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ON THE TAXONOMIC RESOLUTION OF POLLEN AND SPORE RECORDS OF EARTH'S VEGETATION

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Editor: Patrick S. Herendeen

Premise of research.   Pollen and spores (sporomorphs) are a valuable record of plant life and have provided information on subjects ranging from the nature and timing of evolutionary events to the relationship between vegetation and climate. However, sporomorphs can be morphologically similar at the species, genus, or family level. Studies of extinct plant groups in pre-Quaternary time often include dispersed sporomorph taxa whose parent plant is known only to the class level. Consequently, sporomorph records of vegetation suffer from limited taxonomic resolution and typically record information about plant life at a taxonomic rank above species.

Methodology.   In this article, we review the causes of low taxonomic resolution, highlight examples where this has hampered the study of vegetation, and discuss the strategies researchers have developed to overcome the low taxonomic resolution of the sporomorph record. Based on this review, we offer our views on how greater taxonomic precision might be attained in future work.

Pivotal results.   Low taxonomic resolution results from a combination of several factors, including inadequate reference collections, the absence of sporomorphs in situ in fossilized reproductive structures, and damage following fossilization. A primary cause is the difficulty of accurately describing the very small morphological differences between species using descriptive terminology, which results in palynologists classifying sporomorphs conservatively at the genus or family level to ensure that classifications are reproducible between samples and between researchers.

Conclusions.   In our view, the most promising approach to the problem of low taxonomic resolution is a combination of high-resolution imaging and computational image analysis. In particular, we encourage palynologists to explore the utility of microscopy techniques that aim to recover morphological information from below the diffraction limit of light and to employ computational image analyses to consistently quantify small morphological differences between species.

Keywords: palynology, paleoecology, taxonomy, vegetation history, plant evolution.

What Is Taxonomic Resolution?

Palynological research into the history of Earth's vegetation is underpinned by the classification of fossil sporomorphs (pollen and spores) into taxonomic groups (Birks and Birks 1980). This allows researchers to investigate patterns of vegetation diversity and composition on a variety of spatial and temporal scales. The results of these investigations have provided valuable information on subjects ranging from the nature and timing of major evolutionary events, such as the origin and radiation of flowering plants (Heimhofer et al. 2007), to the relationship between vegetation and climate (Jackson and Williams 2004). However, since the earliest attempts to use sporomorphs to reconstruct vegetation history, workers have expressed concern about the taxonomic rank at which spo-

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romorphs are classified. In his classic lecture, von Post remarked, "Although the pollen types enumerated can be identified generically, the species cannot be distinguished. Thus *Betula* species, *Alnus* species, *Salices* etc. can unfortunately only be treated collectively" (von Post [1916] 1967, p. 382). The potential implications of failing to classify sporomorphs at the species level were recognized by Cain, who commented "Nearly all reports of pollen grains in peats and other sediments are based on generic determinations only, despite the generally accepted fact that a knowledge of the species involved would be advantageous in making climatic interpretations and reconstructing vegetational history" (Cain 1940, p. 301).

These comments neatly express the concept of taxonomic resolution in palynology, which is shown schematically in figure 1. In this example, the pollen of six plant species (fig. 1A) has been classified on the basis of morphological criteria. The classifications lie on a continuum from low to high taxonomic resolution (fig. 1B). The classification with the lowest taxonomic resolution in this example is based only on the coarsest
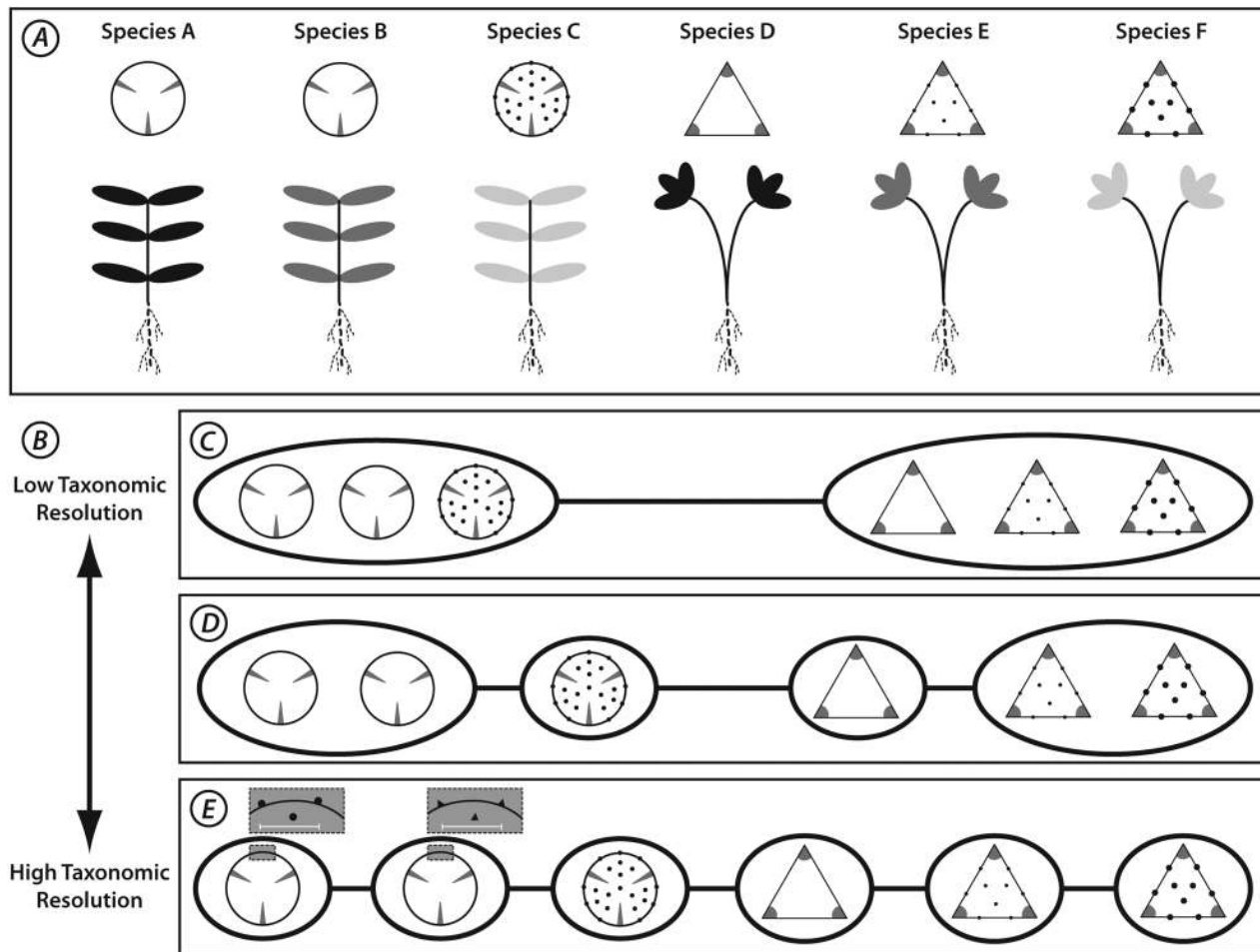
Fig. 1 Schematic diagram to show low and high taxonomic resolution of sporomorph (pollen and spore) classification. *A*, Species A (single main stem with black opposite leaves) produces tricolpate pollen that is circular in polar view and lacks surface ornamentation. Species B (single main stem with dark gray opposite leaves) produces tricolpate pollen that is circular in polar view and has sparse granulate surface ornamentation. Species C (single main stem with pale gray opposite leaves) produces tricolpate pollen that is circular in polar view and has dense granulate surface ornamentation. Species D (two dichotomously branching main stems with black terminal leaves) produces angulaperturate pollen that is triangular in polar view and lacks surface ornamentation. Species E (two dichotomously branching main stems with dark gray terminal leaves) produces angulaperturate pollen that is triangular in polar view and has granulate surface ornamentation. Species F (two dichotomously branching main stems with pale gray terminal leaves) produces angulaperturate pollen that is triangular in polar view and has gemmate surface ornamentation. Descriptive terminology for pollen grains from Punt et al. (2007). *B*, Continuum of taxonomic resolution shown schematically. *C–E*, Classification of the pollen grains of these six species shown at low (*C*), intermediate (*D*), and high (*E*) taxonomic resolution. Scale bars in insets in *E* represent 100 nm. Thick solid lines encircling pollen grains represent morphologically delimited taxa. The pollen of species A and B is separated by the shape of sculptural elements that are less than 100 nm in size (*E*). The pollen of species E and F is separated on the basis of sculptural element size (*E*).

morphological features, aperture arrangement and polar outline, and results in the identification of two taxa, each containing three species (fig. 1*C*). The classification with intermediate taxonomic resolution incorporates an additional character, the presence or absence of surface ornamentation, and results in the identification of four taxa; two containing one species and two containing two species (fig. 1*D*). The classification with the highest taxonomic resolution in this example includes subtle morphological features such as the shape and size of sculptural elements on the pollen surface to distinguish each species (fig. 1*E*). The surface ornamentation of species A and B to the left of the schematic is characterized

by individual sculptural elements that are less than 100 nm in size (fig. 1*E*).

Studies of the morphology of sporomorphs contained within flowers and reproductive structures of extant plants establish the relationship between dispersed sporomorphs and their parent plants. This allows sporomorphs found in modern sediments to be classified in terms of extant plants. For example, an oak pollen grain found in the surface sediments of a modern lake can be classified as a specimen of extant *Quercus*. Fossil sporomorphs of Holocene age (~12,000 yr ago to the present) are also generally classified in terms of extant plants. In the schematic classification shown in figure 1, the classifications

could correspond to the rank of family (fig. 1*C*), genus (fig. 1*D*), and species (fig. 1*E*). Farther backward in time, the phylogenetic position of dispersed sporomorphs is often difficult to establish, and it becomes increasingly unclear whether fossil sporomorphs represent extant plant species, genera, or families. This is partly because certain sporomorphs, such as the pollen grains of seed plants, offer few characters that can be used to place them in a phylogenetic tree and because the flowers, fruits, and seeds of their parent plants, which contain more phylogenetically informative characters, are relatively rare in the fossil record (Pirie and Doyle 2012). This problem is highlighted by an example from Pirie and Doyle (2012), which explores the uncertain phylogenetic position of a fossil that possesses apomorphies *A* and *B*, but in which the organs bearing on apomorphies *D* and *E* are not preserved (fig. 2). The fossil could represent either a stem relative in which apomorphies *D* and *E* had not evolved (as shown in fig. 2), a later stem relative in which *D* and *E* had evolved, or a crown group member lacking apomorphy *C* (fig. 2). Despite uncertainties of this nature, it is thought that many Paleogene–Neogene (~65–2.6 Ma) sporomorphs can be classified in terms of extant genera or families (Chaloner 1968), but this is not the case for the sporomorphs produced by extinct lycophytes and seed-ferns of the Carboniferous, for example (~359–259 Ma). In situations such as this, where sporomorphs cannot be classified in terms of extant species, genera, or families, the relationship between dispersed sporomorphs and their parent plants can be established by studies of sporomorphs found in situ in fossilized reproductive structures (e.g., Balme 1995).

In this review, we define taxonomic resolution as the ability to classify a sporomorph at the taxonomic rank of species. We make the assumption that the vast majority of fossil sporomorphs found in sediments of Quaternary age (~2.59 Ma to the present day) were produced by plant species that are extant, although there is evidence for plant species extinction (Jackson and Weng 1999; Willis and Niklas 2004) and morphological evolution (Birks and Birks 1980) during this period. Accordingly, we acknowledge "Quaternary time," in which our definition implies that a fossil sporomorph has been classified as an extant species (e.g., Punyasena et al. 2012). We also assume that many fossil sporomorphs found in sediments older than the Quaternary were produced by extinct plant species. Consequently, we acknowledge "pre-Quaternary time," in which our definition of taxonomic resolution implies the recognition of characters that are sufficient to classify a fossil sporomorph as an extinct species.

Low taxonomic resolution, the classification of sporomorphs above the rank of species, remains a key obstacle in both Quaternary and pre-Quaternary palynology (Birks and Birks 2000; Jackson and Booth 2007; Mander 2011; Punyasena et al. 2011, 2012; May and Lacourse 2012; Mander et al. 2013). In this review, we aim to examine the factors that can reduce taxonomic resolution and to appraise existing ways in which researchers have approached the problem of low taxonomic resolution. We also offer our views on how greater taxonomic precision may be attained in the future.

Factors That Reduce Taxonomic Resolution

There are several primary factors that, either singly or in combination, prevent the classification of sporomorphs to the
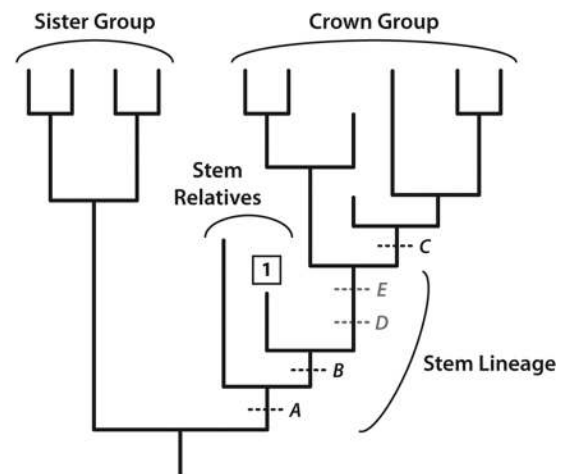

Fig. 2 Cladogram to highlight the difficulty of placing a fossil in a phylogeny. The fossil is labeled 1. Apomorphies are labeled A–E. In this example, apomorphies D and E, highlighted in gray, relate to plant organs that were not preserved in the fossil record. Cladogram topology and example based on Pirie and Doyle (2012).

species level and thus reduce the taxonomic resolution of sporomorph records of ancient vegetation. The most conspicuous of these is the morphological similarity of sporomorphs among many species within a single genus of plants and among many genera within certain families (Jackson and Booth 2007). In Quaternary time, the pollen of *Pinus* (pine) and *Quercus* (oak) are examples of morphological similarity among species within a genus (fig. 3*A*), and the pollen of Poaceae (grasses) is an example of morphological similarity among genera within a single family. Quaternary pollen diagrams typically report fluctuations in the relative abundance of these three taxa through time rather than fluctuations in the relative abundance of their constituent species. Morphological similarity between taxa either can result from a lack of morphological information on which to base a classification, such as Poaceae (Wodehouse 1935; Beug 2004; Mander et al. 2013), or can occur when the natural range of morphological variation within two or more species overlaps considerably, such as the pollen of *Palaua* (Malvaceae), a genus of plants endemic to the coastal deserts of Chile and Peru (Schneider et al. 2009).

There are numerous similar cases of morphological similarity among species of sporomorphs in pre-Quaternary time. In these cases, a single sporomorph morphotaxon has been found in situ in more than one taxon of fossilized reproductive structure and therefore has botanical affinities to more than one parent plant (e.g., Mander 2011). Examples of this include *Punctatisporites*, which has affinities to the Sphenophyllales and the fern families Marratiaceae and Osmundaceae as well as to certain progymnosperms and other Mesozoic gymnosperms of uncertain systematic position (Balme 1995; Traverse 2007; Mander 2011). Other examples of sporomorphs with affinities to several higher plant taxa include the smooth trilete spore *Deltoidospora*, Mesozoic bisaccate pollen of the *Alisporites* morphotype, and the smooth or scabrate "boat-shaped" monocolpate pollen grains of the *Cycadopites/Monosulcites* and *Chasmatosporites* morphotypes (Balme 1995;
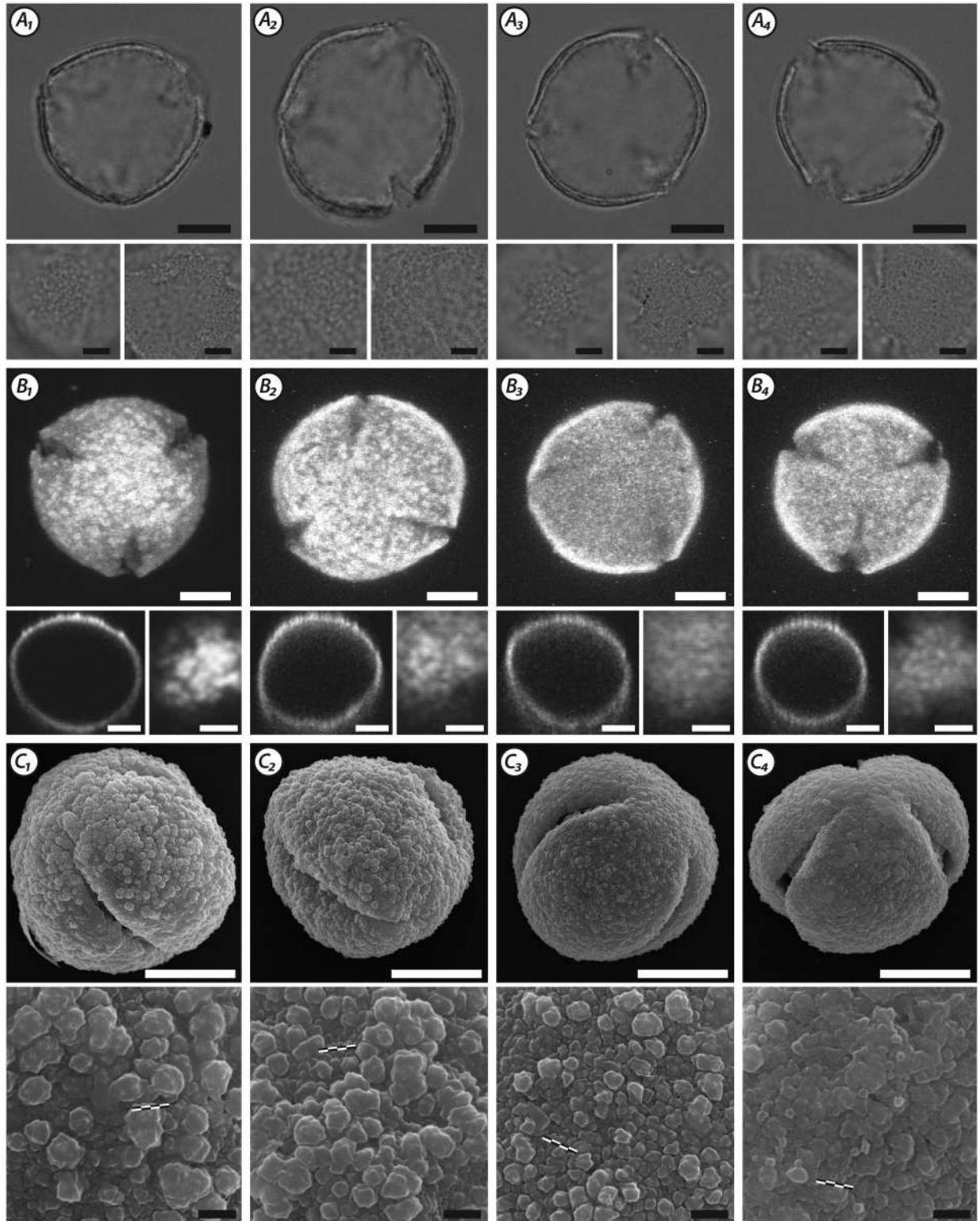
Fig. 3 Pollen of four *Quercus* (oak) species, sampled from herbarium sheets at the Royal Botanic Gardens, Kew. These pollen grains highlight morphological similarity among several species within a single genus. The species are *Quercus englemannii* (A_1–C_1), *Quercus lobata* (A_2–C_2), *Quercus macrocarpa* (A_3–C_3), and *Quercus virginiana* (A_4–C_4). Specimens were imaged with bright-field (transmitted-light) microscopy (*A*), confocal (reflected-light) microscopy (*B*), and SEM (*C*). *A*, Bright-field images show the outline of each specimen (scale bar = 10 μm), together with two smaller insets showing the surface ornamentation at two different focal planes (scale bars = 5 μm). *B*, Confocal images show a

Traverse 2007; Mander 2011). Each of these sporomorphs is a morphotaxon, and although, from a nomenclatural point of view, "it is perfectly legitimate for the taxon to include items that come from totally unrelated plants" (Traverse 2007, p. 247), the situation is clearly problematic when attempts are made to reconstruct vegetation history from sporomorph assemblages that contain these taxa.

In Quaternary palynology, the availability and coverage of pollen and spore reference collections based on vouched herbarium specimens is critical to the process of classifying fossil sporomorphs (Jarzen and Nichols 1996). This is because collections that cover the full range of the natural variation in a taxon's morphology allow the palynologist to compare the morphology of sporomorphs in a fossil assemblage with a comprehensive spectrum of extant taxa (Birks and Birks 1980). Monographic catalogs of reference collections also establish sporomorph types, which anchor the concept of which taxa are morphologically distinct and provide a sound basis for comparative work (Punt et al. 2003). The effects of inadequate reference collections can be particularly acute in highly diverse tropical settings that can contain many rare plant species or in remote areas that have not been subject to intensive botanical research activity. The record of sporomorphs found in situ in fossilized reproductive structures is the pre-Quaternary analog to reference collections based on herbarium specimens. Failure to locate a sporomorph in situ in a fossilized reproductive structure can mean that the parent plant of a fossil sporomorph in pre-Quaternary time is entirely unknown, which obscures the relationship between the source vegetation and assemblages of dispersed sporomorphs (Mander 2011). The in situ record is clearly more comprehensive in some parts of the stratigraphic column than in others. For example, as a result of the intensive study of petrified and compressed fossil plants in the Carboniferous Period (~359–299 Ma), the relationship between dispersed sporomorphs and their parent plants is understood more completely in this period than in the Cenozoic (~66 Ma to the present day; Traverse 2007). Such factors exert a major control on the taxonomic resolution of sporomorph records through time.

The process of fossilization can result in damage to the exine of a sporomorph (Mander et al. 2012). Mechanical damage, such as breakage of the exine that can result from exposure to wet and dry cycles (Campbell and Campbell 1994), and chemical damage, such as corrosion of the exine that can result from exposure to oxidizing conditions or microbial attack (Havinga 1967), can prevent the classification of sporomorphs by obscuring morphological details (Birks and Birks 1980). As an example, the pattern of microbial attack on *Convolutispora* follows the pattern of the rugulae on the surface of the spore (Moore 1963), which could lead to an erroneous classification.

Finally, because the morphological differences among the sporomorphs of closely related plant species are often very small relative to the morphological diversity of sporomorphs among all plant taxa, they are typically identified conservatively at the genus level or higher to ensure that classifications can be reproduced between samples and between researchers (Punyasena et al. 2012). This is frequently the case where morphological differences at the species level are very subtle and difficult to convey with the terminology of descriptive palynology (e.g., Punt et al. 2007).

Existing Solutions to the Problem of Low Taxonomic Resolution

Low taxonomic resolution prevents the accurate reconstruction of ancient vegetation using sporomorphs, and classifications at the genus or family level mask paleoecological, biogeographic, and evolutionary patterns at the species level. In this section of our review, we outline the strategies that researchers have developed to overcome the taxonomic limitations of the sporomorph record. In general, these have been designed to cope with specific factors that reduce taxonomic resolution, such as morphological similarity among many species of sporomorphs or the inconsistency of human analysts, and range from examining other fossil groups to exploring different microscopy techniques and analyzing the morphology of sporomorphs computationally.

The Use of Plant Macrofossils

The extinction of species is one of the most alarming consequences of climate change, and the fossil record is filled with past extinction events that provide context for the current biodiversity crisis (Pereira et al. 2010). However, low taxonomic resolution presents a major barrier to the investigation of the dynamics of plant extinction using sporomorphs (Jackson and Weng 1999; Mander et al. 2010; Mander 2011). For example, the Peltaspermaceae (a clade of seed ferns) suffered global extinction during the Triassic–Jurassic mass extinction, which coincided with major environmental change around 200 Ma and is recognized as the third-greatest extinction event in the history of life (McElwain and Punyasena 2007). The extinction of this plant family is marked by the disappearance of the fossil leaf morphospecies *Lepidopteris ottonis* at the end of the Triassic period (McElwain et al. 2007). However, this extinction event is masked in the sporomorph record because the pollen type produced by the Peltaspermaceae (*Cycadopites*) was also produced by at least three other orders of coexisting plants (Cycadales, Ginkgoales, and Bennettitales; Mander et al. 2010; Mander 2011). In addition, the local extinction or emigration of four cycad leaf genera during the Triassic–Jurassic mass extinction in East Greenland (*Doratophyllum*,

maximum-intensity projection of the entire specimen (scale bar = 10 μm), together with two smaller insets showing an orthogonal projection of the specimen (*left*; scale bar = 10 μm) and a single optical section to highlight surface ornamentation (*right*; scale bar = 5 μm). Bright-field and confocal images were taken with a ×60 1.4-NA (numerical aperture) oil immersion objective. C, SEM images show an entire specimen at ×2000 magnification (scale bar = 10 μm), together with an image of the surface ornamentation at ×6000 magnification (scale bar = 1 μm). The dashed black and white 1-μm scale bars in ×6000 images are divided into 250-nm intervals to highlight individual sculptural elements that are close to or smaller than the diffraction limit of light (~200–250 nm; Weiss 2000; Heintzmann and Ficz 2006). Each image shows a different specimen of pollen.
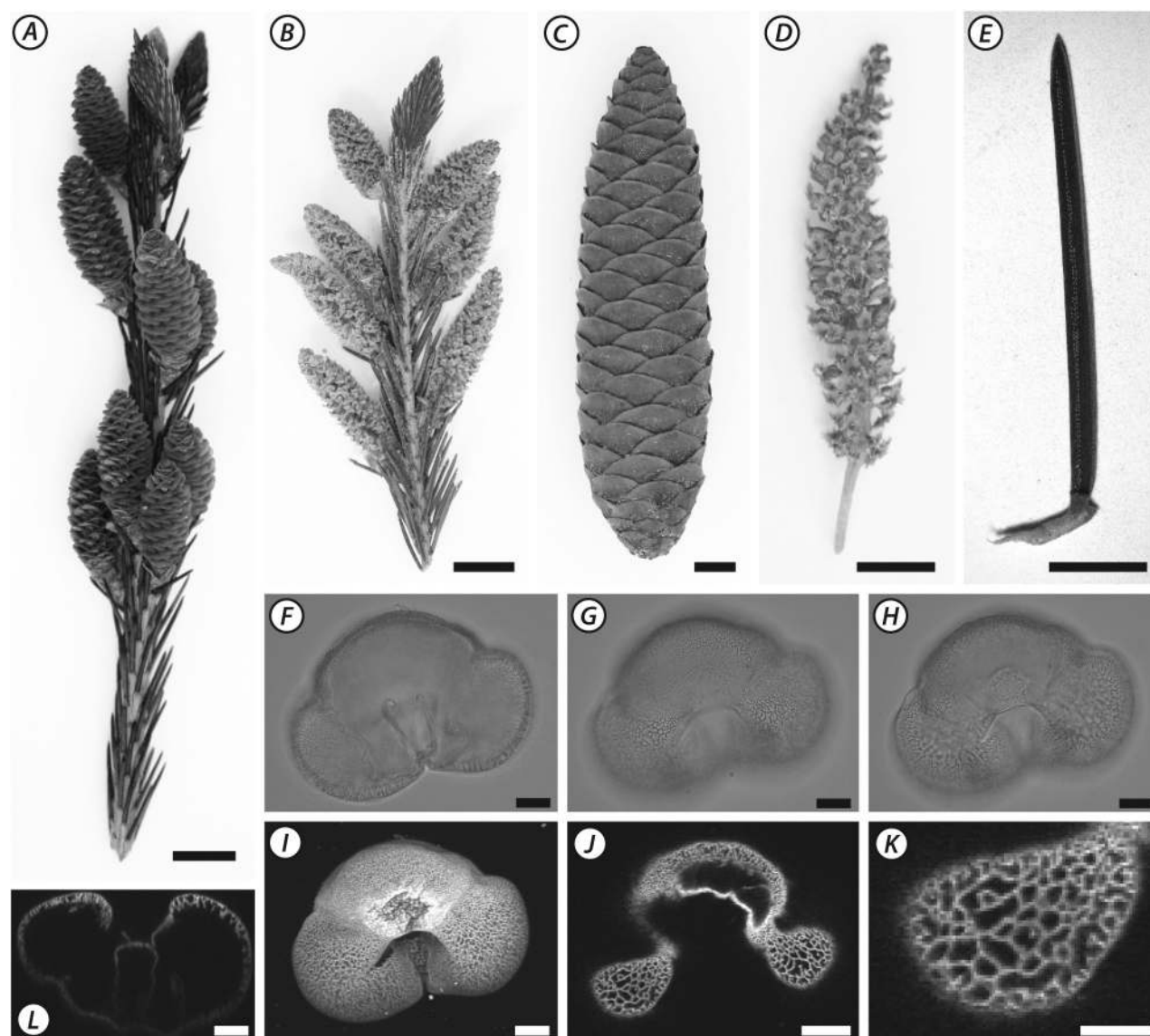
Fig. 4 Aspects of the anatomy of *Picea morrisonicola* (Taiwan spruce), showing potential macrofossils (*A–E*), and a specimen of pollen produced by this plant (*F–L*). *A*, Branch tip with immature ovulate cones. *B*, Branch tip with mature microsporangiate cones. *C*, Mature ovulate cone. *D*, Mature microsporangiate cone with clumps of undispersed pollen. *E*, Mature needle with wax plugs filling each stomatal pit. *F–H*, Bright-field (transmitted-light) images of a *P. morrisonicola* pollen grain, showing its outline (*F*) and the diversity of saccus and corpus ornamentation patterns (*G, H*). *I–L*, Confocal (reflected-light) images of the same pollen grain, showing a maximum-intensity projection of the entire specimen (*I*), saccus and corpus ornamentation (*J*), details of the saccus endoreticulations (*K*), and an orthogonal projection of the specimen to highlight the shape of the pollen grain (*L*). Bright-field and confocal images were taken with a × 60 1.4 NA (numerical aperture) oil immersion objective. Scale bars = 10 mm (*A, B*), 5 mm (*C, D*), 2.5 mm (*E*), 20 μm (*F–J, L*), or 10 μm (*K*).

Ctenis, *Pseudoctenis*, and *Nilssonia*; McElwain et al. 2007) is not recorded by fossil pollen grains in this region because the classification of the pollen grains produced by these plants is currently too coarse to document vegetation change at the genus level (Mander 2011). This example of plant species extinction in the Mesozoic is mirrored by the late Quaternary extinction of *Picea critchfieldii*, a species of spruce that was widespread in the southeastern United States during the last glacial maximum (Jackson and Weng 1999). Morphometric analyses of fossil ovulate cones and fossil foliage needles in-

dicate that *P. critchfieldii* disappeared during the last deglaciation (16,000–9,000 yr ago), but this extinction event is not expressed in pollen spectra from this region because of the difficulty of classifying spruce pollen to the species level (Jackson and Weng 1999).

These examples show that plant macrofossils, which include reproductive structures such as cones as well as vegetative structures such as twigs, needles, and leaves (fig. 4*A*–4*E*), generally record vegetation at higher taxonomic resolution than do sporomorphs (Birks and Birks 2000; Jackson and Booth

2007). This is because interspecific morphological variation of sporophyte plant organs is typically large and because most fossil plant species are described on the basis of sporophyte morphology (although sporomorph morphology is an important character in some groups, such as hornworts [Villareal et al. 2007]; Birks and Birks 2000; Jackson and Booth 2007). In addition, plant macrofossils can have a greater number of characters than sporomorphs, which increases the likelihood of finding differences between specimens. As a result, plant macrofossils have been used extensively in paleoecology to supplement vegetation reconstructions based on sporomorphs and typically fill the taxonomic gaps that are created by the low taxonomic resolution of the sporomorph record (Birks and Birks 2000; Jackson and Booth 2007). The leaves of *Quercus* are an example of a situation in which macrofossils can be used to overcome the difficulty of classifying sporomorphs that are morphologically similar among several species within a single genus (fig. 5), and fossil leaves are essential in order to discriminate between evergreen and deciduous oaks in the fossil record (Liu et al. 2007). As the Mesozoic and late Quaternary examples of plant species extinction show, the low taxonomic resolution of the sporomorph record can mask vegetation change at the species level, and in the absence of macrofossil control this can lead to the impression of compositional stability in time and space. This raises the possibility that many plant clades are more volatile than is currently thought on the basis of sporomorph records of their evolutionary history. For example, sporomorphs produced by tropical rainforest taxa are frequently classified only to the family or genus level (Haselhorst et al. 2013), and this low taxonomic resolution may conceal ancient extinctions or extirpations at the genus or species level, respectively. In this context, it is useful to note that the low taxonomic resolution of the sporomorph record prevents the introduction of Type I statistical errors into paleoecolgical analyses based on sporomorphs. This positive aspect of low taxonomic resolution means that when vegetation change is detected in the sporomorph record, such as compositional change or extinction, it is likely that the true magnitude of the change is underestimated.

The Use of Nonmorphological Characters

There are instances in palynology where even the broad biological affinity of a dispersed sporomorph is questionable because of a lack of diagnostic morphological characters. In such cases, nonmorphological characters can be used to understand the affinity of a dispersed sporomorph. A particularly successful approach has been to examine the chemical composition of the exine to generate chemotaxonomies of problematic groups. Cryptospores, for example, represent the earliest evidence for land plants (e.g., Steemans 2000). In situ occurrences of cryptospores in sporangia provide strong evidence that cryptospores were produced by embryophytes (land plants occupying terrestrial habitats) with possible affinities to liverworts (e.g., Wellman et al. 2003), but in the absence of in situ occurrences, the affinity of dispersed cryptospores is controversial. In the case of Late Silurian (~425 Ma) cryptospores from Sweden, Fourier transform infrared spectroscopy has been used to demonstrate that the wall of these fossils contains sporopollenin and is of similar composition to the
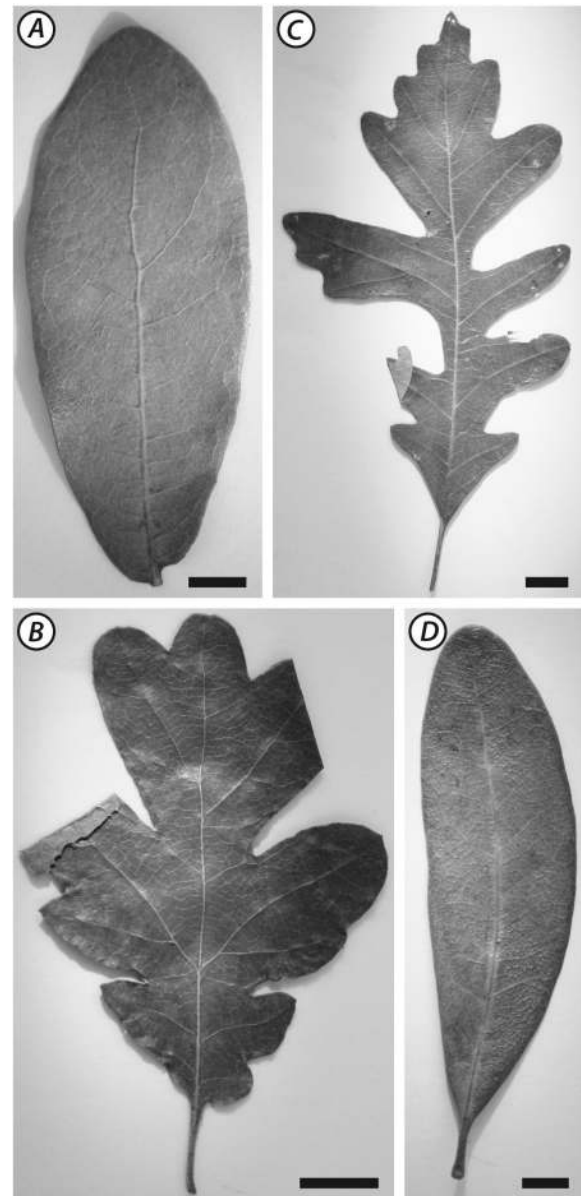

Fig. 5 Leaves of four *Quercus* (oak) species, sampled from herbarium sheets at the Royal Botanic Gardens, Kew: *Quercus englemannii* (*A*), *Quercus lobata* (*B*), *Quercus macrocarpa* (*C*), and *Quercus virginiana* (*D*). These specimens highlight that macrofossils can be useful in situations where species-level classifications using sporomorphs are difficult (fig. 3*A*). Scale bars = 5 mm (*A*, *D*) or 1 cm (*B*, *C*).

wall of fossilized trilete spores but is chemically distinct from the wall of chitinozoans and leiospheres (Steemans et al. 2010). Such spectroscopic techniques have also found application in the monitoring of airborne allergenic pollen (Dell'Anna et al. 2009; Zimmerman 2010). In a similar but geologically younger example, the carbon isotopic composition of fossil grass pollen has been used to show that grasses using C_4 photosynthesis were present on the Earth in the Early Oligocene (~33 Ma; Urban et al. 2010). Such chemotaxonomy is possible because

the tissues of plants that utilize the C_4 photosynthetic pathway, which has independently evolved at least 18 times in grasses (Edwards et al. 2010), have a heavier carbon isotopic composition than the tissues of plants that use the C_3 photosynthetic pathway (Amundson et al. 1997; Vogts et al. 2009). Techniques based on the fluorescence properties of the exine have also been explored, and the fluorescence spectra emitted from the exine following excitation can differ between certain taxa, which has been used to classify pollen grains (Mitsumoto et al. 2009; Pan et al. 2011).

Seeing More: Microscopy in Palynology

The morphological features that form the basis of sporomorph classifications are inspected with a microscope. These features are described in standardized terminology and include aspects of a sporomorph's exine organization, the number and arrangement of apertures, and the nature of any surface ornamentation (e.g., Punt et al. 2007). Palynologists have long recognized the central role of the microscope in the process of recovering detailed morphological information from individual specimens: "In investigating the pollen flora I have generally found that a magnification of about 200 × is most convenient. If one is studying only the coniferous pollen, 50–60 × is sufficient. For investigating finer details, sculpturing, perforations and the like a greater magnification should be available, for instance 400–500 × " (von Post [1916] 1967, p. 382).

This comment alludes to the idea that an increase in the resolution of a microscope (not to be confused with magnification) can increase the amount of morphological information that is recovered from individual specimens, which can in turn improve the classification of pollen and spores (Sivaguru et al. 2012). This idea is highlighted by the example of grass pollen, which is a classic case of low taxonomic resolution (Mander et al. 2013). Grass pollen is spheroidal and monoporate and when viewed with bright-field microscopy possesses a faint scabrate surface ornamentation (Wodehouse 1935; Fægri et al. 1992; Beug 2004). There is no departure from this gross morphological pattern across Poaceae, which provides palynologists with very few morphological characters on which to base classifications of grass pollen (e.g., Strömberg 2011).

However, the resolution of conventional optical microscopes is limited by the diffraction of light to around 200–250 nm in most practical situations (Weiss 2000; Heintzmann and Ficz 2006), and this represents a barrier to the amount of morphological information that can be recovered from individual specimens. This "diffraction limit" prevents conventional optical microscopes from imaging morphological features of sporomorphs that are less than 200–250 nm in size, and this is critical in the case of grass pollen. This is demonstrated by SEM images of grass pollen, which reveal that pollen morphology across Poaceae is characterized by a diverse suite of surface ornamentation patterns (Andersen and Bertelsen 1972; Page 1978; Peltre et al. 1987; Chaturvedi et al. 1998; Mander et al. 2013; fig. 6). Oak pollen is a similar example, and while neither bright-field nor confocal microscopy reveals morphological differences in the surface ornamentation of four species (fig. 3A, 3B), SEM recovers subtle nanoscale features that

could be used for the purpose of species-level classification (fig. 3C).

Schematically, grass and oak pollen could be thought of as species A and B in figure 1E, which are morphologically indistinct except for differences in the shape of sculptural elements that are less than 100 nm in size. The pollen of these two species would appear morphologically indistinct when viewed with bright-field microscopy (fig. 1D) but would look different when viewed with the SEM (fig. 1E). The SEM has been used extensively in palynology because of its ability to image structures that are just a few nanometers in size, and although specimen preparation and image acquisition time have been barriers to the use of the SEM in routine palynological studies (e.g., Fægri et al. 1992), recent work has shown that SEM images of surface ornamentation can be used by human analysts to classify species of grass pollen (Mander et al. 2013, 2014).

Von Post's comment also foreshadows the idea that different microscopy techniques are suited to the analysis of different morphological features of sporomorphs (Sivaguru et al. 2012). Continuing the example of grass pollen, phase-contrast microscopy has been employed to acquire information on the organization of the exine (Fægri et al. 1992; Beug 2004), and this has been used to generate broad groupings of grass pollen morphotypes (Beug 2004) and to distinguish *Tripsacum pilosum*, which has clumped tectal columellae, and *Zea mays* ssp. *parviglumis* (Balsas teosinte), which has uniformly distributed tectal columellae (Holst et al. 2007). Such features of the grass pollen exine cannot be viewed with an SEM because this instrument provides information only from the surface of a specimen (Sivaguru et al. 2012). TEM also provides data on exine organization, and this technique has been useful in pre-Quaternary time for clarifying the systematic position of sporomorphs that have not been found in situ in a fossilized reproductive structure (e.g., Doyle et al. 1990; Taylor 1995; Mander et al. 2012). Once the systematic position of a problematic sporomorph has been clarified by use of TEM, the composition of the source vegetation can be inferred from assemblages of dispersed sporomorphs in greater detail.

Other optical microscopy techniques provide similarly complementary data on the morphology of sporomorphs. Differential-interference-contrast microscopy provides enhanced contrast of morphological features that have high spatial frequency (Inoué and Spring 1997) and has been adopted by palynologists working with pollen and spores in tropical settings that are characterized by many species separated by subtle morphological differences (e.g., Pardo-Trujillo et al. 2003). Reflected-light (fluorescence) techniques, such as confocal and apotome microscopy, have been used to generate taxonomically significant data on the three-dimensional shape of entire pollen grains (Ronneberger et al. 2002a, 2002b, 2007; Punyasena et al. 2012; figs. 3B, 4L) and structures within pollen grains (Hochuli and Feist-Burkhardt 2013), which can be difficult to acquire with transmitted-light techniques because of their relatively poor optical-sectioning capabilities (Sivaguru et al. 2012).

These examples highlight that palynologists have continually explored advances in microscopy for the purpose of acquiring more morphological information from sporomorphs and generating more accurate classifications. Despite this,
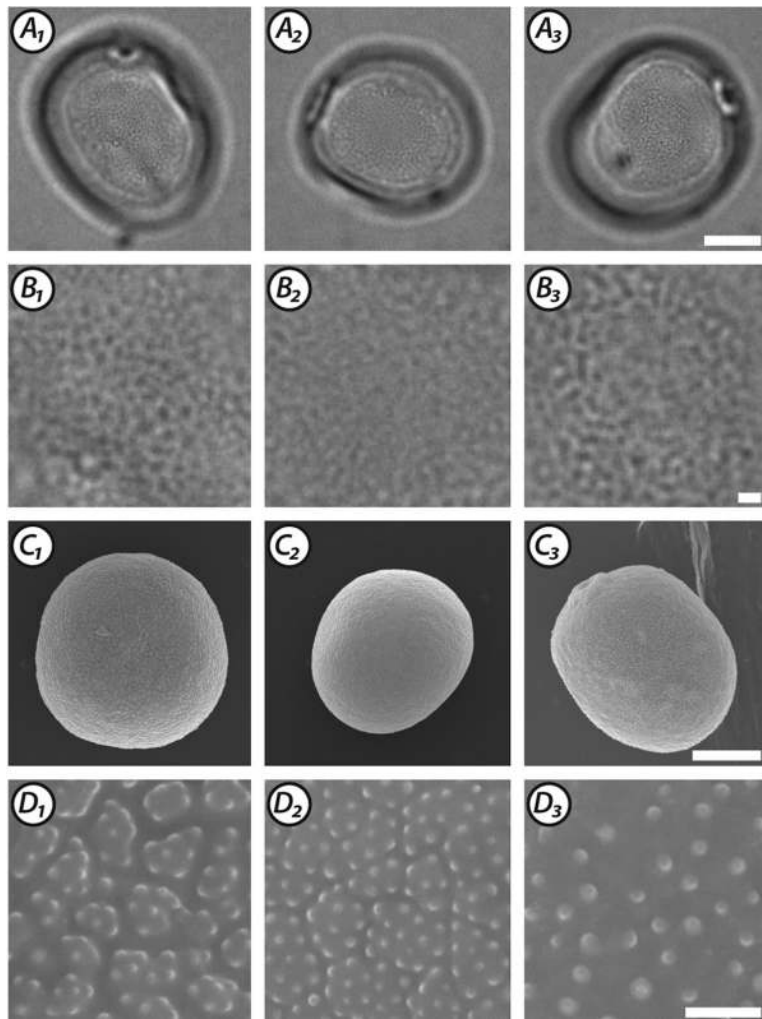
Fig. 6 Pollen of three species of grass from the Poodieae subfamily: *Dactylis glomerata* (A_1–D_1), *Poa australis* (A_2–D_2), and *Stipa tenuifolia* (A_3–D_3). Images highlight morphological differences between these three species that cannot be seen clearly with bright-field microscopy because they are smaller than the diffraction limit of light (*A*, *B*) but can be imaged with SEM (*C*, *D*). Bright-field images were taken with a ×63 1.4 NA (numerical aperture) oil immersion objective. SEM images are shown at ×2000 magnification (*C*) and ×12,000 magnification (*D*). Scale bars = 10 μm (*A*, *C*) or 1 μm (*B*, *D*).

bright-field microscopy remains the most widely used technique for routine palynological investigations. There are sound reasons for this, including the relatively low cost of a compound microscope, the ease of using one of these instruments, the fast rate of data acquisition, and the development of an extensive and functional descriptive terminology guided by morphological features that can be readily observed with this technique (Punt et al. 2007). However, as shown by the example of grass pollen, the diffraction limit of light results in a critical loss of morphological information from sporomorphs, and we believe that this exerts a first-order control on the taxonomic resolution of sporomorph assemblages. Accordingly, we urge palynologists to keep pace with developments in microscopy and to actively explore, test, and evaluate the suitability of new techniques for palynological investigations as and when they arise. The family of fluorescence techniques known as superresolution techniques (because they

achieve a spatial resolution that is not limited by the diffraction of light) are particularly promising because they allow researchers to recover subdiffraction morphological information on sporomorphs that are mounted in standard palynological strew slides rather than on an SEM stub (Sivaguru et al. 2012). Since the exine of sporomorphs is autofluorescent, a technique known as superresolution structured-illumination microscopy (Gustafsson 2000; Huang et al. 2009) appears well suited to palynological requirements.

Morphometric Approaches

In situations where descriptive terminology fails to adequately describe subtle morphological differences between taxa, as is often the case at the species level in Quaternary time, palynologists have made efforts to move away from classifications based solely on the qualitative language of descrip-

tive palynology (e.g., Punt et al. 2007) and have instead attempted to analyze sporomorph morphology quantitatively. A widely adopted approach involves the measurement of taxonomically significant morphological features. Measurements are usually taken from digital images by means of computer software, and this is akin to measuring attributes of macroscopic biological specimens with a ruler or calipers. This approach has been applied to various groups in which species-level classification is difficult, including the pollen of *Pinus* (Hansen and Cushing 1973), *Picea* (Hansen and Engstrom 1985; Lindbladh et al. 2002), Poaceae (Joly et al. 2007; Schüler and Behling 2011*a*, 2011*b*), and *Alnus* (May and Lacourse 2012). Different morphological features are measured in each case, and investigations of bisaccate pollen may include measurements of the total specimen size, the corpus breadth and height, the saccus height and width, the thickness of the corpus cap, and the depth of saccus attachment (Lindbladh et al. 2002). Measurements of Poaceae pollen include the total specimen length and width, the diameter of the pore, and the diameter of the annulus (Schüler and Behling 2011*b*), while measurements of *Alnus* pollen include the specimen diameter, the thickness of the exine, the height and width of the annulus surrounding one of the five pores, and the width of the arcus (May and Lacourse 2012).

Such measurements help to quantify morphological differences between taxa that can be difficult to observe with a microscope, which in turn allows for classifications at a finer taxonomic resolution than is possible by eye. This allows researchers to attempt to track individual species through time, which is particularly useful when there are marked autecological differences between congeneric species, such as *Picea mariana* and *Picea glauca* (Lindbladh et al. 2002; Punyasena et al. 2012), or in cases, such as *Alnus*, where individual species are intimately associated with processes such as forest fires and ecosystem disturbance (Lantz et al. 2010; May and Lacourse 2012). Even in situations where morphometric measurements fail to separate individual species, this approach can be used to track certain ecosystems in time and space, and the length of grass pollen grains, for example, has been used to characterize different grassland ecosystems in South America (Schüler and Behling 2011*a*). Measurements in such studies are usually undertaken manually (e.g., Schüler and Behling 2011*b*; May and Lacourse 2012), and tests of anthropometric measurements taken manually by humans using devices such as calipers, anthropometers, and measuring tape are characterized by considerable intra- and interobserver variance (Gavan 1950; Munro et al. 1966; Feathers et al. 2004). However, to our knowledge there has been no published research designed specifically to investigate the accuracy and consistency of measurements taken from sporomorphs by human analysts using imaging software, and the extent to which morphometric studies of sporomorph morphology are undermined by inconsistencies in measurements made by human analysts is currently unclear.

Computational Image Analyses and Machine Learning

Routine palynological classifications undertaken by human experts involve the qualitative assessment of specimen morphology. Typically, palynologists examine by eye the sizes and shapes of attributes that are thought to have taxonomic significance, such as the sculptural elements on the sporomorph surface, and then use these attributes to qualitatively compare unknown specimens to classified reference material. Experiments designed to measure the consistency of classifications undertaken in this way raise doubts about the ability of human analysts to consistently repeat such classifications over the course of an entire study that may encompass tens of thousands of specimens (e.g., Culverhouse 2007; MacLeod et al. 2010; Mander et al. 2014). For example, seven human subjects classified 120 SEM images of 12 grass pollen species with 68%–82% accuracy (10 images per species), but the classification schemes of each subject were very different from one another, and consistency between subjects was just 28% (Mander et al. 2013). A more recent analysis of human performance on this data set also highlighted considerable differences between analysts and notable self-inconsistency as well (Mander et al. 2014). Similar investigations of human classification consistency with a marine microfossil group revealed irreconcilable differences between analysts (Ginsburg 1997).

In our view, palynologists are acutely aware that classification consistency is difficult to achieve. As we have already mentioned, palynologists attempt to ensure consistency among researchers and between laboratories by classifying at a taxonomic rank above the species level and accept that low taxonomic resolution is an inevitable trade-off of this approach (Punyasena et al. 2012). To our knowledge, there are no published tests of human classification performance in "easy" palynological classification problems at low taxonomic resolution, such as the distinction between the pollen of Poaceae, *Quercus*, Asteraceae, and *Pinus*, which are substantially different from each other. We suspect that expert palynologists would fare well in such a test and, by extension, that they generate consistent classifications at the genus or family level in Quaternary time. However, as testing suggests (Mander et al. 2013, 2014), we have little doubt that palynologists struggle to generate species-level classifications of problematic groups that are both self-consistent and consistent between workers, and this view has led to calls for an algorithmic approach to classification that employs computational image analyses and machine learning to quantify small differences between morphologically similar species (MacLeod 2010; MacLeod et al. 2010; Punyasena et al. 2012; Mander et al. 2013).

This approach is at an early stage of development but is, in our opinion, characterized by three key components. The first is the acquisition of images of the sporomorphs in question, which involves choosing an appropriate microscopy technique. This choice is largely driven by the nature of the morphological characters (which should ideally be phylogenetically informative) that the palynologist aims to use as the basis for classification. For example, the surface ornamentation of grass pollen can be imaged effectively with an SEM (e.g., Andersen and Bertelsen 1972; Page 1978; Peltre et al. 1987; Chaturvedi et al. 1998; Mander et al. 2013; fig. 6), but the sacci of bisaccate pollen appear rather smooth under an SEM, and it is necessary to use an optical microscopy technique, such as confocal microscopy (fig. 4*I*–4*L*), to image the characteristic endoreticulations of these structures accurately (e.g., Punyasena et al. 2012). The second component is the development of

features that describe aspects of sporomorph morphology. These can be relatively abstract and only indirectly related to the primary morphological characters of sporomorphs. Such abstract features include the intensity distribution of an image, measures of gross shape based on low-resolution image projections, and representations of ornamentation by sign changes in the first derivative of pixel intensity values in lines drawn across an image (Punyasena et al. 2012). Less abstract features that aim to describe sporomorph morphology as accurately as possible are akin to morphometric measurements, and they include computational measures of the size and number of sculptural elements on a specimen and measures of the complexity of the surface patterning that such elements form (Mander et al. 2013). The third component of an algorithmic approach to sporomorph classification is the methodology that is used to analyze these descriptive features. These methods can be relatively simple, such as reducing the dimensions of feature vectors using eigenanalysis (Mander et al. 2013), or relatively complex, such as the analysis of Gabor transforms and moment invariants with a multilayer perceptron neural network and other machine-based approaches (Zhang et al. 2004; Holt et al. 2011; Punyasena et al. 2012).

There is considerable scope for experimentation with different configurations of these three components, and in our own research we have tried to explore a spectrum of approaches. At one end of this spectrum is the idea that "measured morphological values do not need to have any biological significance; they only need to define the range of morphological variation observed" (Punyasena et al. 2012, p. 943), which has been successfully applied to the classification of extant and fossil black and white spruce (*P. mariana* and *P. glauca*, respectively; Punyasena et al. 2012). At the opposite end of this spectrum is an approach that is "rooted in the accurate description of morphology" and develops features that "can be directly related to biologically significant morphological characters" (Mander et al. 2013, p. 6), which has been successfully applied to the classification of the pollen of 12 extant grass species (Mander et al. 2013). At present, there have been too few studies to make any definitive statement about which approach is best, or most appropriate for certain situations. Nevertheless, there are clear signs that an algorithmic approach to classification can increase the taxonomic resolution of the sporomorph record (Punyasena et al. 2012; Mander et al. 2013), and we believe that the incorporation of quantitative computational morphometric methods (e.g., Mander et al. 2013) within supervised and unsupervised machine-learning frameworks (e.g., De Sá-Otero et al. 2004; Zhang et al. 2004; Chun et al. 2006; Ranzato et al. 2007; Costa and Yang 2009; Holt et al. 2011; Punyasena et al. 2012) is an area that requires further investigation. Certain approaches that have been designed to fit the requirements of a specific clade (e.g., Mander et al. 2013 for grass pollen) are unlikely to be suitable for the generation of standard paleoecological counts of thousands of sporomorphs from many different higher taxa through time. Partly this is because the optimal recovery of taxonomically significant morphological information from certain groups of sporomorphs requires the use of different microscopy techniques, e.g., SEM for grass pollen (fig. 6) but an optical method, such as apotome (Punyasena et al. 2012) or confocal (fig. 4I–4L) microscopy, for

spruce pollen. The integration of such clade-specific data with standard paleoecological counts, which could be generated by supervised machine-learning systems (e.g., Holt et al. 2011; Punyasena et al. 2012), represents a challenge to be explored as this field of research matures. An additional problem centers on the dissemination and archiving of the morphological data sets and algorithms that are central to a computational approach to sporomorph classification. We are currently using the Illinois Digital Environment for Access to Learning and Scholarship (http://ideals.illinois.edu) and the web-based source code repository SourceForge (http://sourceforge.net) to share images, libraries, and code that we have generated. We are not aware of any cyberinfrastructure that has been specifically designed for the sharing of such information among the palynological research community, and we believe that there is scope for coordinated activity in this domain.

Synthesis and Outlook

Sporomorphs provide an exceptional window into the evolution and diversity of plant life on Earth. However, they are frequently classified above the taxonomic rank of species, and palynological studies often present data at the genus or family level. As a result, sporomorph records of Earth's vegetation are characterized by low taxonomic resolution (Birks and Birks 2000; Jackson and Booth 2007; Mander 2011; Punyasena et al. 2011, 2012; May and Lacourse 2012; Mander et al. 2013). Palynologists classify sporomorphs at a taxonomic rank above species for several reasons. First, sporomorphs are typically morphologically similar among many species within a single genus of plants and occasionally among genera within certain families (Jackson and Booth 2007). In such cases, low taxonomic resolution can arise both from the inability of conventional optical microscopes to recover certain taxonomically significant morphological features and because it can be challenging to describe the very small morphological differences between species using the terminology of descriptive palynology (e.g., Punt et al. 2007). There are several examples of this in Quaternary and pre-Quaternary time, but grass pollen is an extreme and classic case (Mander et al. 2013; fig. 6). In situations such as this, where classification at the species or genus level is extremely difficult, palynologists classify sporomorphs conservatively at the genus or family level to ensure that their classifications are reproducible between samples and between researchers (Punyasena et al. 2012). Other causes of low taxonomic resolution include inadequate reference collections of vouched herbarium specimens, which prevents researchers from comparing Quaternary fossil sporomorphs to the morphological variability of extant plant taxa (Birks and Birks 1980), and the absence of sporomorphs in situ in fossilized reproductive structures (Mander 2011), which blurs the relationship between dispersed sporomorphs and their parent plants in pre-Quaternary time (Mander 2011). Taphonomic factors also play a role, and exine damage as a result of fossilization can prevent the classification of sporomorphs by obscuring key morphological details (Birks and Birks 1980).

Low taxonomic resolution hampers the investigation of several critical issues in vegetation science. Some of these issues are based around evolutionary questions, including the nature

and timing of plant species extinction (Jackson and Weng 1999; Mander et al. 2010), the diversification history of major clades, such as grasses (Mander et al. 2013), and the reconstruction of large-scale biogeographic patterns, such as the latitudinal diversity gradient (Qian and Ricklefs 2007; Jardine et al. 2012). Others are concerned with the reconstruction of paleoecological conditions, which is confounded when congeneric species have widely different autecological preferences, such as spruce, alder, and oak (Lindbladh et al. 2002; Lantz et al. 2010; May and Lacourse 2012; Punyasena et al. 2012). In situations where the constituent species of a genus have considerably variable bioclimatic preferences, the use of genus-level sporomorph data as an indicator of paleoclimate, such as the use of *Podocarpus* pollen as a proxy for cool temperatures, is clearly questionable (Punyasena et al. 2011). Low taxonomic resolution also affects the link between ecology and paleoecology, which is of concern in light of current conservation efforts (Dawson et al. 2011). In particular, the Holocene sporomorph record provides a potential bridge between ecological studies of present-day vegetation and shallow- and deep-time paleoecological studies of past vegetation (Harrington 2004; Jaramillo et al. 2006; Jardine et al. 2012), but low taxonomic resolution prevents the robust linkage of real-time species-level dynamics observed in ecological data and the patterns observed in paleoecological archives that take place over timescales longer than a human life (Brewer et al. 2012).

Improving the taxonomic resolution of the sporomorph record is a key current challenge in palynology, but there are, of course, some causes of low taxonomic resolution that palynologists can do little about. Neither homoplasy nor the process of fossilization can be reversed, and perhaps the best that can be done in these situations is to recognize a robust example of convergent evolution and note potential biases due to poor preservation. Similarly, certain plants are absent or poorly represented as dispersed sporomorphs, which creates marked gaps in the sporomorph record. The causes of these gaps are now fairly well understood in both Quaternary and pre-Quaternary time, and it is thought that they result from the low production of sporomorphs by certain plants and the poor preservation of certain sporomorph taxa (e.g., Davis 1963; Chaloner 1968; Birks and Birks 1980, 2000; Prentice and Webb 1986; Dunwiddie 1987; Jackson and Booth 2007; Traverse 2007; Mander et al. 2010). Plant macrofossils (and other fossil groups, such as phytoliths; e.g., Piperno 2006; Strömberg 2011) can be extremely valuable as a source of additional information on vegetation composition (e.g., Birks and Birks 2000), but some plants, such as *Carya* and *Corylus*, are poorly represented as macrofossils (Jackson and Booth 2007). Consequently, neither the sporomorph record nor the macrofossil record is necessarily more complete or more accurate than the other, and "each gives an incomplete picture of the contemporaneous flora; the two pictures have large areas of overlap, but each complements the omissions of the other" (Chaloner 1968, p. 129). The immediate challenge for palynologists is to develop methods and technologies to improve the taxonomic accuracy of the picture that sporomorphs provide us.

In our view, the most promising approach to this challenge is the combination of high-resolution imaging and computational image analysis. The resolution of the optical microscopes that are used as the workhorses of palynological data collection is limited to 200–250 nm in most practical situations by the diffraction of light (e.g., Weiss 2000; Heintzmann and Ficz 2006). This represents a barrier to the amount of morphological information that can be recovered from individual specimens, and we sense that this exerts a first-order control on the taxonomic resolution of sporomorph assemblages. There are many examples, including focused studies of a single group, such as grasses (Andersen and Bertelsen 1972; Page 1978; Peltre et al. 1987; Chaturvedi et al. 1998; Mander et al. 2013; fig. 6), individual genera, such as *Tilia* (Moore et al. 1991) and *Quercus* (fig. 3), and whole floras, such as northwest Europe (Punt et al. 1995), where SEM studies highlight that morphological information that could be used for the purpose of classification is hidden from the palynologist by the diffraction limit of light. SEM cannot capture all aspects of sporomorph morphology (Sivaguru et al. 2012), but we believe that these examples show that it is essential for palynologists to continue to explore other technologies, such as superresolution microscopy techniques (e.g., Gustafsson 2000; Huang et al. 2009), that aim to recover morphological information from below the diffraction limit of light.

Computational image analyses allow researchers to quantify small morphological differences between taxa and can translate qualitative observations of sporomorph morphology into robust and repeatable measurements (Punyasena et al. 2012; Mander et al. 2013). This can allow a wider range of features to be incorporated into the process of classification, including both abstract (Punyasena et al. 2012) and morphometric (Mander et al. 2013) features, and it can prevent researchers from defaulting to conservative classifications in an effort ensure repeatability (Punyasena et al. 2012). The diversity of approaches that have been explored to date (e.g., De Sá-Otero et al. 2004; Zhang et al. 2004; Chun et al. 2006; Ranzato et al. 2007; Costa and Yang 2009; Holt et al. 2011; Punyasena et al. 2012; Mander et al. 2013) suggests an emerging distinction between researchers who employ computational image analyses, often with machine learning, in order to reduce the amount of time expert palynologists spend undertaking routine work (e.g., Holt et al. 2011), and those whose primary aim is to increase taxonomic resolution with less regard for the amount of time spent doing so (e.g., Mander et al. 2013). Exploring this distinction promises exciting avenues of future research and may lead to the development of clade-specific palynology, in which certain combinations of imaging techniques and analytical approaches are tailored to suit different sporomorph groups.

Appendix

Voucher Information for *Quercus* Specimens Sampled from the Herbarium at the Royal Botanic Gardens, Kew

Quercus englemannii, Epling and Robinson, s.n. (catkin). *Quercus englemannii*, Epling, s.n. (leaf). *Quercus lobata*, Purpus, 4102 (catkin and leaf). *Quercus macrocarpa*, Bebb, 39 (catkin and leaf). *Quercus virginiana*, Chapman, s.n. (catkin and leaf).

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