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Aberrant *Classopollis* pollen reveals evidence for unreduced (2n) pollen in the conifer family Cheirolepidiaceae during the Triassic–Jurassic transition

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Polyploidy (or whole-genome doubling) is a key mechanism for plant speciation leading to new evolutionary lineages. Several lines of evidence show that most species among flowering plants had polyploidy ancestry, but it is virtually unknown for conifers. Here, we study variability in pollen tetrad morphology and the size of the conifer pollen type *Classopollis* extracted from sediments of the Triassic–Jurassic transition, 200 Ma. *Classopollis* producing Cheirolepidiaceae were one of the most dominant and diverse groups of conifers during the Mesozoic. We show that aberrant pollen *Classopollis* tetrads, triads and dyads, and the large variation in pollen size indicates the presence of unreduced (2n) pollen, which is one of the main mechanisms in modern polyploid formation. Polyploid speciation may explain the high variability of growth forms and adaptation of these conifers to different environments and their resistance to extreme growth conditions. We suggest that polyploidy may have also reduced the extinction risk of these conifers during the End-Triassic biotic crisis.

1. Introduction

Polyploidy (or whole-genome doubling (WGD)) is a key mechanism for plant speciation leading to new evolutionary lineages [1,2]. Several lines of evidence show that most species among flowering plants had polyploidy ancestry [3,4]. Molecular dating of ancient whole-genome doubling events suggests that the rise of seed plants and angiosperms were associated with a series of WGD events at around 319 and 192 Ma [5]. While polyploidy is ubiquitous among flowering plants, it is virtually unknown for extant gymnosperms [6]. It is only reported within one species of the Cupressaceae, *Sequoia sempervirens*, and one species of Cycads, *Encepharlatos hildebrandtii*, while it is more common among species of *Ephedra* [7,8]. In angiosperms, the formation of unreduced gametes and pollen is regarded as the main mechanism leading to polyploids [9,10]. In modern gymnosperms, unreduced gametes are less common and have been reported for only one species of the Cupressaceae, *Cupressus dupreziana* [11]. However, extant gymnosperms represent only a small group of a much more diverse group of gymnosperms which were outcompeted by angiosperms during the Cretaceous [12]. It has been suggested that differences in life strategies and genome dynamics may have favoured the evolutionary success of angiosperms over conifers [3,6,13,14]. However, the Cheirolepidiaceae represent one of the most prominent and diverse groups of Mesozoic conifers. This extinct conifer family is presumably related to the Cupressaceae or Araucariaceae and occupied a wide range of ecological niches and possessed a highly variable growth habit [15,16]. It had an exceptional long geological record ranging from the Late Triassic until the End-Cretaceous, from approximately 225 Ma until 65 Ma.

In this paper, we test the hypothesis that this conifer family produced unreduced pollen. Polyploidy may have been an important evolutionary mechanism
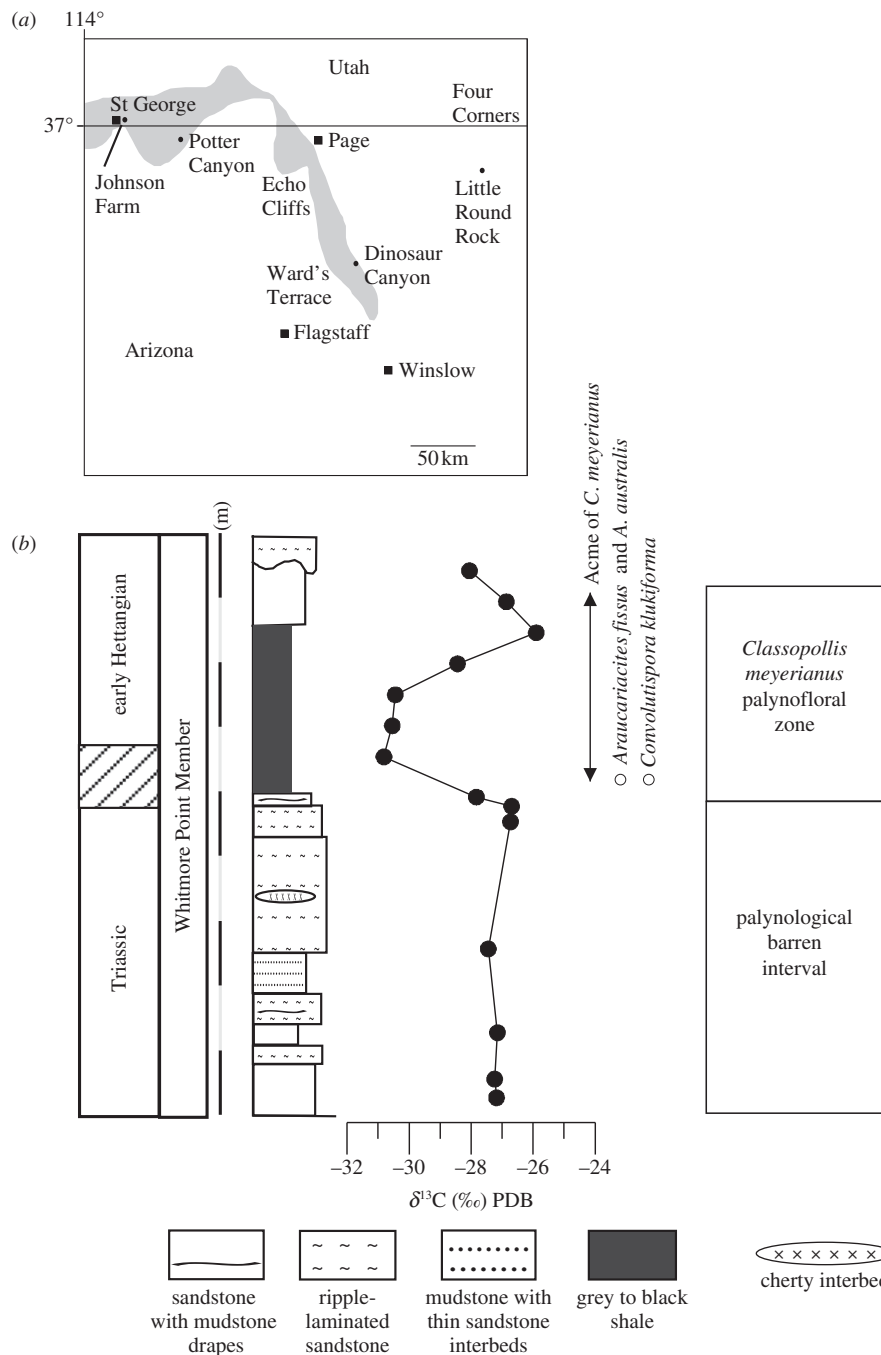
Figure 1. (a) Location map of the Potter canyon section in AZ, USA and (b) summary diagram with lithology, C-isotope ratios of bulk sedimentary organic matter and main palynological events from the lower Whitmore Point Member of the Moenave Formation. C-isotope ratios are reported in standard delta notation relative to Vienna PDB standard (PDB, Pee Dee Belemnite).

explaining the exceptional ecological diversity of this successful conifer family. As cell size correlates with the DNA content [1], stomatal size of living and fossil leaf remains has been used to infer ploidy history in angiosperms during the Caenozoic [17,18]. Similar to the stomatal size, pollen size and volume also varies with the ploidy level of a plant [9,10,19–22]. In order to test our hypothesis, we examined the morphology of the fossil pollen *Classopollis*, which was produced by the Cheirolepidiaceae.

While polyploidy is a widespread phenomenon in flowering plants, it is in modern gymnosperms, despite their large genome size, rare [1,6]. We show that tetrad and pollen size analysis of the fossil conifer pollen *Classopollis* reveals evidence of hitherto unknown WGD events within gymnosperms. Our results suggest that WGD events may be more widespread within this clade than has been inferred before

and that palaeobotanical and palynological analysis may be a key to elucidating this history of genome evolution.

2. Material and methods

The Potter Canyon section is a south-facing promontory in Mohave County in northern Arizona at 36°52.872′ N, 112°52.083′ W. Fourteen rock samples were taken from the lower part of the Whitmore Point Member for palynological and bulk C-isotope analysis according to Utrecht laboratory standard protocols [23,24].

Classopollis-dominated pollen assemblages were extracted from rocks of the Whitmore Point Member of the upper part of the Moenave Formation at the Potter Canyon section on the Southern Colorado Plateau, northwest Arizona (figure 1). Bio- and magnetostratigraphic data suggest that the Whitmore Point Member of the upper part of the Moenave Formation

encompasses the Triassic–Jurassic transition [25]. The C-isotope record shows in the middle part of Whitmore Point Member a marked negative C-isotope excursion of −4‰ (figure 1). We correlate this part of the Whitmore Point Member with the lower part of the *Classopollis meyerianus* palynofloral zone of the Newark Supergroup [26]. In accordance with previous stratigraphic correlations [25], we correlate the negative C-isotope excursion with the main isotope excursion at the Triassic–Jurassic transition [23,24].

Classopollis pollen diameters were measured (in total $n = 3754$) with an image analyser (ANALYSIS program of Olympus Soft Imaging Solutions), attached to a light microscope from one interval with *Classopollis*-dominated pollen assemblages extracted from the same sediment samples. Also, pollen tetrad configurations were analysed in these *Classopollis*-dominated pollen assemblages. Histograms of *Classopollis* size-frequency distributions were constructed using R [27]. Statistical analyses were performed using the R package 'moments' [28]. In a first series, 1400 *Classopollis* pollen grains were measured. The size-frequency distribution of the first series of measurements on *Classopollis* pollen ($n = 1400$) indicates that the majority of specimens measure 28–38 μm and is positively skewed which was statistically significant using a D'Agostino test of skewness (skew = 1.140; $p < 0.0001$) [29]. In order to examine the frequency of pollen grains in the right-hand tail of the distribution, a second round of measurements ($n = 2354$) were undertaken on specimens of *Classopollis* pollen larger than 34 μm from the same rock sample.

As all measurements were carried out from the same population, we can exclude the confounding bias of pollen size during sample preparation. If pollen size was eventually influenced by shrinking during fossilization, then all *Classopollis* pollen should be affected in the same way because they all possess the same basic shape, structure and morphology. *Classopollis* is a robust and thick-walled pollen grain that is well preserved in the sediments and approximates the shape of an ellipsoid. The volume can be calculated by the formula $V = 4/3 \times \pi \times a \times b \times c$, in which a, b and c represent the axes of the ellipsoid.

3. Results

(a) *Classopollis* pollen and tetrad morphology

Well-preserved dispersed pollen of *Classopollis* dominated the palynofloral assemblages (figure 2a–c). Also, clustered pollen tetrads, triads and dyads were encountered, which show a remarkable variation. We found three types of tetrads. The normal multiplanar *Classopollis* clusters form tetrahedral tetrads in which each *Classopollis* pollen grain is in contact with three others (figure 2d). A second type of multiplanar tetrads shows *Classopollis* pollen arranged in two pairs lying at right angles across one other (figure 2e,f). One pollen pair in these unbalanced tetrads is usually larger than in the normal tetrahedral tetrads, whereas the second pollen pair is frequently smaller. There are also uneven-sized tetrads with three small *Classopollis* attached to one giant *Classopollis* pollen grain (figure 2g). A third variant of tetrads shows four evenly reduced *Classopollis* pollen grains (figure 2h). As a third kind of pollen clusters, we also found pollen triads with even and uneven pollen size in several combinations of different-sized *Classopollis* pollen (figure 2i–k). As a fourth category of pollen cluster, we found pollen dyads (figure 2l).

Another striking feature of the dispersed *Classopollis* population is their variability in diameter. No other conifer pollen from this palynofloral assemblage shows a similar variability of size. Variability in size and morphology of *Classopollis* pollen from this location has previously been used to identify different

morphological 'species' of *Classopollis*, such as *C. meyerianus*, *Classopollis torosus* as well as *Classopollis itunensis* [29]. According to our light microscopy and scanning electron microscopy analyses, however, most of the morphotypes of *Classopollis* exactly resemble the morphology and surface structure of *C. meyerianus*, except for the variability in size (see the electronic supplementary material). The surface structures of both the normal and the giant *C. meyerianus* pollen are rather smooth and show no ornamentation or striation. The relative size proportions of these pollen types are equal. As small and large *Classopollis* morphotypes form together tetrads or triads, they cannot be separate taxa as suggested by previous authors [30], but belong to the same mother plant.

(b) *Classopollis* pollen size analysis

We systematically measured the variability of *C. meyerianus* pollen diameter with an image analyser in order to assess its size distribution (figure 3). The size-frequency distribution of *Classopollis* pollen from the Whitmore Point Member indicates that the majority of specimens measure 28–38 μm (figure 3). However, several specimens are much larger, measuring in excess of 50 μm, and the distribution is positively skewed (figure 3). This observation is statistically significant using a D'Agostino test of skewness (skew = 1.140; $p < 0.0001$) [29].

The right-hand tail of the frequency distribution of *C. meyerianus* pollen with diameters larger than 34 μm was further investigated with a second round of measurements (figure 4a). Within the frequency distribution of the larger diameters, distinct peaks can be observed (figure 4a). Using the main peak in the pollen diameter frequency diagram of 32 μm as the base value, we calculated the pollen volume as a function of the measured diameters. Some of the distinct increments in the pollen diameter histogram (figure 4b), such as the peaks at 45, 49, 55 and 60 μm, represent discrete multiplications of the pollen volume such as two, three and five times the average pollen volume.

Pollen tetrad formation has been previously described for *Classopollis* pollen [31]. Typically, *Classopollis* forms multiplanar tetrahedral tetrads in which each *Classopollis* pollen grain is in contact with three others (figure 2d). The pollen diameter of the individual *Classopollis* pollen in these normal tetrads is constant and is equal to the main diameter of the dispersed *Classopollis* pollen of 32 μm. In this study also aberrant, uneven-sized multiplanar tetrads occur where *Classopollis* pollen grains are arranged in two pairs lying across one other (figure 2e,f). Pollen grains in the abnormal tetrads are notably larger than in the normal tetrahedral tetrads. One pollen pair is frequently significantly reduced in size (figure 2e,f). A second type of uneven-sized multiplanar tetrahedral tedrads is formed by a combination of three small and one giant *Classopollis* pollen grain (figure 2g). Besides these combinations of uneven-sized tetrads, we also found tetrads with equally reduced *Classopollis* pollen grains (figure 2h). Triads occur in combination of three normal-sized pollen grains (figure 2i) or in uneven-sized combinations of two normal-sized and one smaller pollen grain (figure 2j) or one normal-sized pollen grain and two smaller pollen grains (figure 2k). It is possible that these pollen triads and dyads represent damaged pollen tetrads where one or two pollen grains were lost. However, in a multiplanar tetrahedral configuration, pollen grains are aligned at a different angle to each other than in a planar triad or dyad configuration. The orientation
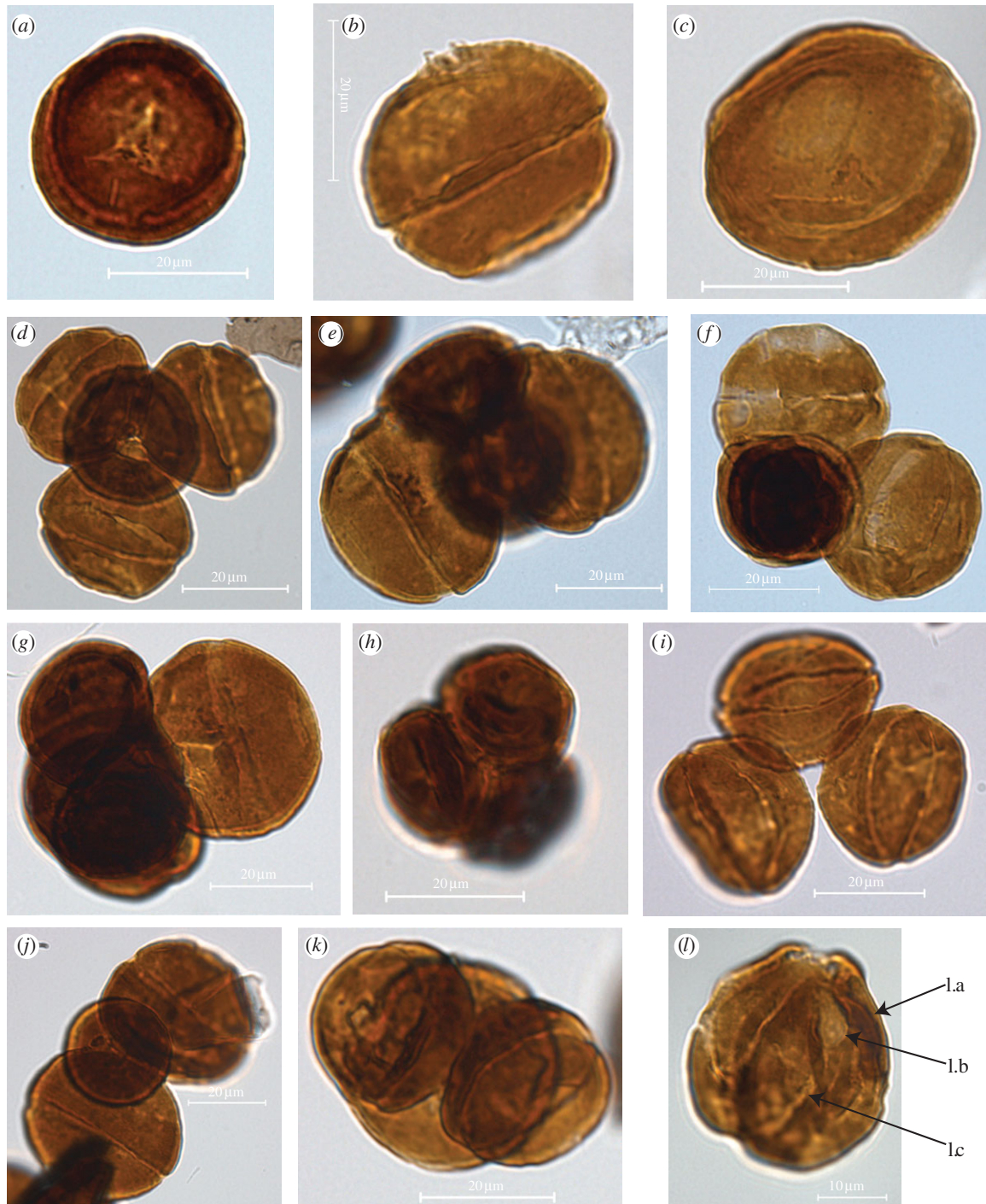
Figure 2. Light-microscope photographs of *Classopollis meyerianus* pollen: (*a,b*) dispersed normal-sized pollen and (*c*) giant variant, (*d*) normal-sized multiplanar *Classopollis* clusters form tetrahedral tetrad, (*e,f*) multiplanar tetrads with two uneven-sized pairs lying at right angles across one other, (*g*) uneven-sized tetrad with three small *Classopollis* attached to one giant *Classopollis* pollen grain, (*h*) small even-sized *Classopollis* cluster form tetrahedral tetrad, (*i*) even-sized planar triad, (*j*–*k*) uneven-sized triads. (l) Dyads showing the furrow (l.a), the proximal tetrad scar (l.b) and the distal cryptopore (l.c). (Online version in colour.)

of the openings or the furrow of the *Classopollis* pollen indicates the alignment of the pollen and helps to distinguish a primary planar or multiplanar orientation. In the studied material, the orientation of the furrow indicates the planar alignment of the *Classopollis* pollen in most of the triads and dyads.

The observed variability in the formation of *Classopollis* tetrads is unusual and has not, to our knowledge, been described before. Principally, the observed pattern can be ascribed to the formation of anucleate, haploid and unreduced $2n$ pollen. In modern plants, pollen size is related to the number of chromosomes with increasing pollen volume at higher ploidy levels [9,19–22,32–35]. While the number of pollen openings has been reported to vary in some polyploidy angiosperms [36],

in this study, the number of pores and the furrows of *Classopollis* remains constant.

The pollen diameter in the normal even-sized *Classopollis* tetrads is in agreement with the pollen measuring 28–38 μm, with the mode at 32–33 μm, in the dispersed *Classopollis* assemblages. They represent the majority of the specimens measured in this study (figure 3). Pollen grains from this size class are interpreted as haploid (*n*) pollen grains dispersed at maturity following successful meiosis. Therefore, the normal even-sized *Classopollis* tetrads (figure 2*d*) are interpreted as clusters of unripe haploid (*n*) pollen that were released before separation. Specimens of *Classopollis* pollen smaller than the majority, around 25 μm and below,
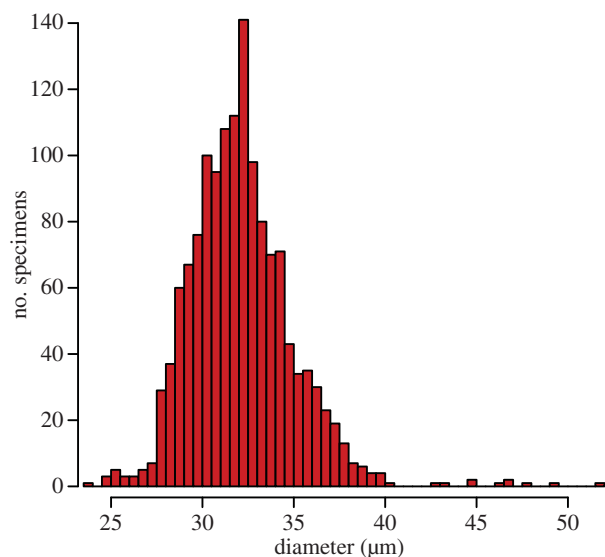
Figure 3. Histogram showing the overall distribution of *Classopollis* pollen size from the Whitmore Point Member ($n = 1400$). Data binned at 0.5 μm intervals. (Online version in colour.)

are interpreted as anucleate pollen grains (figure 3). Aborted pollen development is also recognized in the tetrahedral *Classopollis* tetrads with evenly reduced pollen (figure 2*h*) of the same size, approximately less than or equal to 25 μm. Specimens of *Classopollis* pollen from the right-hand tail of the positively skewed distribution are interpreted as diploid ($2n$) and higher polyploid pollen grains (figure 3).

In order to examine the frequency of pollen grains in the right-hand tail of the distribution, a second round of measurements were undertaken on specimens of *Classopollis* pollen larger than 34 μm from the same rock sample. These measurements increase the maximum size of *Classopollis* pollen found in the Whitmore Point Member from 52 to 75 μm (figure 4*a,b*). They also highlight a group of specimens that measure between 43 and 53 μm (figure 4*b*). *Classopollis* specimens in this group are interpreted as diploid ($2n$) pollen grains. Specimens that are larger than this may represent higher orders of polyploidy, including $4n$ and perhaps even $8n$ although a linear relationship between pollen volume and ploidy level needs to be tested.

Subpopulations within larger populations can be difficult to separate and define [37]. Partly, this is because the upper and lower tails of each subpopulation can overlap with one another. Such overlap has been observed in studies of pollen diameter from polyploid *Arabidopsis* plants, in which the right-hand tail of pollen from triploid ($3n$) plants overlaps with the left-hand tail of pollen from hexaploid ($6n$) plants [22]. Such overlap in the diameter of pollen from polyploids can be shown clearly in studies of modern plants because the ploidy of the parent plant is known. The boundaries between different populations in fossil material are much harder to define, although a Mann–Whitney test indicates that the population of *Classopollis* pollen shown in figure 3 and the population of *Classopollis* pollen shown in figure 4*b* have significantly different size-frequency distributions ($p < 0.0001$).

4. Discussion

Unreduced gametes have been observed in many modern polyploid mutants and can be generated by a variety of cytological mechanisms [9,10,32]. In plants where unreduced gametes were found, unreduced pollen is formed by anomalies occurring during meiotic cell division. These aberrations include abnormal spindle orientation, defective synapsis, omission of meiosis I or II and impaired cytokinesis [33–35]. Cytological studies also frequently document aberrant unequal-sized tetrads, triads and dyads during the formation of unreduced pollen. The exact cytological mechanism leading to unreduced pollen in the Cheirolepidiaceae awaits further investigations. It should be noted, however, that unbalanced tetrads, normal and unbalanced triads, as well as dyads were also found as a result of abnormal spindle orientation at male meiosis II in *Arabidopsis thaliana* (AtPS1) mutants leading to diplogamete formation and diploid pollen grains. The presence of aberrant pollen tetrads, triads and dyads is one line of evidence for unreduced pollen in the fossil *Classopollis* assemblage.

While polyploidy is a widespread phenomenon in flowering plants, it is uncommon in modern gymnosperms [1,6]. Yet, gymnosperms have a large genome size compared with most other land plants, and ancient polyploidy events may have played an important role in their evolution [6]. Two lines of evidence in our study, the presence of aberrant pollen tetrads, triads and dyads as well as the distinct variation in pollen size within the dispersed *Classopollis* pollen assemblage suggests the formation of unreduced $2n$ pollen by the Cheirolepidiaceae about 200 Ma. Our data represent the oldest evidence for unreduced pollen yet documented from the fossil record of vascular plants. We suggest that polyploidy events did not only play a pivotal role in the evolution of flowering plants but were also a more widespread phenomenon in the evolutionary history of extinct gymnosperms.

It should be noted that Cheirolepidiaceae are probably related to the Araucariaceae or Cupressaceae [15,16], the latter representing one of the few modern conifer families reported to have polyploids [6]. They represent an exceptional successful group of gymnosperms including growth forms from small shrubs to large trees, which are adapted to a wide range of environments, from wet to arid (sub)tropical environments including mangroves [15,16]. During their long evolutionary history starting in the Late Triassic and lasting until the end of the Cretaceous, they inhabited many of the niches now dominated by angiosperms. The unusual exine structure of the *Classopollis* pollen has attracted much interest because its tectate collumellate structure resembles to some degree the pollen wall structure of angiosperms [38,39]. It has been suggested that the complex wall structure may have served as a biochemical recognition mechanism for taxonomical compatibility [40]. Moreover, seeds of the Cheirolepidiaceae are covered by a double integumentum that opened proximally on the scale [41]. This feature, uncommon in gymnosperms would imply that the pollen did not have direct access to the ovule micropyle and may have germinated on the scale. Further studies need to investigate whether the unusual pollen structure and pollination biology of the Cheirolepidiaceae may have had an effect on the formation of unreduced pollen and polyploids.

Polyploid plants are more stress tolerant than their diploid relatives [1,6,42]. Growth experiments showed that $2n$ pollen production is stimulated by environmental factors such as temperature, herbivory, wounding, and water and nutrient stress [43,44]. Plants in their natural habitats experience many of the environmental factors that influence $2n$ gamete
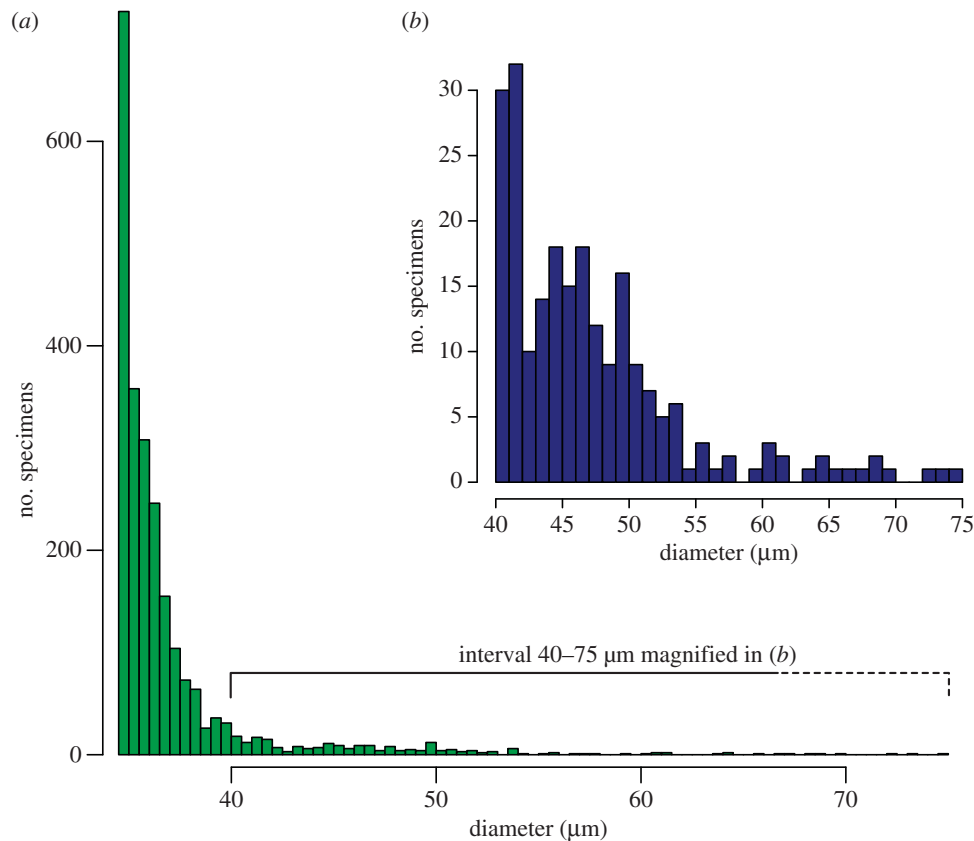
Figure 4. Histograms showing the size-frequency distribution of *Classopollis* pollen greater than 34 μm measured in a detailed investigation of large *Classopollis* from the Whitmore Point Member. (*a*) The overall distribution of *Classopollis* pollen greater than 34 μm (*n* = 2354). Data binned at 0.5 μm intervals. (*b*) Magnification of the 40–75 μm interval to show the shape of the distribution of *Classopollis* pollen around 43–53 μm, and to highlight the frequency of very large *Classopollis* pollen grains (*n* = 226). Data binned at 1 μm intervals. (Online version in colour.)

production in growth experiments. This suggests that natural environmental variation, as well as large-scale climate change, could substantially alter the frequency of polyploid evolution. The high incidence of polyploidy at high latitudes, high altitudes, and recently glaciated areas may be related to the tendency of harsh environmental conditions to induce 2*n* gametes and polyploid formation [45]. Moreover, several geological periods during the Phanerozoic are characterized by large-scale environmental stress, which caused unusual high extinction rates [46]. WGD events may have lowered extinction risks during mass extinction events, such as at the Cretaceous–Paleogene boundary some 65 Ma [47]. We suggest that similar to aberrant lycophyte spores [48] and conifer pollen [49], during the end-Permian mass extinction aberrant *Classopollis* pollen may indicate environmental mutagenesis during the end-Triassic mass extinction. Extensive volcanism of the Central Atlantic magmatic province during the Triassic–Jurassic transition is generally viewed as a trigger mechanism of this global biotic crisis by extreme climate transitions [50]. Evidence for rapid climate cooling followed by extreme warming comes from geochemical proxy [51] as well as biological proxy records [52]. Palynological records across the Triassic–Jurassic transition become completely dominated by *Classopollis* pollen in many parts of the world [53] indicating a widespread proliferation of the Cheirolepidiaceae forests in the aftermath of the biotic crisis. Based on our findings, we suggest that the unusual evolutionary success of this conifer family may be related to their ability to form unreduced 2*n* pollen and thus polyploid hybrids, which were more stress tolerant. Polyploidy may have reduced the extinction risk of the

Cheirolepidiaceae during the end-Triassic environmental crisis. To corroborate the concept of polyploidy within this conifer family, we need to collect additional morphological evidence of polyploidy within the sporophyte. Complementary to pollen, variation in stomatal size of Cheirolepidiaceae shoots, such as *Brachyphyllum* and *Pagiophyllum* is another potential source of palaeobotanical evidence that verifies the presence of polyploidy in the sporophyte. It should be noted that on *Brachyphyllum/Pagiophyllum* shoots from the Newark Supergroup show a distinct trend from small to large leaf sizes across the Triassic–Jurassic transition (B. Cornet 1977, unpublished data). These preliminary observations await quantification by cuticle analysis but points to the existence of polyploidy among the conifer sporophyte generation. Finally, our results imply that the fossil plant record holds key evidence for a much more prominent role of WGD events in extinct lineages which is of importance because, aside angiosperms, so much of green plant diversity and disparity is unrepresented in the modern flora.

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