR and KBr pellet (Zimmermann 2010, Bağcıoğlu et al. 2015). FTIR microscopy spectra are noisier or show scatter artifacts, but in general FTIR microscopy produces spectrum under most circumstances. Spectral noise can be addressed by averaging spectra (Zimmermann 2018) and scattering effects can be suppressed by embedding or analytically, which paper IV demonstrates. With Raman, there are a number of undesired effects that may be produced, rendering the spectra unusable. For The near-IR absorption of the sample can pose a problem, because absorption bands in the near-IR region can weaken the incident laser and unequal absorption can reduce Raman band intensities. Another common

problem for pollen is intense heating and in some cases thermal decomposition, caused by absorption at the laser wavelength (Chase 1986, Baranski et al. 2005, Kairyte et al. 2012). FT-Raman can alleviate some of these issues, as we have seen in paper II where I encountered some problems with sample heating, but reduction of laser power solved these issues.

Nevertheless, flourescense and thermal decomposition are a significant problem when measuring pollen grains with the labile components removed, such as acetolysised modern pollen or fossil pollen from the Quaternary. Interestingly, Raman spectroscopy may be viable again for very old or thermally matured pollen and microfossils (Marshall et al. 2005, Marshall and Marshall 2015, Bernard et al. 2015). These studies are examples of Raman microscopy used to record spectra from microfossil algae (acritarch) (Marshall et al. 2005), which made up of a complex biopolymer, which is similar to sporopollenin. As for pollen from the Quaternary, the problem of flourescense and thermal decomposition remain significant and require novel solutions (such as Joseph et al. 2011). Here, purified modern pollen was embedded in silver nanoparticles that enabled the vibrational spectroscopic access to the sporopollenin biopolymer based on surface-enhanced raman scattering.

Another significant challenge for Raman is the development of suitable Raman microscopy technique to address autofluorescence and sample heating. Innovative new sample preparations or embeddings may solve the heating issue for Raman, while other adjustments to laser type or laser power may alleviate challenges further. I think more experiments with Raman microscopes on modern and fossil pollen are a way to improve measurement techniques. Our results have shown the successes of FT-Raman for pollen analysis, which suggests studies with FT-Raman microscopes on modern pollen material as a first step to investigate pollen chemistry.

FTIR microscopy on the other hand has made very promising developments in the past five years, which allows the simultaneous capture of multiple fossil pollen spectra as evidenced in my work in paper III, where we record single grain spectra of fossil pollen from sediment cores. Paper IV shows several approaches that can address scattering anomalies in FTIR spectra. In fact, for taxonomic purposes, a certain amount of scattering may be beneficial, because scattering does hold taxonomic information (see results in

paper IV). For other applications, such as proxy reconstructions (UV-B), scatter suppression by embedding or filtering of scatter effects by analytical means may improve signal recovery.

In general, FTIR remains a promising method for modern and fossil studies, because of its versatility in measuring both sporopollenin and labile components of pollen chemistry. For modern pollen Raman is already a viable alternative that adds important information on carotenoids and sporopollenin composition. I was able to show the potential of Raman, FTIR and FTIR microscopy for pollen chemical studies in this thesis. Application of Raman for fossil or purefied sporopollenin remains challenging, but given enough time and investment, I remain convinced that Raman can "catch up" to FTIR. More work on fossil material is needed to refine these methods.

4.5 Perspectives for chemical palaeoecology

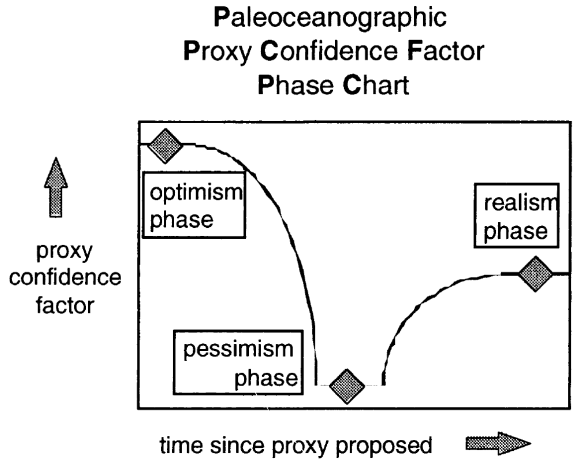

Figure 10: Proxy confidence graph first published in Elderfield (2002).

The development of new methods in palaeoecology is always connected to large risks and new challenges that will reshape the goals of the development. Elderfield (2002) provides a schematic representation of how confidence in palaeoecological proxies can evolve over time (Fig. 10) and move through three phases: optimism, pessimism and realism. As the development of the proxy continues, it moves through the different phases, where more and more knowledge is learned about the controlling factors of the proxy and interactions

to other systems in the pessimism phase, before it reaches the realism phase. They are using the Mg/Ca palaeotemperature proxy as an example, which had the potential to also provide an estimate of ocean δ ^{18}O, which lead to new knowledge about dissolution dynamics and factors controlling trace element incorporation. It is important to note that this confidence graph is not a scientific fact but merely an illustration of proxy development by one scientist.

4.6 Are we at the realism phase yet?

If we apply Elderfield's graph to pollen chemistry and specifically palaeochemistry, I would suggest the optimism phase is behind us. There have been multiple studies that show the possibilities of classification on modern material and we have begun to accumulate more and more knowledge about the chemical composition of pollen. The field has begun to explore the sources of chemical variation, because it was necessary to understand modern pollen chemical variation. The first half of this thesis is a contribution to this effort, exploring chemical variation of *Quercus* with FTIR and FT-Raman. Studies in chemical palaeoecology have also begun to explore fossil pollen chemistry and how it is affected by, e.g., extraction methods, diagenesis, etc.. Papers III and IV of this thesis contribute to this aspect. Paper III explores fossil pollen chemistry and extraction methods alternative to acetolysis, while paper IV demonstrates the possibilities of scatter correction techniques and shows that some taxonomic information is stored in scattering signals. We, as a field, have begun to understand more and more about the variations underlying pollen chemistry, but I think there are still too many known unknowns (e.g. chemical structure of sporopollenin, etc), and unknown unknowns to have reached the realism phase. I would place chemical methods in the pessimism phase, with quite a few unanswered questions and challenges.

In order to move into the realism phase, we have to address the challenges I described earlier. I outlined the importance of examining commonly used chemical extraction methods and their impact on (sub)-fossil pollen chemistry as a crucial point to consider as a result of paper III. The development of methods to explain or bridge the differences between modern and fossil pollen chemistry may be the main challenge for chemical

palaeoecological methods. An important step is to measure additional untreated fossil pollen species to examine the composition of sporopollenin in the fossil setting. The work in paper III is a first step in that direction and spectra published in Jardine et al. (2021) also show promising insights into the chemistry of sporopollenin and how it may be affected by diagenetic processes and chemical extractions. Further studies on untreated fossil and modern material may give more insight. Our use of density separation is one method of acquiring untreated fossil pollen. We outlined the advantages: no chemical alteration, and challenges: requires fine-tuning of density and sufficient washing after extraction. Furthermore, the exploration of the stepwise degradation of modern pollen material until they resemble fossil pollen, may further our understanding of the processes affecting fossil pollen and our knowledge on the chemistry of sporopollenins. It would also allow for artificial fossilization of larger amounts of pollen, easing the analysis with destructive methods, such as pyrolysis GC-MS or more work intensive methods, such as nuclear magnetic resonance (NMR) spectroscopy.

This is a truly exciting and oftentimes challenging moment, in which I look forward in the future to contribute to moving chemical methods into the realism phase.

4.7 Perspectives of deep learning for chemical palynology

One of the most promising applications of chemical palynology is the combination with deep-learning methods. Deep-learning, computer-vision methods and CNN architectures have evolved and steadily improved over the course of my PhD. Newer deep-learning models are able to locate objects of interest in images (Ren et al. 2015) and assign descriptions or finer classifications via vector embeddings (Xu et al. 2018, Yu et al. 2019). These methods are all image-based and, after some exploratory work in the beginning of my PhD with applying neural networks and deep learning on pollen images (Appendix A), I think these techniques have great potential for the future of palynology. I applied some, at the time, new neural network architectures on a small set of *Eucalyptus* pollen, to compare to traditional approaches and found higher classification success. Due to Covid-19 and some technical challenges, I was unable to pursue this work further, but I see potential for deep learning in palynology and also for combination of image based-

with chemical approaches.

New applications for palynology are exciting. One could design a network that describes pollen the same way a palynologist would using morphological characteristic by utilizing vector embeddings (Xu et al. 2018, Yu et al. 2019). This approach would also allow for pollen types that are not part of the training set to be described if certain morphological characteristics that the network knows are detected. In traditional CNNs, classes the network does not know are always assigned a class even if it is not similar to any class the network knows. This is problematic for palynology, where unexpected pollen types could end up as mis-identifications with other common taxa and remain undetected mis-identified. The before mentioned vector embeddings can also be used as a dissimilarity estimation to check if a pollen is too dissimilar to pollen that the network knows before identification. The prospects of new applications of deep-learning models for palynology are very promising and could finally fulfill the demand by Stillman and Flenley (1996) for automation of palynology.

The transfer of computer-vision methods to chemical palynology would be valuable and open new research possibilities. In principal, an image with spectra for pixels is just a picture with 100s of channels instead of three in convential image-files (RGB). Localization, segmentation and identification of objects on images are tasks that deep-learning models have performed well at (Long et al. 2015, Ren et al. 2015, Xiang and Fox 2017). The application of these models to spectra images for palynological purposes is still a challenge, but not an insurmountable one. In theory, these models could be applied to spectra images to, for example, extract and identify pollen grains from images taken with a FTIR microscopes equipped with a FPA detector. Data created from FPA-FTIR microscopes produce large amounts of data, and deep-learning models are excellent at processing such large amounts of data. In addition, with deep-learning models, combining spectral information with images is also possible as shown in Kang et al. (2020) where spectra and images-stacks of bacteria were used together to train the deep-learning model. This would allow the combination of chemical and morphological information for classification or other applications. The combination of chemical and traditional palynology is a promising development for the future of palaeoecology.

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Received: 12 August 2019 | Revised: 3 December 2019 | Accepted: 14 January 2020

DOI: 10.1111/jbi.13817

RESEARCH PAPER

Journal of Biogeography | WILEY

Chemical variations in *Quercus* pollen as a tool for taxonomic identification: Implications for long-term ecological and biogeographical research

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Funding information
Research Council of Norway; L. Meltzer Høyskolefond, Grant/Award Number: 2017/05/LMH; Olaf Grolle Olsen Legat, Grant/Award Number: 2017/52/FOL; EU H2020, Grant/Award Number: 741413

Handling Editor: Mark Bush

Abstract

Aim: Fossil pollen is an important tool for understanding biogeographical patterns in the past, but the taxonomic resolution of the fossil-pollen record may be limited to genus or even family level. Chemical analysis of pollen grains has the potential to increase the taxonomic resolution of pollen analysis, but present-day chemical variability is poorly understood. This study aims to investigate whether a phylogenetic signal is present in the chemical variations of *Quercus* L. pollen and to assess the prospects of chemical techniques for identification in biogeographical research.

Location: Portugal.

Taxon: Six taxa (five species, one subspecies) of *Quercus* L., *Q. faginea*, *Q. robur*, *Q. robur* ssp. *estremadurensis*, *Q. coccifera*, *Q. rotundifolia* and *Q. suber* belonging to three sections: *Cerris*, *Ilex* and *Quercus* (Denk, Grimm, Manos, Deng, & Hipp, 2017).

Methods: We collected pollen samples from 297 individual *Quercus* trees across a 4° (~450 km) latitudinal gradient and determined chemical differences using Fourier-transform infrared spectroscopy (FTIR). We used canonical powered partial least squares regression (CPPLS) and discriminant analysis to describe within- and between-species chemical variability.

Results: We find clear differences in the FTIR spectra from *Quercus* pollen at the section level (*Cerris*: ~98%; *Ilex*: ~100%; *Quercus*: ~97%). Successful discrimination is based on spectral signals related to lipids and sporopollenins. However, discrimination of species within individual *Quercus* sections is more challenging: overall, species recall is ~76% and species misidentifications within sections lie between 18% and 31% of the test set.

Main Conclusions: Our results demonstrate that subgenus level differentiation of *Quercus* pollen is possible using FTIR methods, with successful classification at the section level. This indicates that operator-independent FTIR approaches can surpass traditional morphological techniques using light microscopy. Our results have implications both for providing new insights into past colonization pathways of *Quercus*,

and likewise for forecasting future responses to climate change. However, before FTIR techniques can be applied more broadly across palaeoecology and biogeography, our results also highlight a number of research challenges that still need to be addressed, including developing sporopollenin-specific taxonomic discriminators and determining a more complete understanding of the effects of environmental variation on pollen-chemical signatures in *Quercus*.

KEYWORDS

chemical composition, ecology and environmental sciences, Fourier-transform infrared spectroscopy, palynology, partial least squares regression, pollen

1 | INTRODUCTION

Subfossil pollen remains preserved in lake sediments or peat bogs have been important tools to reconstruct past floristic, vegetational and environmental changes for over 100 years. The biogeographical applications of such reconstructions are varied and wide-ranging. Palaeoecological studies based on fossil pollen have made vital contributions to understanding the broad-scale range dynamics through time, the rates and directions of spread of different plant species, and the location of glacial-stage refugia (see Birks, 2019 for a review). Fossil pollen data can also be used to track relative-niche shifts in association with the emergence of no-analogue climates (Veloz et al., 2012) and forecast future range shifts as a result of climate change (e.g. Nogués-Bravo et al., 2016, 2018).

The basis of all such studies is reliable identifications of fossil pollen to the lowest taxonomic level possible. With detailed identifications, reconstructions and answers to particular biogeographical and ecological questions can similarly be detailed. Indeed, many advances in historical plant geography (e.g. Birks, 2008; Birks, 2014; Godwin, 1975; Lang, 1994; Magri et al., 2006) have been made because of advances in the identification of plant fossils. However, although Quaternary botany (sensu Birks, 2019) has been dominated for over 100 years by pollen analysis, identifications can only be made to the genus or family level for many taxa. This is limiting the biogeographical information gained from fossil pollen to relatively coarse taxonomic levels.

This issue is of particular relevance for understanding the past, present and future distributions of the genus *Quercus* (oak). *Quercus* contains 22 native species in two subgenera and three sections in Europe (Denk, Grimm, Manos, Deng, & Hipp, 2017; Tutin et al., 1993), several of which have striking and often distinct geographical distributions today (e.g. Iberia, Balkans, eastern Mediterranean, widespread Mediterranean, Apennine Peninsula, widespread to about 60°N; Jalas & Suominen, 1976). However, what is known about the history of *Quercus* is almost entirely based on pollen and is thus only at the genus level. Although three pollen-morphological types can, with care, be distinguished by conventional light microscopy (LM) (Beug, 2004) and scanning election microscopy (SEM) (Denk & Grimm, 2009), fossil pollen of *Quercus* is usually determined as either *Quercus* Deciduous or *Quercus* Evergreen types.

This situation has several implications for biogeographical research. Maps of the changing distribution and abundance of oak pollen in the late-glacial and Holocene of Europe (Brewer et al., 2017; Huntley & Birks, 1983) can only confidently be made using the two broad pollen morphotypes (i.e. *Quercus* Deciduous or *Quercus* Evergreen). Palaeo-biomization methods used to forecast the future responses of Mediterranean ecosystems to climate change have used the same distinction between these two morphotypes (Guiot & Cramer, 2016), while a recent attempt to model future responses of *Quercus* in Europe using fossil pollen were based on *Quercus* pollen resolved to the genus level (Nogués-Bravo et al., 2016). As the sensitivity and response of *Quercus* to recent environmental changes is species-specific in Mediterranean ecosystems (Acácio, Dias, Catry, Rocha, & Moreira, 2017), and because *Quercus* macrofossils are very rarely found, any improved understanding of its historical and future biogeography clearly depends on consistent pollen identifications at levels lower than is presently available.

One potential approach lies in the chemical analysis of pollen. Fourier-Transform Infrared Spectroscopy (FTIR) is a non-destructive method which is used to infer the chemical composition of a sample based on the fact that different molecular-functional groups have different wavelength-specific absorbances of infrared radiation due to differences in vibrational patterns (Bağcıoğlu, Zimmermann, & Kohler, 2015; Gottardini, Rossi, Cristofolini, & Benedetti, 2007; Ivleva, Niessner, & Panne, 2005; Pappas, Tarantilis, Harizanis, & Polissiou, 2003; Parodi, Dickerson, & Cloud, 2013; Schulte, Lingott, Panne, & Kneipp, 2008; Zimmermann, 2010; Zimmermann & Kohler, 2014). Evidence suggests that the analysis of pollen using FTIR may be a useful tool for differentiating between pollen types extracted from sediment sequences (Jardine, Gosling, Lomax, Julier, & Fraser, 2019; Julier et al., 2016; Woutersen et al., 2018), because pollen-grain chemistry may show biogeographical patterns related to phylogeny and environmental conditions (Bağcıoğlu, Kohler, Seifert, Kneipp, & Zimmermann, 2017; Depciuch, Kasprzyk, Roga, & Parlinska-Wojtan, 2016; Zimmermann et al., 2017).

However, although taxonomic differentiation of pollen based on the chemical variations inferred by FTIR shows considerable promise,

widespread application of FTIR in biogeography and palaeoecology remains limited. One potential for this limitation is because lipids and proteins can be major discriminants of variation in FTIR spectra (Bağcıoğlu et al., 2017; Zimmermann & Kohler, 2014), but these may not be preserved in sediment sequences alongside the sporopollenin-based exines (Zimmermann, Tkalčec, Mešić, & Kohler, 2015). Therefore, the relative importance of the different chemical structures (e.g. lipids, proteins, sporopollenins) that are responsible for discrimination between many modern pollen types still needs to be established. Moreover, influence of various abiotic stressors on pollen chemistry can hinder taxonomic differentiation of pollen samples by FTIR (Depciuch et al., 2016; Lahlali et al., 2014; Zimmermann et al., 2017), and this needs to be researched further. In addition, the majority of studies investigating pollen chemistry have used pollen from herbaria, botanical gardens or university campuses, with a limited number of replicates per location and large number of different species and families. Although this sampling design encourages ease of access, reliable identification, and a broad species range, the number of replicates remains a limiting factor for understanding chemical variations in response to both environmental and taxonomic variations.

This study aims to address the challenges related to understanding the taxonomic variations of pollen chemistry by investigating the relative importance of within- and between-species chemical differences in *Quercus*. Our dataset is unique because it represents the largest collection of closely related species sampled from populations outside botanic gardens across a large bioclimatic and biogeographical gradient in Portugal. We use multivariate-discriminant analysis to (i) investigate the potential for FTIR as tool to differentiate six taxa of *Quercus* based on pollen, and (ii) determine the main chemical-functional groups responsible for chemical variation observed in the dataset. Addressing these questions represents the first step if we are to successfully use pollen chemistry as a tool to improve our understanding of past biogeographical patterns and the history of oaks in Europe.

2 | MATERIALS AND METHODS

2.1 | Sample collection

We collected pollen samples from 294 individual trees belonging to five *Quercus* species across a 4° (~450 km) latitudinal gradient in Portugal (Figure 1). The *Quercus* taxa in this study belong to the sections *Cerris*, *Ilex*, and *Quercus* according to Denk et al. (2017) and have different geographical distributions (Table 1). Trees were sampled along gradients of temperature and precipitation to cover a wide range of environmental conditions. A detailed summary of the number of trees sampled at each location is in Table S1.

All samples were collected in spring 2018 by taking whole-tree composite samples of ca. 30 catkins per individual tree. Several branches were sampled for catkins up to 5 m in height. The catkins were air-dried at room temperature (23°C) for at least 24 hr and the pollen was separated from the anthers by light shaking. Pollen was also sieved through 60 µm sieves to remove excess plant material before analysis.

2.2 | Pollen-chemistry measurements

Reflectance-infrared spectra were recorded using a Vertex 70 FTIR spectrometer (Bruker Optik GmbH) with a single reflectance-attenuated total-reflectance (SR-ATR) accessory. The ATR IR spectra were

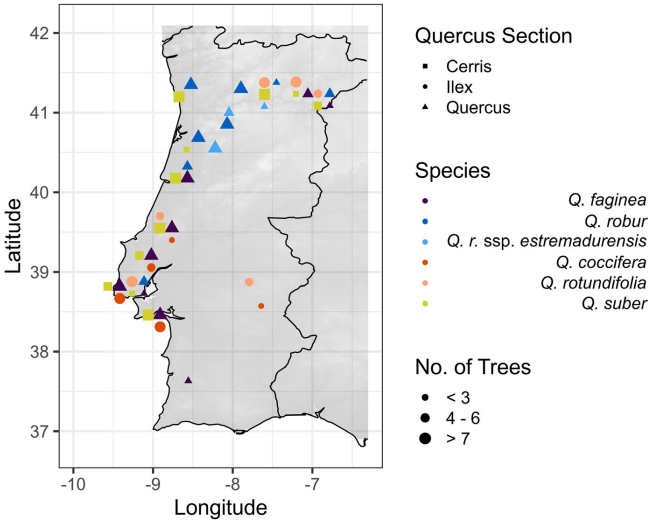

FIGURE 1 Map of Portugal displaying the locations where the *Quercus* trees where sampled. Colour indicates species, shape indicates *Quercus* section following Denk et al. (2017) and size of the symbol indicates number of trees sampled per location

TABLE 1 Taxonomy of sampled Quercus trees and total number of trees sampled (*n*). Sections are according to Denk et al. (2017)

Subgenus	Section	Species	Subspecies	*n*	Distribution notes
Cerris	*Ilex*	*Q. coccifera*		36	*Q. coccifera* and *Q. rotundifolia* prefer xerophytic conditions and are often co-occurring species. Both are indifferent towards bedrock conditions, but prefer soils without waterlogging, although *Q. coccifera* is a thermophilous species and is less tolerant of winter cold.
Cerris	*Ilex*	*Q. rotundifolia*		38	
Cerris	*Cerris*	*Q. suber*		69	*Q. suber* is distributed across the Mediterranean Basin on siliceous bedrock but is absent from areas with winter cold (Amigo, 2017; Matías, Abdelaziz, Godoy, & Gómez-Aparicio, 2019).
Quercus	*Quercus*	*Q. faginea*		60	*Q. faginea s.l.* has a broad distribution in the Iberian Peninsula (Tschan & Denk, 2012) and is more abundant on limestone with higher summer precipitation, tracking the sub-Mediterranean bioclimatic belt (Sanchez, Benito-Garzon, & Ollero, 2009).
Quercus	*Quercus*	*Q. robur*		76	*Q. robur* has a temperate distribution and is more abundant in north-west Portugal (Jalas & Suominen, 1976). It occurs in regions with sufficient summer rain, and is absent from areas with summer drought (Amigo, 2017; Ülker, Tavsanoglu, & Perktas, 2018).
Quercus	*Quercus*	*Q. robur*	*estremadurensis*	15	

recorded with a total of 32 scans and spectral resolution of 4 cm^{-1} over the range of 4000–600 cm^{-1}, using the horizontal SR-ATR diamond prism with 45° angle of incidence on a High Temperature Golden gate ATR Mk II (Specac). Approximately 1 mg of dried pollen was deposited onto the ATR crystal for each measurement (three replicate measurements). Between each measurement a background (reference) spectrum was recorded using the sample-free setup. The OPUS software (Bruker Optik GmbH) was used for data acquisition and instrument control.

We pre-processed the spectra since multivariate-regression methods (e.g. partial least squares; PLS) have been shown to perform better with pre-processed spectra in other studies (Woutersen et al., 2018; Zimmermann & Kohler, 2013). The processing of the spectra consisted of smoothing and calculation of the second derivative using the Savitzky–Golay algorithm, as implemented by the extended multiplicative signal correction (EMSC) package (Liland, 2017). The settings of the Savitzky–Golay smoothing algorithm (Edwards & Willson, 1974; Savitzky & Golay, 1964) were: second degree polynomial and a window size of 11. The second-derivative spectra were constrained between 700 and 1,900 cm^{-1} and normalized using EMSC, a multiplicative signal correction model extended by a linear and quadratic component (Liland, 2017). For further analyses, the mean of the measurement replicates (three) was calculated for each tree (resulting in one spectrum per tree). We follow peaks of interest that have been attributed to chemical-functional groups according to Pappas et al. (2003), Gottardini et al. (2007), Schulte et al. (2008), Zimmermann (2010) and Zimmermann and Kohler (2014) (Table 2).

2.3 | Statistical analyses

For the exploration of within- and between-species chemical variability, we fitted a PLS model combined with canonical correlation analysis (CPPLS) to the processed mean spectra (second derivative) to predict species identity. This analysis was implemented in the 'pls' package (Mevik, Ron Wehrens, & Liland, 2019) in R version 3.6.0 (R Core Team, 2019). The PLS family of models has been shown to be powerful in multivariate analyses of FTIR-spectral data (Liland, Mevik, Rukke, Almøy, & Isaksson, 2009; Telaar, Nürnberg, & Repsilber, 2010; Wold, Sjöström, & Eriksson, 2001; Zimmermann et al., 2017). The CPPLS method improves the extraction of predictive information by estimating optimal latent variables in comparison to standard PLS regression (Mehmood & Ahmed, 2016). Unlike standard PLS, CPPLS weights the contribution of the explanatory variables (wavenumbers), which weakens the contribution of non-relevant wavenumbers to optimize the covariance between response (species) and explanatory variables (wavenumbers). Indahl, Liland, and Næs (2009) show improved accuracy and increased explained variance of CPPLS compared with conventional PLS regression using spectral data.

To assess the classification performance of the CPPLS, the dataset was randomly split into training and test sets using a 60%/40% split. This split was repeated 100 times to create 100 versions of the dataset (folds) with different training/test splits. A CPPLS model was fitted for each fold and the extracted component scores were used to predict species identity using limited discriminant analyses. The performance of the classifier in predicting the test set was averaged over the folds and summarized in a confusion matrix (Table 3).

3 | RESULTS

3.1 | Chemical variations in *Quercus*

Assessment of the mean spectra of the five *Quercus* species and one subspecies reveals clear differences in absorbance between the major intrageneric lineages (sections) at wavelengths associated with specific chemical functional groups (Figure 2). For example, the lipid peak absorbance at ~1,745 cm^{-1} is weaker in section *Ilex* compared with the other sections, while the sporopollenin and carbohydrate absorbance bands (at 1,516, 1,171, 833 and 985 cm^{-1} respectively) are noticeably lower in absorbance in the taxa belonging

TABLE 2 Wavenumber of peaks attributed to specific functional groups in spectra of fresh pollen and their representative compounds (Pappas et al., 2003; Gottardini et al., 2007; Schulte et al., 2008; Zimmermann, 2010, p. 20109; Zimmermann & Kohler, 2014 compiled in Zimmermann, Bağcıoğlu, et al., 2015)

Compounds	Wavenumber (cm^{-1})	Functional group
Lipids (Triglycerides and Phospholipids)	1,745 1,462 1,171* 721; 995*	C=O stretch CH_2 deformation C–O–C stretch CH_2 rocking
Proteins	1,641; 1,651 1535; 1,551	Amide I: C=O stretch Amide II: NH deformation and C–N stretch
Carbohydrates (Cellulose and Amylose)	1,200–900 1,171*; 1,107; 1,055; 1,028 1,076; 995*	C–O–C and C–OH stretch
Sporopollenins	1,605; 1,516; 1,171*; 852; 833 and 816	Aromatic rings in phenylpropanoid subunits

Note: A (*) marks wavenumbers which are shared by more than one compound (Bağcıoğlu et al., 2015). The peak at 1,171 cm^{-1} is an indicator for C-O-C stretching, that can be present in various types of lipids (triglycerides and phospholipids) and sporopollenins as well as some types of carbohydrates.

TABLE 3 Confusion matrix of linear discriminant analysis on the test sets using four components of the fitted canonical powered partial least squares (CPPLS) model

Pred/Ref	*Q. faginea*	*Q. robur*	*Q. r. estr.*	*Q. coccifera*	*Q. rotund.*	*Q. suber*
Q. faginea	64 ± 12	18 ± 8	11 ± 13	0	0	1 ± 1
Q. robur	30 ± 12	76 ± 10	81 ± 18	0	0	1 ± 2
Q. r. estr.	1 ± 2	5 ± 6	6 ± 13	0	0	0
Q. coccifera	1 ± 3	0	0	75 ± 14	25 ± 11	0
Q. rotund.	1 ± 2	0	0	25 ± 14	75 ± 11	0
Q. suber	3 ± 4	1 ± 3	2 ± 6	0	0	98 ± 2

Note: Predictions as rows and reference as columns. Values given as % of spectra that were predicted as species and sum to 100% column wise, for example, for *Q. robur* 18% of spectra were predicted as *Q. faginea*, 76% were correctly classified as *Q. robur* and 5% as estremadurensis. Blue signifies section Quercus; red is section Ilex and yellow is section Cerris.

Abbreviations; *Q. r. estr*, *Q. robur* ssp. estremadurensis; *Q. rotund.*, *Q. rotundifolia*.

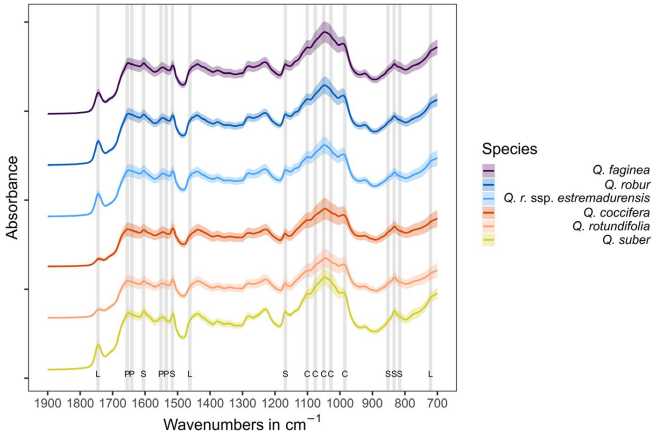

FIGURE 2 Mean absorbance spectra of *Quercus* species and notable peak locations. Lipids (L), protein (P), sporopollenin (S), and carbohydrates (C) using wavenumbers given in Table 2. Spectra are offset. Shaded area represents the standard deviation of the mean spectra for each corresponding *Quercus* species or subspecies. The *Quercus* taxa are colour-coded according to their section: shades of blue indicate section *Quercus*; shades of red indicate section *Ilex* and section *Cerris* is in yellow

to section *Ilex*. Note, however, that although clear spectral differences exist at the section level, it is more difficult to separate variations between species *within* different sections (Figure 2).

The observations made following assessment of the mean spectra are confirmed by the analysis using CPPLS (Figure 3a). Here, the three sections of *Quercus* can be clearly separated using

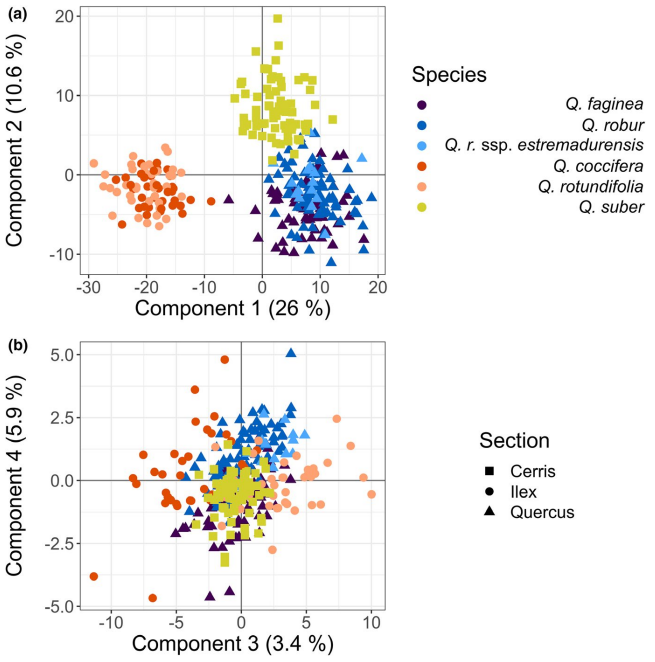

FIGURE 3 Ordination of components 1–4 of canonical powered partial least squares (CPPLS) models (100-fold cross-validation). Each point represents a sampled *Quercus* tree. The mean training scores for each sample over the 100-folds were calculated. Proportion of variance explained by each component in parentheses. Colour indicates species, while symbol indicates *Quercus* section

the variance explained by two CPPLS components (Figure 3a). For example, the section *Ilex* scores are negative along the first component (26% of the variation), while individuals of sections *Quercus* and *Cerris* have positive scores along this axis. The sections *Quercus* and *Cerris* can mainly be separated along the second principal component (section *Cerris* with positive scores and section *Quercus* with negative scores). However, between-species level variations within species in the same section are harder to differentiate, because there is a large overlap between species of the sections *Ilex* and *Quercus*. This overlap is reduced on later components, where the different species separate within their respective sections. On components 3 and 4 *Q. coccifera* and *Q. rotundifolia* can be separated along the third component, while *Q. robur* and *Q. faginea* show some separation along the fourth component (Figure 3b). In total, the two first components explain ~37% of the variation in the dataset and separate the samples into the *Quercus* sections, while the next two components explain a further ~9.7% (Figure S1).

Since specific absorbance peaks in the FTIR spectra can be related to chemical functional groups (summarized in Table 2), it is possible to identify which chemical functional groups have the highest influence in explaining patterns of variation observed in our dataset (Figure 4). In general, lipids and sporopollenins have high loadings (i.e. greater influence) on component 1, followed by carbohydrates. On component 2, the 852 cm^{-1} sporopollenin peak shows the highest positive loadings in contrast to the other sporopollenin peaks (816, 833, 1,516 and 1,605 cm^{-1}), followed by the 995 cm^{-1} peak, which ca be attributed to carbohydrates or phospholipids (Bağcıoğlu et al., 2015). Components 3 and 4 have higher loadings for carbohydrates and protein peaks. Taken together, these results indicate that sporopollenin and lipids are strong drivers of the main sources of variation in the dataset and congeneric discrimination is achievable based on FTIR at least at section level.

3.2 | Discriminant analysis

Using four components (explaining ~45% of the variance) the confusion matrix of the classification CPLS model shows clear differentiation between the *Quercus* sections, with some misidentified spectra (<~2 ± 3) from section *Quercus* and *Q. suber* (Table 3). *Quercus robur* ssp. *estremadurensis* has by far the worst accuracy in the model and is most often identified as its parent species *Quercus robur*, possibly due to the limited number of samples in the dataset (Table 1). In general, species misidentifications are contained within the different *Quercus* sections and lie between 18% and 30% of the test-set samples (within-section misidentifications: 18% of *Q. robur* as *Q. faginea*; 30% of *Q. faginea* as *Q. robur*; 25% of *Q. coccifera* as *Q. rotundifolia*; 25% of *Q. rotundifolia* as *Q. coccifera*). Overall species accuracy within sections ranges from 64% to 76% in the Ilex and Quercus sections. Increasing the components available to the model to 10 (~57% explained variance) increases species accuracy by 5–10 percentage points in both sections (*Quercus*, *Ilex*) (Table

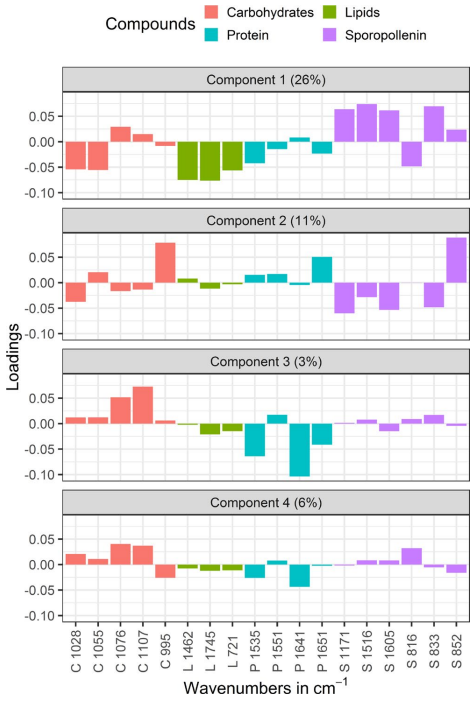

FIGURE 4 Loadings plot of classification of canonical powered partial least squares (CPPLS) model. Lipids (L), protein (P), sporopollenin (S), and carbohydrates (C) using wavenumbers given in Table 2. High absolute loading indicates a high importance of a given wavenumber for the corresponding component. Loadings are chosen in such a way as to describe as much as possible of the covariance between the variables (wavenumbers) and the response (species). Proportion of variance explained by each component in parentheses

S2). As demonstrated with our ordination plots (Figure 3), differentiation of the three sections of *Quercus* is possible using 37% of the variance in the spectral data, but these results indicate difficulties in differentiation between species of the same section.

4 | DISCUSSION

4.1 | Separation of *Quercus* according to chemical variation

Recent research has shown that spectroscopic methods such as FTIR are effective at differentiating pollen taxa between distantly related families and/or genera using their chemical composition (Dell'Anna et al., 2009; Gottardini et al., 2007; Jardine et al., 2019; Julier et al., 2016; Woutersen et al., 2018; Zimmermann, 2010; Zimmermann, Bağcıoğlu, Bağcıoğlu, Sandt, & Kohler, 2015; Zimmermann et al., 2017; Zimmermann & Kohler, 2014; Zimmermann, Tafintseva, Bağcıoğlu, Berdahl, & Kohler, 2016). Our results build on these previous studies to reveal the potential for chemical variations in pollen to distinguish infrageneric variation between species in pollen samples from 297 individuals from Portugal, which belong to three different *Quercus* sections (*Cerris*, *Ilex*, *Quercus*). We identify a clear separation at the *Quercus* section level (Figure 3 and Table 3) using two components of a PLS model and 37% of the explained variance in the spectral data. One component (component 1) can be used to differentiate the sections *Ilex* and *Quercus*, while component 2 can be used to separate section *Cerris*. Combined, these two components achieve the performance equivalent to SEM methods, where *Quercus* pollen can be confidently identified to section level.

Despite finding that classification at the section level is possible using FTIR approaches, there is considerable overlap in variation between species of the same section. Furthermore, classification performance does not improve when using a more complex model in which the number of components used increases from 4 to 10. Using this more complex model, which explains ~57% of the variance (compared with ~45% in the four-component model), classification accuracies remain roughly similar within *Quercus* sections (Table S2). For example, *Q. coccifera* and *Q. rotundifolia* have a recall of ~75% with both 4 and 10 components. Similarly, approximately one-third of *Q. robur* and *Q. faginea* samples (both belonging to section *Quercus*) are misclassified as the other species. Thus, while our results indicate that subgeneric classification of *Quercus* pollen is possible at the section level using FTIR, we still find it difficult to distinguish between more closely related (i.e. within-section) pollen types.

These findings are approximately in line with other studies that have performed species classification using FTIR. For example, both Julier et al. (2016) and Jardine et al. (2019) report classification successes of ~80% and ~85%, respectively, using an FTIR analysis of cryptic morphospecies within the family Poaceae. Their studies are based on a combination of specimens of mainly non-congeneric grass species (except two species of *Oryza*, Julier et al., 2016, and four species of *Triticum*, Jardine et al., 2019). In both these studies, classification success is lower for the samples belonging to congeneric species and higher for the more-distantly related pollen types (i.e. those species belonging to different genera). In another study, Woutersen et al. (2018) report ~95% recall on largely congeneric species in the Nitrariaceae family using single-grain FTIR, but also note that lack of environmental variability (pollen from one individual per species) could have led to an overestimation of classification success. In contrast, Zimmermann et al. (2017) achieve ~100% accuracy on species identification and 75% accuracy on identification of origin using hierarchical PLSR on pollen from three species of Poaceae (*Festuca ovina*, *Anthoxanthum odoratum*, *Poa alpina*) of different genera and origins (Sweden, Norway, Finland) grown under controlled conditions (45 individuals per species). Such a high classification success on taxa grown in controlled conditions demonstrates the strong phylogenetic signature that can be observed using FTIR. Our results also demonstrate strong phylogenetic

differences in FTIR spectra (i.e. the ability to differentiate between *Quercus*-section level variability), but our study also demonstrates the difficulty of distinguishing between-species level variability, even when relatively large subsets of samples are used.

4.2 | Key chemical drivers of variation within *Quercus* spp. pollen

Given the result that identification is possible at the section level, a key question that follows is which of the chemical components of the pollen grain are mostly responsible for the difference between the three sections of *Quercus* under FTIR? In our study, we find that lipids are one of the most important functional groups in diagnosing samples belonging to section *Ilex*. The wavebands at 1,462 and 1,745 cm^{-1} are particularly important in this regard. Previous research has shown that these wavebands are indicators for triglyceride lipids (Bağcıoğlu et al., 2017). Our results also confirm previous findings by Zimmermann and Kohler (2014), who show extreme variations in the relative content of triglycerides and find this waveband to be an important separator between *Iris*, *Quercus*, and *Pinus* pollen types. Indeed, our results extend the inferences made in that previous study by demonstrating a subgeneric level variability of the relative lipid content. Specifically, we identify relatively fewer lipids in pollen sampled from individuals within the Ilex section (Figure 2).

In addition to the importance of triglyceride lipids as a tool for chemical separation, we also find that wavebands representing building blocks of sporopollenin (Table 3) are important for differentiating taxa on the first two components of our CPPLS analysis. For example, the peaks at 833, 852, 1,516 and 1,605 cm^{-1} are associated with building blocks of sporopollenin (Bağcıoğlu et al., 2015) and have relatively high loadings on component 2, which is used in this study to isolate *Q. suber*. Peaks at 833 and 852 cm^{-1} are related to different types of phenylpropanoid building blocks and our results suggest relative differences in their abundance within the sporopollenin of *Q. suber* compared with the other species. In addition to lipid variation, aromatic peaks at 833, 852, 1,516 and 1,605 cm^{-1} also have a high loading on component 1, which can be used to separate the *Ilex* section pollen from the other sections. Thus, both lipids and sporopollenins are important functional groups to different pollen between the three sections in our dataset.

Our observations of different chemical compositions of sporopollenin mirror the sequence of the development of the pollen wall in different *Quercus* sections described by Solomon (1983a, 1983b) and Denk and Grimm (2009). Evolutionary, pollen of section *Ilex* represent the earliest, primitive state of *Quercus* pollen, with a microrugulate pattern on the pollen exine surface. A set of secondary sporopollenins are then added to this surface during exine formation in pollen of sections *Cerris* and *Quercus* (Denk & Grimm, 2009). It is possible that these key differences in structure and formation of the exine between the section *Ilex* and other *Quercus* pollen grains are responsible for the differences in sporopollenin chemistry identified using FTIR. More detailed work on the composition of sporopollenin of different genera, and how this affects pollen grain structural elements (e.g. Li, Phyo, Jacobowitz, Hong, & Weng, 2019) is needed for this finding to be confirmed.

Finally, protein and carbohydrate peaks (Carbohydrates: 1,107, 1,028, 1,076 cm^{-1}; Proteins: 1,535, 1,641 cm^{-1}) have the highest loadings on components 3 and 4 and are partly responsible for the partial distinction of species within the same section. These peaks represent amylose and cellulose as carbohydrates and amide functional groups within proteins (Table 2). For example, variation along component 3 contributes to the separation of *Q. robur* from *Q. faginea*. However, overall, protein and carbohydrate peaks have the least influence for explaining the variance of classification success, and most of the taxonomically important information we used to distinguish between the species is stored in the lipids and sporopollenin components of the pollen chemistry.

4.3 | Implications for understanding past and future *Quercus* dynamics

We investigated the potential for chemical separation of *Quercus* pollen because, despite the high diversity (22 species) of *Quercus* in Europe, fossil pollen of this genus is still most commonly determined as either *Quercus* Deciduous or *Quercus* Evergreen types (e.g. Brewer et al., 2017; Huntley & Birks, 1983). As a result, there may be much detail missing in our current understanding about past *Quercus* dynamics, which could be improved through methods that result in refined taxonomic resolution. Indeed, our results indicate the potential for FTIR to surpass traditional LM methods used in palynology, and work at a comparable level to SEM (Denk & Grimm, 2009; Denk & Tekleva, 2014; Grímsson, Grimm, Meller, Bouchal, & Zetter, 2016; Grímsson et al., 2015). However, the extensive automated-classification possibilities offered by future IR analysis (Mondol et al., 2019), and in the ease of sample preparation and data collection, may mean it will be easier to expand these technologies compared with the more time-consuming SEM methods in the long term.

The ability to differentiate at higher taxonomic resolution would enhance our understanding of past trajectories of co-occurring *Quercus* sections, in particular for understanding the expansion of *Quercus* since the Last Glacial Maximum (e.g. Brewer, Cheddadi, de Beaulieu, & Reille, 2002). FTIR techniques may also be useful for older interglacial sequences, where identification of *Quercus* pollen to section is often not possible due to degradation (Tzedakis, 1994). This would complement studies that use genetic methods on modern samples to reconstruct colonization pathways, which have higher taxonomic resolution and compliment the palynological data, but lack the temporal resolution that pollen records provide (Petit et al., 2002).

In addition, a number of studies have highlighted the need to incorporate long-term ecological information to improve biodiversity forecasts of environmental change (Dawson, Jackson, House, Prentice, & Mace, 2011). Rates of temperature increases in the Mediterranean are projected to outpace the rest of the temperate

regions in Europe and are predicted to rapidly change the associated biomes in the region (Giorgi & Lionello, 2008; Guiot & Cramer, 2016; Guiot & Kaniewski, 2015), but the consequences of this change for Mediterranean oak forests remain uncertain (Acácio et al., 2017; Lindner et al., 2014). A number of studies have integrated pollen data in order to reduce uncertainties when forecasting the biotic responses of *Quercus* to climate change in the future (Nogués-Bravo et al., 2016; Guiot & Cramer, 2016). However, like the palaeoecological studies discussed above, the limited taxonomic resolution used may bias projections. For example, species distribution models based solely on *Quercus* pollen were only able to estimate niche-environment relationships at the genus level. Extensive application of FTIR techniques may therefore provide a bridge between long-term ecological and modern biogeographical approaches.

Nevertheless, despite the potential shown in our FTIR approach, our findings reveal a number of challenges before vibrational methods can be rolled out across biogeographical and palaeoecological applications. First, our results are still unable to resolve at the species level, and so although taxonomic resolution would be refined using FTIR approaches, in many cases palaeoecological studies would still lack the taxonomic precision of other biogeographical tools (e.g. phylogenetic analysis). Second, our results are based on fresh pollen sampled from modern taxa, and lipids were some of the main functional groups used to differentiate between taxa in this study, in addition to by sporopollenins (Figure 4). Although the preservation and stability of sporopollenins in fossil sequences are well established (Fraser et al., 2012), the extent to which lipids are preserved in chemical sequences in subfossil pollen sequences remains uncertain. Variations in sporopollenin functional groups were still responsible for differentiation between the three main *Quercus* sections, but the ability for these functional compounds to be used as taxonomic tools in isolation is yet to be established. In the future it may be more beneficial to focus on variations of sporopollenins in pollen, perhaps through the use of Raman spectroscopy, which preferentially targets the vibration of non-polar bonds in sporopollenins and so may be able to achieve finer-scale differentiation of sporopollenin building blocks (Merlin, 2009).

Third, in this study we used bulk pollen samples to infer differences using FTIR, but fossil-pollen samples would require single-grain measurements since pollen grains are difficult to separate from other organic material within the sediment matrix. Single-grain FTIR spectra are less reproducible than bulk, mostly due to spectral anomalies caused by scattering and by non-radial symmetry of certain pollen types (Zimmermann, 2018; Zimmermann, Bağcıoğlu, et al., 2015). Although these issues have been addressed by adjusting experimental settings and by implementing numerical correction methods (Zimmermann, 2018; Zimmermann et al., 2016), future work is needed to test whether the patterns we observe at the bulk level can be replicated using single-grain FTIR measurements.

Finally, our study shows the importance of using large numbers of replicates in the pollen samples to account for the large amounts of chemical variation present in the chemical spectra, even within replicate species. The large numbers of samples and high levels of replication here (i.e. 50 ± 23 tree replicates per species) are a major advantage over previous studies, which have featured either fewer replicates (<5) (Jardine et al., 2019; Julier et al., 2016; Woutersen et al., 2018) or fewer/no congeneric species (Julier et al., 2016; Zimmermann et al., 2017). Although we do find clear signals in the data linked to systematics (Figure 3 and Table 3), we also find that ~60% of the total variation remains unexplained. One probable reason for the unexplained variation observed in our study may be linked to the environmental controls on pollen chemistry. Previous studies have suggested plasticity of pollen chemistry to climate and other environmental variables (Bağcıoğlu et al., 2017; Depciuch et al., 2016; Depciuch, Kasprzyk, Sadik, & Parlińska-Wojtan, 2017; Zimmermann et al., 2017; Zimmermann & Kohler, 2014). The other probable reason is the intra-species variation between the genotypes of different populations as well as within populations (Zimmermann et al., 2017). This suggests it will be critical to understand the other factors which can account for this variation if these pollen-chemistry techniques can be successfully applied to fossil sequences.

5 | CONCLUSIONS

We investigated the chemical variation in pollen sampled from 294 individuals of *Quercus* using FTIR to investigate whether this technique could enable taxonomic discrimination of modern *Quercus* pollen. Our results achieved excellent (~97%) recall to section level, showing that subgenus level differentiation of pollen samples is possible using IR methods. However, despite these promising results at the section level, more detailed, species-level differentiation was complicated by overlapping variation in the chemical composition of closely related species.

We also aimed to identify which specific functional groups are responsible for the taxonomic discrimination in the data. Here, we found lipids and sporopollenins to be key determinants between different *Quercus* sections. Although the sporopollenin functional groups are identified as important for discrimination between *Quercus* taxa, isolating the effect of these sporopollenin groups from the effects of other functional groups which may not be preserved in sediment sequences (e.g. lipids) still present a challenge. In addition, testing the application on single-grain *Quercus* samples, and developing a more complete understanding of the effects of environmental variation on pollen-chemical signatures in *Quercus* is required. Taken together, our findings build on previous studies and show that, while FTIR approaches on modern *Quercus* pollen can perform at a similar level to SEM techniques, future work on the discrimination of sporopollenin components is required before FTIR can become a more widespread tool in long-term ecology and biogeography. Thus, our study represents a valuable step forward in improving our understanding of variation in pollen chemical composition and its application in long-term ecology and biogeography.

ACKNOWLEDGEMENTS

We dedicate this paper to the memory of John Flenley (1936-2018) who pioneered so many exciting and novel aspects of pollen analysis and vegetation history. One of us (HJBB) knew John since 1963 and was always stimulated by John's latest ideas and palaeoecological

studies. John would have been fascinated by the potential of pollen chemistry in palaeoecology and biogeography. This work is part of the PollChem project funded with a FRIPRO Grant from the Research Council of Norway (PollChem 249844); the extensive field work was possible through funding from the L. Meltzer Høyskolefond (2017/05/LMH) and the Olaf Grolle Olsen Legat (2017/52/FOL). HJBB is supported by EU H2020 grant 741413 HOPE Humans on Planet Earth.

DATA AVAILABILITY STATEMENT

Data and code for analysis and reproduction of the figures are available as supplementary material on a dryad repository (Muthreich et al. 2020). URL: https://doi.org/10.5061/dryad.73n5tb2sx

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BIOSKETCH

Florian Muthreich is a palaeoecologist interested in developing new methods of pollen classification. This work represents a component of his PhD work at the University of Bergen University within the PollChem project (https://www.uib.no/en/rg/EECRG/98775/pollchem). In this project he and other authors collaborate to explore pollen-chemistry applications in biogeography and long-term ecology.

Author contributions: FM, AWRS, BZ and HJBB conceived the idea. FM and CMVV conducted the fieldwork and collected the data. FM, BZ and AWRS analysed the data. FM and AWRS led the writing with assistance from BZ, HJBB and CMVV.

SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

How to cite this article: Muthreich F, Zimmermann B, Birks HJB, Vila-Viçosa CM, Seddon AWR. Chemical variations in *Quercus* pollen as a tool for taxonomic identification: Implications for long-term ecological and biogeographical research. *J Biogeogr.* 2020;00:1–12. https://doi.org/10.1111/jbi.13817

A Appendix A

A.1 Exploring neural networks

I began my PhD in September 2016, which meant that trees were not in flower for at least 6 months in Europe. During this time, I researched and learnt about neural networks in addition to working with the classifynder system in our pollen laboratory.

A.2 Background

Computer vision deals with identifying objects on images and their position, to make this information accessible for computers to process images and video for a range of applications: e.g. hand-written text recognition, self-driving cars, etc. These models are very powerful at identifying objects and areas of interest in images, which is similar to pollen counting, where objects of interest are randomly located among other objects that have to be ignored. All of these approaches are based on artificial neural networks (ANN), which were a digital recreation of neural pathways, connections, albeit simplified (McCulloch and Pitts 1943, Rosenblatt 1959). In ANNs neurons are organized in layers and receive inputs, which are transformed using an activation function and the result is sent to the next layer of neurons or output. Each neuron receives every input and sends the output to each neuron in the next layer, where each connection is weighted and the weights are learned during training. The advantage of this model is that it can model non-linear responses.

A big breakthrough for the application of ANNs to computer-vision was the introduction of convolutional layers which are kernel filters that reduce the size of the image and transform it. Kernel filters are transformation windows (3x3 pixels), which are applied to each pixel of the input image and perform a transformation. Convolutional layers were first implemented by LeCun et al. (1989) for recognition of handwritten digits (e.g. zip-codes) and improved the performance of neural networks drastically. NN that utilize convolutions would be called convolutional neural networks from now on (CNN).

It would take until the 2010s for the next evolutionary step in NN architechture. The

advent of increasing computation performance and dedicated graphic cards that specialize on matrix multiplications used during training of NN provided the infrastructure for deeper architectures, increasing classification performance significantly. One of the breakthrough models is Alexnet (Krizhevsky et al. 2017), which utilized more convolutional layers (8) and made improvements to activation function and pooling layers that increased classification performance. In the wake of Alexnet, a number of new variants and improvements were made: Resnet introduced skipping layers of the network to improve generalization (He et al. 2015), while Inception introduced the convolution module (Szegedy et al. 2015b), which performed several size convolutions at the same time instead of in dedicated layers. These are the more prominent examples of CNN models, which inspired me to use them for classification of pollen images.

Several people in our group had used Classifynder to capture pollen images using the automated microscope, which is quite reliable at taking images of pollen from ordinary microscope slides and calculating a number of morphological parameters of the pollen. The built-in neural network (NN) classifier of the classifynder is a single hidden layer NN, which was a simpler architecture than the state of the art at the time of my PhD (Szegedy et al. 2015a, Ren et al. 2015). These deeper neural networks need large amounts of training and testing data, which would be time consuming to collect by hand. My goal was to use the Classifynders excellent image capture capabilities to take images of pollen from a set of *Eucalyptus* species relevant for the Bega Swamp record. Further, I trained a number of models to classify this dataset:

- i. the built-in classifynder classifier,
- ii. random forest,
- iii. LeNet,
- iv. Resnet,
- v. Inception. The first two models would use the 50 morphological features extracted by Classifynder, while the CNNs would use the images.

A.3 Methods and Materials

The pollen was collected from the herbarium at the KEW Botanic Gardens. *Eucalyptus* flowers/anthers were removed from the herbarium sheets and acetolysed, using the standard protocol (Erdtman and Praglowski 1959, Fægri and Iversen 1989), to extract the pollen grains. I successfully extracted pollen from three species and one sub-species: *E. fraxinus*, *E. pauciflora*, *E. pauciflora* ssp. *niphophila* and *E. stellulata*. The pollen was stained, embedded in glycerol and mounted on microscope slides. Using Classifynder, approximately 400-600 images of pollen were captured for each species/sub-species. The set of images was split into training, validation and test set at 70%/15%/15% ratio of the full data-set.

I used the Pollen software, that controls Classifynder, to extract the morphological parameters from the images and used a random forest model (4000 trees) to classify the pollen based on the ~50 morphological parameters.

For training with the CNNs, I converted the images to black and white and reduced the resolution to 28x28 pixel to save training time. For LeNet the model was trained from scratch, while I used models for Resnet and Inception that were pre-trained and implemented in the MXnet package in R. The architectures for Resnet and Inception were reduced in depth to allow for the training of smaller images.

A.4 Results and Discussion

Overall, the CNNs performed better than methods based on morphological parameters, achieving betwen ~70% recall for LeNet and 75% recall for Inception bn. The ANN of Classifynder performed worst at 62% recall. CNNs were also capable of differentiating between *E. pauciflora* and its sub-species at the same confidence level as the other species. These results are quite promising, considering the reduced resolution of the images used (black and white 28x28 pixel) compared to the original images (500x500). The advanced CNNs I used (Inception and Resnet) were reduced versions, where some of the layers were removed to allow for images of smaller sizes to be usable. The full networks use larger images (e.g. 299x299 for Resnet) and may increase the performance of

the models. This test with a mini *Eucalyptus* training set showed that there is potential for the use of CNNs in pollen identification and I planned to use the full images and additional *Eucalyptus* species to train a full-scale model at a later time in my PhD. The successes of CNNs in other field of research demonstrated that this was a very promising approach for pollen identification and later results by Sevillano et al. (2020) showed precise classifications of pollen from 46 species. They used a similar approach that I had planned. The training images were captured using a Autostage Classifynder system and a pretrained version of Alexnet (Krizhevsky et al. 2017) was used, which is one of the deepest CNN architectures.

Pollen Indentification Using Neural Networks

Performance of different neural network architectures in Identifying Pollen based on images. Feature extraction using Classifynder and new approaches using convolutional neural Networks are tested.

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ABSTRACT

Traditional morphological methods for pollen identification in Quaternary palaeoecology are time consuming and can be limited in taxonomic precision. The automated Classifynder system (CFS; Holt et al. 2011) has the potential to use detailed morphological measurements and machine learning techniques to distinguish pollen types at higher levels of taxonomic resolution. In addition convolutional neural networks (CNNs) are powerful tools in classifying images. These approaches open new and exciting prospects for the classification of pollen.

Classifynder System.
Image Source:
http://www.massey.ac.nz/massey/fms/Massey%20News/2016/G/images/classifynder-scope.jpg

Methods & Materials

The automated CFS was used to gather 2700 images and morphological information of 4 Eucalyptus pollen species collected from the Herbarium Collection at Kew, London. I have tested several classification methods on the dataset, by comparing the performance of the CFS to random forest and different CNNs. LeNet (Lecun et al. 1998) was developed for written character recognition, while Resnet (He et al. 2016) and Inception bn (Ioffe et al. 2015) were developed for image classification.

During classification the dataset was split into a 70% training and a 15% validation and test set. The images were scaled to 28x28 pixels.

Results

Overall the CNNs performed better than the classifications based on the Classifynder extracted morphology data. Accuracy was higher when comparing species as well as total accuracy.

Classification Results

Classifier	E. frax.	E. pauc.	E. p. niph.	E. stell.	Acc.
Classifynder	65%	46%	73%	64%	62%
R. Forest	63%	50%	69%	70%	63%
h2o	75%	51%	70%	59%	64%
LeNet	69%	70%	69%	72%	70%
Resnet	66%	70%	64%	77%	69%
Inception bn	76%	73%	71%	80%	75%

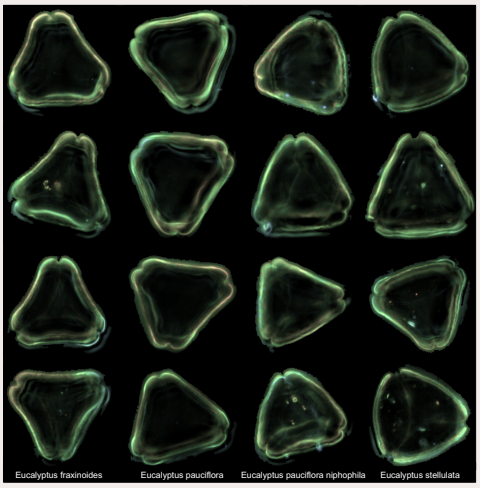
Confusion matrices

Classifynder	E. frax.	E. pauc.	E. p. niph.	E. stell.	Recall
E. frax.	60	10	12	11	65%
E. pauc.	7	41	27	14	46%
E. p. niph.	6	10	90	18	73%
E. stell.	6	13	16	64	64%

Inception bn	E. frax.	E. pauc.	E. p. niph.	E. stell.	Recall
E. frax.	71	10	7	5	76%
E. pauc.	7	65	7	10	73%
E. p. niph.	13	17	88	6	71%
E. stell.	9	9	2	79	80%

Future

CNNs offer a promising tool in identifying pollen independent of predetermined morphological parameters, through their unique feature extraction and flexibilty.



Feature extraction and Classification

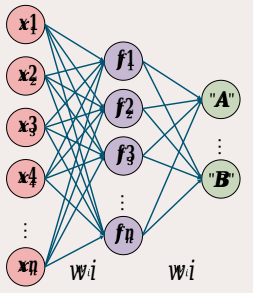
Classifynder

The Classifynder extracted 50 features from the scanned images. Spanning simple geometric attributes, such as area, size, circumference, etc. and additional features aimed at capturing pollen surface structure and texture. These are fed into a Neural Network. During the training the data is split into two and trained seperately.

Convolutional Neural Networks

In CNNs the feature extraction is based on low level image manipulations (Kernel Convolutions) directly on the source images. The source images are turned into abstracted lower resolution versions through several convolution and pooling rounds. Every pixel from these features is treated as an input into a Neural Network.

Neural Network Structure

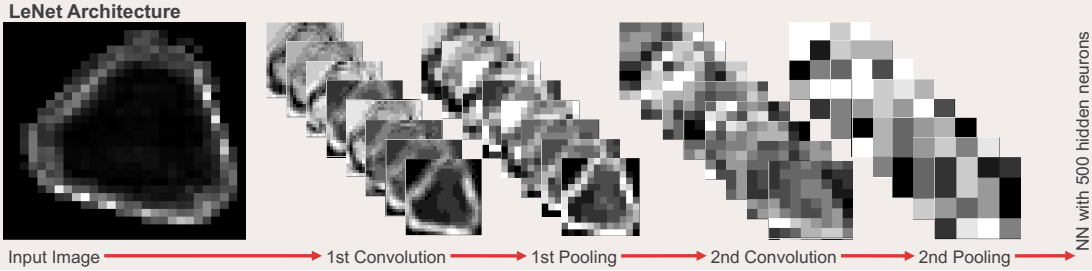


How Neural Networks work

The neurons in the hidden layers are transforming inputs using activation functions (i.e sigmoid).

During training the connection weights (w_i) are adjusted to achieve the wanted result in the output layer.

LeNet Architecture



ACKNOWLEDGEMENTS

The work covered on this poster was possible through funding from Norwegian Research Council and help from Sally Dawson (Kew), Arild Breistøl and Alistair Seddon.

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Graphic design: Communication Division, UiB / Print: Skipnes Kommunikasjon AS

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ISBN: 9788230865071 (print)
9788230854532 (PDF)