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Harry Sanders

University of Nebraska at Kearney, sandersh@lopers.unk.edu

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POLYPLOIDY AND POLLEN GRAIN SIZE: IS THERE A CORRELATION?

HARRY SANDERS

Department of Biology, University of Nebraska at Kearney

Mentor: **DR. BENJAMIN PELISSIE**

ABSTRACT

Pollen grain size has been regularly used as an indicator of the ploidy level in plants. It has been demonstrated in some plants and assumed in others that the larger the pollen, the higher the level of ploidy. However, very little work has been done to confirm that this rule is accurate in at least the majority of cases. Most correlations of pollen grain size to ploidy level have been a side note in other research and have not considered the theoretical and genomic implications involved in such a measurement. While much of the literature presents ploidy level as being correlated with pollen grain size, there are some hints that the association might actually be with C-value. It is thus important to ensure that pollen grains are an accurate indicator of ploidy level if they are to continue to be used as such in the literature.

INTRODUCTION

Polyploidy, sometimes referred to as whole-genome duplication (WGD), is a common occurrence in plants. Exactly how common is debated, but conservative figures estimate that somewhere between 24 (Barker et al., 2016) and 35 percent (Wood et al., 2009) of extant plant taxa are recently formed polyploids. Polyploidy is largely clustered within angiosperm plants. Because a whole-genome duplication increases the amount of DNA in each cell, the size of the cells often increases to fit the additional DNA (Cavalier-Smith, 1978, Zhang et al., 2019). This increase in cell size is so well accepted that it has been used to estimate ploidy levels in fossil plants by looking at the size of the fossil plant's guard cells (Masterton, 1994).

Following Masterton's line of thinking with fossil pollen grains, Kurschner et al. (2013), concluded that polyploidy was possibly a mechanism of diversity in the late Triassic conifer family Cheirolepidiaceae. Studies like those of Masterton and Kurschner et al. illustrate the importance of ensuring pollen grains are a reliable indicator of ploidy level. If they are not, theories about past diversity may be incorrect, and even some phylogenetic trees may be affected. Thus, it is critical that pollen grain correlation to ploidy level be established firmly in the present before attempting to use pollen grain size to interpret past events.

Pollen grains are typically haploid when produced by diploid plants. When affected by polyploidy, the natural n haploid state can be increased to $2n$ or even higher levels. Such an increase in DNA size should cause an increase in the size of the pollen grains as well, at least in principle. Therefore, theoretically, polyploidy should increase the size of at least half of the pollen grains in any plant species, and almost all pollen grains in even-numbered polyploids. This argument is common sense and is sometimes assumed in the literature (see Chung et al, 2013) but

will not be assumed for the purposes of this paper. Instead, this paper attempts to determine if there is a correlation between pollen grain size and ploidy level.

ODD PLOIDY LEVELS

Triploid plants, like other plants with odd numbers of chromosome copies, produce pollen during normal meiosis slightly differently than a diploid or higher even-numbered ploidy level plants. Instead of producing pollen that is solely n or $2n$, odd levels of ploidy produce gametes in equal measure. In other words, triploids produce some gametes that are normal haploid gametes, and some that are the potentially enlarged diploid gametes. Odd-numbered ploidy levels higher than three would have a similar effect, producing some pollen that would be at an odd ploidy level, and some at an even ploidy level.

As an example, Martin et al. (2019) found that diploid plants of genus *Annona* produced pollen smaller than their triploid cousins on average. However, the margin of error on both the diploids and the triploids was large enough that both averages fell within the margin of error of the other average. This result would seem to argue that changes to pollen grain sizes are random and not correlated to ploidy level. However, Martin et al. did not distinguish between triploid produced pollen that was $2n$ and n . Including the n pollen with the $2n$ meant that the average was likely lower than the $2n$ pollen alone would have been had the two been measured separately. Thus, this study cannot be taken as strong evidence that ploidy level does not correlate with pollen grain size.

A study on *Parnassia palustris* appears to establish triploid produced pollen as the bridge between tetraploid produced and diploid produced pollen grains. Funamoto et al. (2006) found that n pollen on average was the smallest, followed by triploid produced, then $2n$ pollen. Both tetraploids and diploids were within the margin of error of the triploid pollen, but not each other. Like Martin et al., Funamoto et al. did not differentiate between $2n$ and n pollen grains from the triploids. The triploid results therefore likely represent a mix of n and $2n$ pollen and thus the mean size being between the mean size for exclusively n and exclusively $2n$ pollen is not surprising.

Because of its nature, being part n and part $2n$, triploid pollen cannot be a reliable indicator of whether pollen size correlates with genome size. The same would be true of any odd level of ploidy. The reason why odd level pollen is not a reliable indicator is simple. Theoretically, half the pollen will receive $2n$, and half n . If ploidy level is correlated to pollen size, then the $2n$ pollen will always be larger than the n pollen, meaning that odd ploidy levels will appear to an unsuspecting researcher as if they originated from two different plants with different ploidy levels, when in fact they came from the same plant. This fact would seem to make drawing inferences of ploidy level from fossil pollen much more difficult. It also might complicate analysis of living plants, with its tendency to fall between and overlap the two even ploidy levels surrounding it.

ARTIFICIAL POLYPLOIDS

Inducing polyploidy by the application of the chemical colchicine is a well-established technique used frequently in the literature. However, colchicine-induced polyploids are not necessarily the same as natural polyploids. Ahmad et al. (2018) points out that pollen grain size increases with the application of colchicine in *Gladiolus grandiflorus*. Since they did not correlate this increase with an increase in ploidy levels, it is safer to assume there is no correlation.

Therefore, the conservative route will be taken and colchicine induced polyploids will be treated separately from naturally occurring ones.

Omidbaigi et al. (2010) examined the common spice basil (*Ocimum basilicum* L.). They applied colchicine to some basil plants and compared cell sizes, including pollen, from the resulting tetraploids as well as the original diploids. $2n$ pollen was much larger than n pollen on average and the margins of error do not overlap. This was also true in *Tragopogon*. Tate et al. (2009) showed that the mean pollen grain size was largest for colchicine induced tetraploids, followed by natural tetraploids. Diploids were the smallest. Standard errors were very small and did not cause overlap between the groups. This paper seems to confirm that colchicine treatments cause greater cell enlargement than occur naturally in polyploids and justifies not including them with the natural polyploids.

NATURAL TETRAPLOIDS AND HIGHER EVEN PLOIDY LEVELS

In naturally produced tetraploids and other even ploidy levels, there is a range of variation in pollen grain size and overlap between ploidy levels. In *Eriotheca* species, pollen grain size differed strongly between diploid, tetraploid, and hexaploid individuals (Oliveira et al., 2013). In *Rosa hybrida*, $2n$ pollen was roughly 30% larger on average than n pollen (Zhou et al., 2019). A second study in *Rosa* confirmed that n and $2n$ pollen do not have overlapping mean diameters, even accounting for the standard deviation (Le Bris et al., 2011). Likewise, while the tetraploid produced pollen was not double the size of the diploid-produced pollen, it was larger in the *Bouteloua curtipendula* complex (Siqueiros-Delgado et al., 2017).

Ramsey (2007) found a similar correlation in *Achillea borealis*. Tetraploid-produced pollen was larger on average than diploid-produced pollen. A study in the Australian grass *Themeda triandra* revealed similar results with the diploids producing pollen significantly smaller than the tetraploid (Godfree et al., 2017). These lines of evidence would seem to indicate that pollen size does increase with increasing ploidy levels.

Other lines of evidence would also seem to point to this conclusion. A study of *Chrysanthemum* species found that $2n$ pollen was larger on average than n pollen, and was outside the margin of error (Tobe et al., 2009). However, very few tetraploid produced pollen were examined in each species, making up just over one percent of the analysis. The limited number of the $2n$ pollen examined makes the *Chrysanthemum* analysis questionable. A similar problem presents itself in an analysis of *Gossypium* where a tetraploid species was found to have significantly larger pollen than a diploid species (Wendel et al., 2017). Because the data was presented graphically, rather than numerically, it is difficult to draw firm conclusions, though helpful error bars do inform us that the tetraploid mean size is outside the margin of error of the diploid mean size. Intriguingly, the wild population had larger pollen grains than the domestic population in tetraploids but not in diploids.

Increasing the ploidy level does not always correlate exactly with increased pollen grain size. While tetraploid-produced pollen was larger than diploid-produced pollen in *Eragrostis tef* with no overlap between the two, octoploid-produced pollen overlapped with tetraploid-produced pollen (Gungsa and Loerz, 2013). This pattern also held in *Physochlaina praealta* (Singhal et al., 2017a). Tetraploid plants pollen was produced in three sizes, with the largest grains being much larger than the diploid plants pollen and outside the margin of error. However, the typical size overlapped and smaller tetraploid plant's pollen was much smaller than the diploid plant's pollen

average and outside the margin of error. The overlapping pattern in *P. praealta* was also seen in *Inula grandiflora* except there was no “small” category in that species (Singhal et al., 2017b).

The results of the study in *Physochlaina praealta* are particularly inconsistent. Tetraploid plants producing pollen the same size, larger, and smaller than the diploid plant should not be the case if increasing levels of ploidy also increases pollen size. Such a result could be seen as refuting any correlation between pollen grain size and ploidy level, at least as an across the board rule. However, a possible solution suggests itself. The sizes of the pollen might be representative of the ploidy level. In other words, the largest pollen might be $2n$, the typical size might be n , and the smallest pollen $0n$ due to errors in meiosis. If that were the case, however, pollen fertility would be expected to drop. It did not do so in tetraploids in *Physochlaina praealta* (Singhal et al., 2017a). Therefore, this explanation seems unlikely in this instance.

A puzzling case comes from persimmons. A study in *Diaspyros kaki* found that a small percentage of the pollen was hexaploid, rather than the expected triploid (Sugiura et al., 2000). The hexaploid pollen was significantly larger than the triploid pollen normally produced. On the surface, this study seems to support the idea that the higher the ploidy level, the larger the pollen grain. However, *D. kaki* is naturally hexaploid and should produce triploid pollen. Producing hexaploid pollen would seem to challenge the validity of using pollen grain size as an indicator of the parental plants ploidy level. However, given the relative rarity of hexaploidy pollen produced, the most likely explanation for this phenomenon is meiotic error.

Another interesting case comes from *Stevia rebaudiana*. Forni-Martins et al. (2004), examined multiple strains of the species, including one triploid, and one tetraploid strain. The triploid strain had the largest pollen on average, followed by the tetraploid strain. Both were significantly larger than the diploid pollen on average. The tetraploid result does not fit well with what is expected if ploidy level correlates with pollen grain size. However, the tetraploid pollen has a high (38%) coefficient of variance, indicating that the sizes varied greatly. This variance hints at meiotic error in that particular *S. rebaudiana* strain. In this instance however, appealing to meiotic error does not solve the issue. Meiotic error does not explain the lack of correlation between ploidy level and pollen grain size.

OBJECTIONS TO PLOIDY LEVEL/POLLEN SIZE CORRELATION

While pollen grain size has been frequently used to determine ploidy levels in the literature, there have been a few questions raised as to its value. Knight et al. (2010) attempted to correlate pollen size to ploidy level with the hope of determining ploidy levels in fossils and was unable to do so. Knight et al.’s analysis was heavily dependent on C-values to determine the ploidy level. These results creates two problems. The first is the possibility that C-value does not correlate with the ploidy level. Given that species with the same ploidy level in the same genus have widely different C-values (see examples in Suda et al., 2006), the assumption that a higher C-value is equivalent to a higher ploidy level cannot be substantiated, at least across all polyploid plants. The second problem comes from the tendency of polyploids to undergo gene loss over time (Buggs et al., 2009). Thus, while C-value might be a fair assessment of the ploidy level when the polyploid lineage is first formed, it becomes a much less useful assessment over time, something Knight et al.’s analysis did not take into account.

Another study looked at ploidy levels, C-value, and pollen grain size in *Plantago* (Wong and Murray, 2012). Like Knight et al., they found that there was no direct correlation between

pollen size and ploidy level. However, they found that C-value correlated directly with pollen size. Given the gene loss polyploids undergo over time, and the fact that *Plantago* is believed to be an older polyploid, this conclusion seems reasonable. It does, however, open the question of how reliable pollen size is as an indicator.

Other studies have noticed that pollen size is not always correlated to the ploidy level. Horandl et al. (2017) discovered that pollen size in *Ranunculus kuepferi* was not an indicator of the ploidy level. In fact, tetraploids produced the majority of the smallest pollen in the study, something that clearly seems to go against what has been found elsewhere. A similar pattern occurred in *Ramonda*, where, despite differing ploidy levels, there was no distinct difference in pollen grain size (Siljak-Yakovlev et al., 2008), though, regrettably, the authors do not provide their data for this claim. While in line with Horandl et al.'s results, these results do not mesh with other results presented above.

A study of *Ipomoea* species likewise found somewhat mixed results. The species with the largest mean pollen size was hexaploid, but the second and third species were diploid and the second largest, *I. purpurea*, was the only one with a margin of error less than $1\mu\text{m}$ (Siljak-Yakovlev et al., 2019). It is possible that selective pressures could have been involved in this difference, especially since multiple populations of the same species are not included in the analysis, but this explanation has not been tested. Interestingly, there was a rough correlation between 2C-value and pollen grain size across the *Ipomoea* species studied, making C-value the more likely explanation for pollen grain size in this genus.

Interestingly, Siljak-Yakovlev et al. (2008) also performed C-value analysis on three species of *Ramonda*. While it was largely consistent across species, there were outliers that did not overlap, even when chromosome numbers were the same. If, as mentioned above, pollen grain sizes were the same across ploidy levels, it adds strength to the argument that C-value, rather than ploidy level, correlates with pollen grain size.

DISCUSSIONS AND CONCLUSIONS

The above-presented data may seem very contradictory and it is at first glance. Some plants produce pollen that correlates nicely with ploidy level. Others produce pollen that does not correlate with the ploidy level. In many cases, there is an overlap between the sizes of the pollen at various ploidy levels. There are several possible explanations for this conflicting mass of data.

The first explanation is that there is a correlation between pollen grain size and ploidy level and that the counterexamples presented above are aberrations, or incorrect. This argument would be in line with the accepted understanding in the literature, along with the observation that cell size increases leaf size in *Populus* (Zhang et al., 2018). However, it fails to provide a comprehensive explanation for variations in pollen grain size. Essentially, this explanation is content with explaining most, but not all the data. As such, other options should be considered.

It is possible that the reason some higher ploidy level plants do not have enlarged pollen is due to a selection-imposed limit to growth. In other words, selection might impose a constraint on the increase in pollen size. Tate and Simpson (2004) propose this idea for plants of genus *Tarsa*. They argue that selection has caused tetraploid pollen to shrink in average size over time, to aid in reproduction. Such a selection constraint might be in place in some of the other, aforementioned

examples, explaining why pollen grains do not always correlate with ploidy levels, such as in Gungsa and Loerz's (2013) work with *Eragrostis*.

Given that it has been proposed that self-compatibility can help jumpstart many polyploid species once they form (Horandl, 2008), it makes sense that pollen grains of polyploids might be under selective pressure to still fit in the stigma of their parent plants. Being able to reproduce is key to the fitness of an organism, so selection should favor pollen grain sizes that will permit the plant to produce offspring. However, as these structures are often enlarged, selection should not strongly impact pollen grain size in a self-compatibility scenario.

The issue with the selection explanation is that self-compatibility has been shown to not be directly correlated with polyploidy (Mable, 2004). Therefore, when a new polyploid arises, it may be self-incompatible. If it is self-incompatible, selection should immediately favor smaller pollen grains to fit its diploid relatives. Yet in general, ploidy does seem to be very loosely correlated with pollen grain size. This would hint at least that selection may not be the driving force across the board in pollen grain size determination.

A third possible explanation is that pollen grain size does not correlate with the ploidy level at all. Instead, pollen grain size is correlated with C-value. Wong and Murray (2012) provide good evidence to support this argument. However, Dewitte et al. (2009) specifically stated that some members of genus *Begonia* have much different pollen grain sizes than other members of the genus with the same genome size. If pollen grain size correlated with C-value, a measure of genome size, then members of the same genus with the same genome size should have very similar pollen.

Another possible explanation is that there is no single universal factor undergirding pollen grain size. Instead, pollen grains size is controlled by a combination of selection constraints and C-value or ploidy level. This explanation seems perhaps the most plausible, but little work has been done to test it. Before concluding it makes sense, empirical testing must be done to ensure it has explanatory power.

There are, of course, limits to this analysis. Working with pollen grains is not easy, requiring careful staining and microscopy. Many species have not been subject to careful study as yet, and thus the results of this analysis must be considered highly tentative. However, given the current body of data, there seems to be reason to doubt that the correlation between pollen grain size and ploidy level is firm.

There is much work to be done in this area. As seen in this paper, pollen grain size does not seem to be explained by any single overriding factor. Further, many species seem to violate the assumption that there is a correlation between pollen size and ploidy level. This being the case, it would be wise for researchers to refrain from using it as an indicator for the ploidy level until a correlation between the two can be established. If it must be used, it would be wise to check the literature to ensure that the ploidy level does correlate to pollen grain size in closely related species. However, even this practice is not a guarantee as the aforementioned *Begonia* study pointed out (Dewitte et al., 2009). Ideally, other markers should be used to determine ploidy level, as pollen grain size does not appear to be as reliable as might be expected.

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