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BRIEF COMMUNICATION

A METHOD TO ESTIMATE POLLEN VIABILITY FROM POLLEN SIZE VARIATION[1]

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The mean diameter of viable pollen grains is approximately 13 μm greater than the mean diameter of inviable grains in *Mimulus guttatus*. We show that this difference is large enough to be detected by particle counters and that these machines can be used to obtain a rapid estimate of pollen viability. While requiring a separate calibration, a size-based statistic is also strongly correlated with pollen viability in *Collinsia verna*. These results suggest that statistics derived from the size distribution of pollen grains may provide an alternative to more labor-intensive methods for estimating pollen viability, particularly in cases where inviability results from inbreeding depression or hybrid failure.

Key words: *Collinsia*; inbreeding; *Mimulus*; particle counters; pollen viability.

The amount and quality of pollen produced by a flower is an important component of fitness. Pollen quality is often equated to pollen viability, i.e., the proportion of pollen grains that are viable. While viability can be measured in a number of ways (Stanley and Linskens, 1974; Heslop-Harrison, Heslop-Harrison, and Shivanna, 1984), a common method to assess both pollen load and pollen viability is by staining and direct count (e.g., Barrett, 1985; Dudash, 1991; Willis, 1999). Anthers are collected and suspended in a solution that contains a dye such as aniline blue. Viable or potentially viable grains absorb the dye while inviable grains do not. The pollen solution is then dispersed onto a hemacytometer slide and the number of stained (viable) and unstained (inviable) grains are counted using a microscope (Kearns and Inouye, 1993, pp. 94–96, 109–111). While this approach can yield highly repeatable estimates of pollen load and pollen viability, it is also very labor intensive.

Electronic particle counters provide an alternative to direct counts (Thomson, McKenna, and Cruzan, 1989; Harder, 1990; Young and Stanton, 1990). These machines count the number of particles within a given size range in a specific volume of solution. Assuming that all particles within this range are individual pollen grains, the resulting counts can be extrapolated to give accurate estimates for the total number of pollen grains produced by a flower. However, particle counters do not distinguish stained from unstained pollen grains and have thus been used primarily to determine total pollen load and not pollen viability.

Here, we suggest that variation in the size of pollen grains, which can be measured with particle counters, can be used to estimate pollen viability. This suggestion is motivated by direct measurements of pollen grain diameter in *Mimulus guttatus* (Scrophulariaceae; $2n = 28$). Figure 1 illustrates the size distributions of viable and inviable pollen grains from 15 *M. guttatus* plants. The anthers were collected from the first flower of each plant and placed in micro-centrifuge tubes containing 60 μL of aniline blue in lactophenol (see Willis, 1999). The tubes were vortexed to allow pollen to fully dissociate from the anthers, and a subsample was analyzed under a microscope fitted with an ocular micrometer. The diameter of up to 30 viable and 30 inviable pollen grains was determined for each of the 15 samples ($N = 343$ for viable and $N = 322$ for inviable). The mean diameter of viable grains is 41.9 μm (SD = 7.1 μm) and the mean diameter of inviable grains is 28.9 μm (SD = 4.3 μm). While the distributions are overlapping, they are clearly distinct. Most viable grains are greater than 35 μm, while most inviable grains are less (Fig. 1).

Can we exploit this difference to estimate the proportion of viable grains in a mixed sample? We addressed this question by comparing direct (manual) estimates of pollen viability with machine counts. We used a Coulter Counter Model Z1 dual (Coulter, Miami, Florida, USA). This machine counts the number of particles between two size thresholds (e.g., 10 and 25 μm) and the number of particles that are larger than the upper threshold (e.g., greater than 25 μm). A set of preliminary trials indicated that the Coulter Counter reads viable *M. guttatus* grains between 25 and 35 μm (these diameters are smaller than suggested by direct measurements [Fig. 1] for reasons that are unclear to us). We used this information to set the lower threshold at 10 μm and the upper threshold at 25 μm. These settings were based on the assumption that viable grains would be included primarily in the upper count (above 25 μm) while inviable grains would be included primarily in the lower count (between 10 and 25 μm).

Anthers were collected from the first flower of 31 *M. guttatus* plants and stored in aniline blue in lactophenol (as described previously). Each plant was derived from a distinct inbred line or from a cross between two inbred lines (as were the plants used to establish Fig. 1). Inbred lines were used

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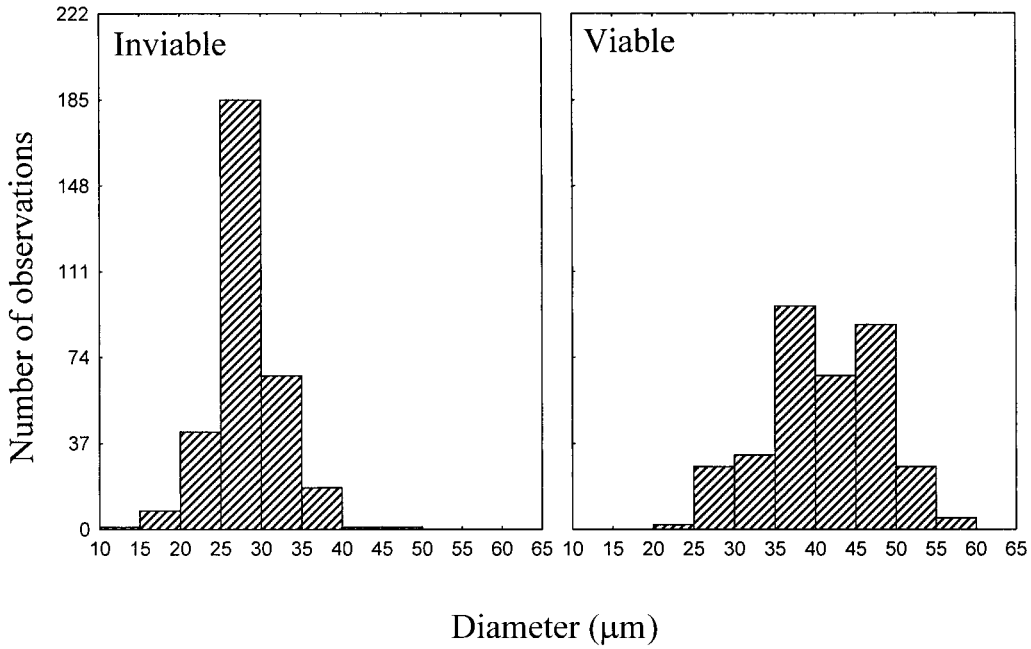

Fig. 1. The distribution of pollen grain diameter for inviable and viable grains.

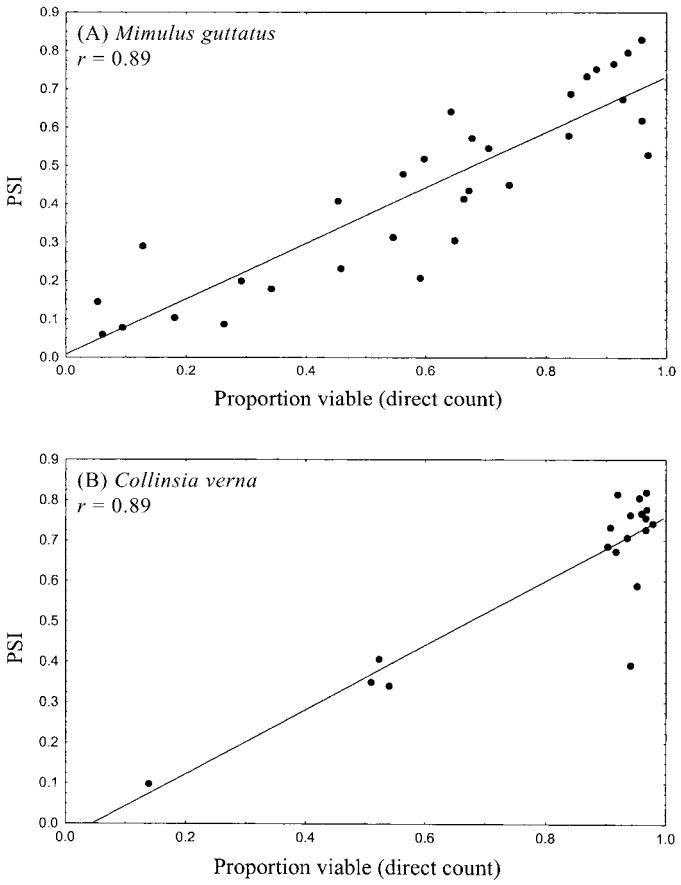

Fig. 2. The relationship between pollen size index (PSI, as determined by the particle counter) and proportion viable (as determined by direct count) for (A) *Mimulus guttatus* and (B) *Collinsia verna*. The Pearson product-moment correlation, *r*, is given for each comparison.

because they exhibit the full range of values for pollen viability, from near 0 to near 100% viable (Willis, 1999). Each sample was first subject to direct counts under a microscope on a hemacytometer. The proportion viable, as determined by this method, is given on the *x*-axis of Fig. 2. Each sample was then diluted into 4 mL of electrolyte solution and run through the Coulter Counter. The two counts (10–25 μm and above 25 μm) were recorded and used to calculate the pollen size index (PSI), i.e., the proportion of grains in the upper size category. The PSI for each sample is given on the *y*-axis of Fig. 2. For *M. guttatus*, the correlation between estimates is 0.89 and highly significant (Fig. 2A; $P < 0.001$).

To determine the potential generality of the method, we performed a similar analysis on pollen samples from *Collinsia verna* (Scrophulariaceae; $2n = 14$). Samples were collected from 19 plants, each with two generations of selfing in their ancestry. Each sample was stained and subject to direct count. The pollen grains of *C. verna* are approximately half the diameter of grains from *M. guttatus*. We thus set the Coulter Counter thresholds at 5 and 12 μm, respectively. Each sample was diluted in 20 mL of electrolyte solution and run through the machine. The PSI was calculated as the number of grains longer than 12 μm divided by the total count. For *C. verna*, the correlation between PSI and direct estimate is 0.89, also highly significant (Fig. 2B; $P < 0.001$).

An important consideration for these analyses is that we use a histological method to obtain our ''direct'' estimate of pollen viability. Aniline blue stains callose and is probably most useful for distinguishing fully formed pollen grains from those aborted during development. Developmental failure is likely to be the primary cause of pollen inviability in inbred plants or those derived from hybridization. In these cases, inviable pollen grains may be larger due to unreduced gametes (e.g., Calvacante, Schifino-Wittmann, and Dornelles, 2000) or smaller (Sharma, Singh, and Lal, 1996, who also found a positive correlation between pollen size and pollen viability). However, there are a number of nongenetic causes of pollen

inviability including pollen age and physical factors such as temperature and humidity. This class of factors would not be expected to generate predictable size differences between viable and inviable grains and detecting their effects would thus require alternative methods (e.g., Heslop-Harrison, Heslop-Harrison, and Shivanna, 1984).

The use of PSI as an indicator of pollen viability requires (1) that inviable grains have a smaller or larger mean diameter than viable grains and (2) that this difference is sufficiently large relative to natural size variation among viable grains. As species may exhibit substantial variation in the size of viable pollen (e.g., Barrett, 1985), the relationship between PSI (or alternative statistics based on the size distribution of pollen grains) and pollen viability will have to be evaluated on a case-to-case basis. However, the effort necessary to establish such a relationship (to construct graphs like Fig. 2), is small relative to that required to stain and directly count thousands of pollen samples.

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