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Practical and cost-effective method for the isolation of pollen grains from various sources

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ABSTRACT. Mock standards, with known concentrations and varied characteristics, when analyzed alongside unknown samples, can provide evaluation, optimization, and validation of scientific methods. Due to the scarcity of commercially available pollen grains, this study introduces a practical and cost-effective method for isolating pollen grains from various sources to be used in a mock pollen standard. Our method was tested using 25 diverse species derived from different sources, including herbarium materials (n, 20; dated from 1941 to 2006), commercially sourced (n, 2), and fresh hand-collected (n, 3), representing a wide range of taxonomic diversity and pollen morphology. Isolation with vacuum filtration, which can be completed in a basic laboratory, easily removes inorganic and organic debris while avoiding lysis of the pollen grains. This paper details the key steps in this method, including a) collecting suitable plant materials containing pollen grains from fresh and herbarium specimens and b) isolating, quantifying and storing the pollen grains. This approach is particularly beneficial for researchers in palynology, plant biology, forensic science and environmental monitoring, offering a practical way to isolate pollen grains for inclusion as a mock standard while preserving both morphological features and genetic material.

KEYWORDS: pollen, herbarium, pollen isolation, pollen mock standard

INTRODUCTION

Pollen has long served as a biological marker across diverse disciplines, including forensic science, environmental research, allergen monitoring, paleontology and archaeology. Despite variations in the sample matrix, identifying the plant species that produced the pollen is a crucial analytical step.

Identification of pollen to determine the source plant species can be done via examination of morphology, spectroscopic chemical analysis (Pappas et al., 2003), DNA analysis, and biochemical assessment using fatty acid profiles (Villagómez et al., 2023). Morphological analysis, which relies on features like polarity, symmetry, size, shape, apertures, and exine ornamentation, is the gold standard approach employed for pollen identification. However, this method has limitations, including that taxonomic resolution is often limited to the genus or family level, and microscopic identifications can be time-consuming with only a limited number of expert palynologists (Keller et al., 2015; Bell et al., 2016). These challenges bring the utility of DNA-based methods into focus. Specifically, pollen contains nuclear DNA and multiple copies of organellar DNA from both plastids and mitochondria (Parducci et al., 2005), making it a suitable candidate for DNA-based identification.

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In DNA-based approaches, particularly those utilizing next-generation sequencing (NGS), positive controls serve multiple functions: a) validate the functionality, efficacy, and operational effectiveness of the experiment, b) detect biases, c) assess sensitivity and quantitative accuracy, d) identify sequencing errors, and e) assist in detecting batch effects, potentially acting as a normalization factor. For instance, pollen grains from different species exhibit varying DNA extraction efficiencies depending on the extraction method (Swenson and Gemeinholzer, 2021; Devriese et al., 2024). By incorporating a pollen mock community with known species and pollen counts, researchers can determine the minimum and optimal detectable pollen quantities while identifying biases introduced at specific stages throughout the workflow.

There is currently a deficit of commercially available pollen grains from diverse species that could be used as a standard. Ideally, a "mock" pollen standard should encompass pollen grains that exhibit a variety of morphological attributes, such as size, shape, aperture and ornamentation. Furthermore, these grains should be taxonomically diverse and relevant to the focus of the research. Previous studies using DNA metabarcoding coupled with NGS to identify and quantify pollen in bulk environmental samples have included mock pollen standards (Kraaijeveld et al., 2015; Bell et al., 2019; Lang et al., 2019; Banchi et al., 2020). However, these standards were specifically prepared to either test their methods or answer a specific research question, and thus are not suitable as a broad pollen standard.

A variety of techniques for isolating pollen grains from plant tissues have been documented. The primary extraction technique in palynology is inherently destructive. The use of potent acids in acetolysis destroys the innermost cell wall of the protoplast and any genetic material within the pollen grains (Kelley et al., 2020). Consequently, acetolysis-free techniques have also been suggested. These range from traditional methods, such as gradient centrifugation (Forster and Flenley, 1993), to more practical homemade approaches utilizing mesh filters heat-sealed to the sample tubes (Kakui et al., 2020), and even extend to advanced technologies like chip sorting (Kasai et al., 2021). In this study, we focused on developing a practical and cost-effective method for isolating pollen grains from both fresh and preserved materials that could: a) be completed within a standard laboratory setting, and b) preserve morphological grain features and genetic material. This method is applicable to various pollen-related studies where developing mock standards is essential, including DNA-based analysis of mixed samples to qualitatively and quantitatively determine species composition, training machine learning models for automated pollen identification, and assessing DNA degradation under different preservation conditions. This paper will outline the following: a) selection of species to include in the mock standard, b) collection of pollen grains from herbarium materials, c) isolation of pollen grains derived from different sources, and d) quantification of isolated pollen grains and storage.

MATERIALS AND METHODS

A schematic of the developed isolation method is shown in Figure 1.

SELECTION OF SPECIES

A total of 25 species were chosen for the mock pollen standard (Supplementary File 1[1], 2[2]). Two species were commercially acquired: *Zea mays* L. (Corn; Carolina Biological Supply, Burlington, NC) and *Populus tremuloides* Michx. (Quaking aspen; Sigma-Aldrich, St Louis, MO), while three were hand-collected fresh anthers in Raleigh, NC from an *Ipomoea hederacea* Jacq. (ivy leaf morning-glory), an *Alstroemeria aurea* Graham (Peruvian-lily) and a *Camellia japonica* L. (Camellia) in bloom. The remaining 20 species were sourced from the North Carolina State University (NC State) Herbarium. Species selected for sampling from the herbarium included those possessing variable pollen morphology (primarily size, apertures, shape and ornamentation) and taxonomic diversity.

[1] Supplementary File 1. Overview of Pollen Samples. All species are classified under the division Magnoliopsida, with the exception of *Tsuga canadensis*, which falls under the division Coniferophyta. The size and Scanning Electron Microscope (SEM) ornamentation information were sourced from PalDat; exceptions denoted by "^" were sourced from Willard et al. (2004).

[2] Supplementary File 2. NCBI Taxonomy Information of Pollen Samples. All species are classified under the kingdom Viridiplantae, phylum Streptophyta, class Magnoliopsida with the exception of *Tsuga canadensis*, which falls under the class Pinopsida. The taxonomy information was extracted from the NCBI taxonomy data updated in April 2024.

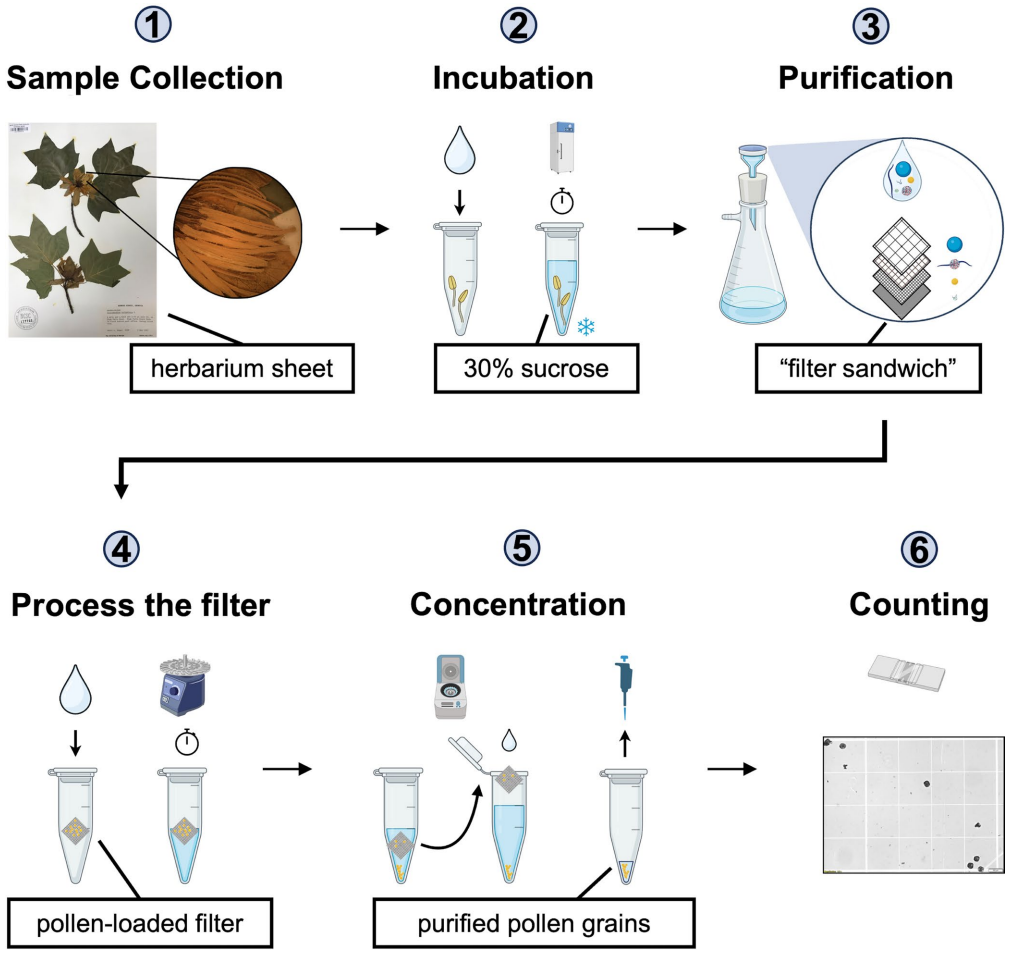

Figure 1. A schematic of the developed isolation process (Created with BioRender.com). **1**. Pollen-bearing tissues were collected from herbarium sheets; **2**. The collected tissues were stored in a 30% sucrose solution at 4°C overnight; **3**. Pollen grains, along with debris, underwent a vacuum filtration process utilizing the ‘filter sandwich’; **4**. The filter material, which contained the pollen grains, was re-suspended in a 30% sucrose solution and placed on a shaker to facilitate the release of the pollen grains; **5**. The samples were centrifuged at 15,000 g for 10 minutes. The excess solution was removed, leaving approximately 50 µl; **6**. A hematocytometer was used for counting the pollen grains

HERBARIUM SAMPLE COLLECTION

The collection of plant material from the NC State Herbarium adhered to the protocols of the NC State Herbarium and the “Herbarium Sample Collection Protocol” from the Florida Institute of Technology (Herbarium Sample Collection Protocol). Briefly, anthers or strobili were carefully harvested using forceps and scissors under the guidance of a dissecting microscope. Tools were sterilized between samples using a 10% sodium hypochlorite solution (NaOCl) followed by a 70% ethanol (EtOH) rinse to prevent cross-contamination. The collected tissues were placed into labelled 1.5 ml Eppendorf tubes and stored at room temperature in a sealed cabinet. Photographs of the herbarium sheets were taken for the purpose of record keeping.

POLLEN GRAIN ISOLATION

Pollen grains frequently coexist with a variety of debris, including remnants of plant tissue, dust, and unidentified organic and inorganic particles. In this study, samples collected from all 25 species, irrespective of their sources, underwent the isolation process. This process aimed to significantly reduce inorganic materials and remove organic plant debris. Furthermore, the isolation steps enhanced the accuracy of pollen counting and the reliability of subsequent analyses.

Resuspension and rehydration of pollen grains in sucrose solution

Various factors, such as pollen grain age, storage solution composition and concentration have been identified as the most critical determinants of pollen grain rupture (Rao and Ong, 1972; Duhoux, 1982; Siriwattanakul et al., 2019). To minimize pollen grain bursting, 30% (w/v) sucrose solution, equivalent to 30 g of commercially available sugar per 100 ml of ddH_2O, was used. Sterilization of the sucrose solution was achieved using syringe filters with a pore size of 0.22 µm. To ensure complete immersion of pollen-bearing tissues, a 1 ml volume of the 30% sucrose solution was dispensed into the original 1.5 ml Eppendorf tubes containing anthers or strobili collected from herbarium material. Given the herbarium specimens had been in a dried state for an extended period (up to 81 years; see Supplementary File 1), an overnight incubation at 4°C was used to ensure sufficient grain rehydration before proceeding with subsequent isolation steps.

Before adding 1 ml of sucrose solution to the commercially purchased pollen, the original storage solution was removed after a brief centrifugation. Sucrose solution (1 ml) was also added to the hand-collected pollen grains to facilitate downstream filtration.

Separation of pollen grains from unwanted particles via vacuum filtration

A vacuum filtration system was established within a biosafety cabinet using a MilliporeSigma glass base and stopper attached to a filtering flask. Nylon mesh filters, with mesh sizes of 80 (177 µm) and 500 (25 µm), were procured from commercial suppliers (Amazon, Seattle, WA). Both the mesh and Millipore 0.45 µm mixed cellulose ester filter were cut into 1.5 cm × 1.5 cm squares and subsequently sterilized by autoclaving at 120°C for 30 minutes. A piece of parafilm was cut to fit the base of the Millipore glass apparatus, and a 1.5 cm × 1.5 cm square was further cut out from this parafilm. This gap accommodated the other filter materials, ensuring proper suction during the vacuum filtration process. As default, a single 0.45 µm mixed cellulose ester filter was positioned over the parafilm opening to avoid grain escape. This was followed by the placement of a 500 (25 µm) mesh filter and then an 80 (177 µm) mesh filter. This configuration was referred as a "filter sandwich" (Fig. 1).

For each sample, the pollen grains suspended in sucrose solution were carefully dispensed onto the filter sandwich in a controlled, drop-by-drop manner using a pipette. To ensure complete recovery of pollen, an additional 100 µl of sterile 30% sucrose solution was used to rinse the residual pollen in the 1.5 ml tube and dispensed onto the filter sandwich. Using this "filter sandwich" method, small organic and inorganic particles would be trapped by the finest filter (in our case, a 0.45 µm filter), whereas larger debris would be captured by the coarser mesh filters (mainly with a 177 µm filter). Given the size of the pollen grains being isolated in this study was known for each species (Supplementary File 1), the filter with a mesh size smaller than the hydrated pollen size was carefully removed from the filter sandwich with sterile tweezers and stored in 1.5 ml Eppendorf tube at 4°C (short-term storage) for elution. For instance, if the smallest dimension of the pollen was 40 µm, only the filter with a 500-mesh (25 µm) was removed for the elution step. To avoid cross-contamination between samples, a new filter sandwich was made for each sample, and the vacuum filtration system was cleaned as follows: parafilm and the glass base were cleaned with 10% NaOCl, followed by 70% EtOH, and then rinsed with sterile ddH_2O. The parafilm was rotated 45° clockwise before processing the next sample. Once the parafilm returned to its original position, it was replaced with a new one after thoroughly cleaning the glass base again.

The 1.5 ml Eppendorf tube, which contained a mesh filter with the pollen grains of interest from the vacuum filtration step, was further processed to elute the grains into 800 µl of fresh 30% sucrose solution. The tube with the filter and the sucrose solution was first placed on a Vortex-Genie® 2 mixer (Scientific Industries, Bohemia, NY) and a Bead Genie™ Horizontal Plastic Clip microtube holder on a low setting for 30 minutes to facilitate the gentle release of pollen grains from the mesh filter into the solution. Following incubation and shaking, the sample was centrifuged at 10,000 g for one minute. The filter was then repositioned at the tube's apex, with a minor corner intentionally protruding. An additional 200 µl of sterile 30% sucrose solution was carefully applied onto the filter, and subjected to centrifugation at 15,000g for 10 minutes. This extra step was employed to ensure: a) the thorough removal of all pollen grains adhering to the filter, b) enable the filter's easy removal from the tube, and c) pellet the pollen grains at the bottom of the tube. After centrifugation, pollen grains were seen at the bottom of the tube. All but ~50 µl of the sucrose solution was carefully removed using a pipette and discarded in order to concentrate the grains prior to counting and storage. The pellet of pollen grains was resuspended in the solution through gentle manual agitation, as opposed to the use of forceful pipetting, which may disrupt the integrity of the grains.

COUNTING OF ISOLATED POLLEN GRAINS

A Neubauer hematocytometer was utilized for counting the isolated pollen grains from each sample. First, the isolated pollen grains from each sample were diluted at a ratio of 1:5. Briefly, the process involved mixing 2 µl of the sample with 8 µl of 0.4% trypan blue to achieve a final volume of 10 µl and mixed thoroughly by gentle pipetting. The entire 10 µl was subsequently loaded onto one section of the hematocytometer with care. Pollen grains in four large squares, each corresponding to an area of 1 mm^2, were counted. The average pollen count derived from these squares was multiplied by the dilution factor to ascertain the final concentration of isolated pollen grains for each sample. Microscope images were taken for the purpose of counting and record keeping.

STORAGE OF ISOLATED POLLEN GRAINS IN SUCROSE SOLUTION

For optimal preservation of pollen grains for both morphological and DNA-based analyses, isolated pollen grains were stored in a 30% sucrose solution at –20°C for long-term storage and at 4°C for short-term storage.

RESULTS AND DISCUSSION

Isolation of pollen grains was carried out on 25 unique species, spanning 24 genera, 21 families, 20 orders, 3 classes and 2 divisions (Supplementary File 1, 2). The pollen grains from these species exhibit a range of shapes and sizes, and could be combined to create a diverse mock pollen standard. The grain sizes fell into three primary categories: small (10–25 µm), medium (26–50 µm) and large (>51 µm). The distribution of sizes among the 25 species was as follows: small (4/25), medium (13/25), and

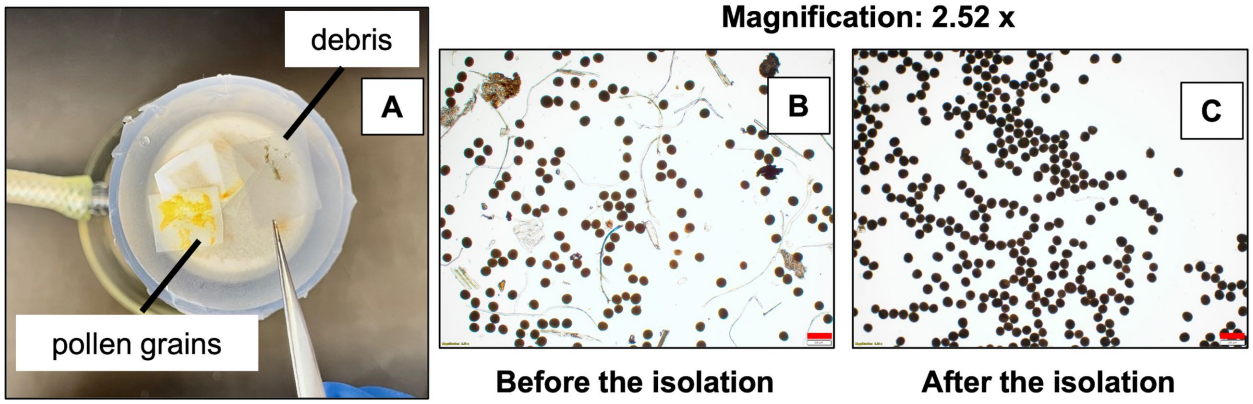

Figure 2. Demonstration of debris removal from the pollen grains of *Zea mays* L. (Corn). **A**. The vacuum filtration setup, equipped with filter layers of varying sizes, captures debris or pollen grains; **B**, **C**. Images, captured by IX83 inverted microscope and cellSens™ software (Olympus Corporation, Center Valley, PA), illustrate *Zea mays* L. samples before (B) and after (C) the isolation process. The images were taken at a magnification of 2.52x, with a red scale bar representing 200 µm

large (8/25). The age of the herbarium species collected for this study spanned from 1941 to 2006. Figure 2 demonstrates the "filter sandwich" vacuum filtration technique (Fig. 2A) applied to *Zea mays* (Corn), with microscope images showing the removal of debris before (Fig. 2B) and after isolation (Fig. 2C). The technique effectively retains intact pollen grains while removing most debris. The filter sizes selected for this study optimize the yield of isolated pollen grains while permitting a minimal amount of small debris.

While this method worked effectively for the species examined and collected in this study (Fig. 2), several steps should be considered by users prior to implementing it in their laboratories. Firstly, conducting a preliminary isolation test is crucial when using the vacuum filtration method to isolate pollen grains. This is especially important for pollen grains with rough, spiky ornamentations, which may adhere to mesh filters larger than their actual size. Similarly, pollen grains with non-spherical shapes may bypass smaller filters, despite being nominally larger, leading to potential pollen escape. To mitigate these issues, employing a diverse array of filter sizes and/or multiple filters of the same size is advisable, with the exact number of filters required being dependent on the shape, size, and ornamentation of the hydrated pollen grains. Secondly, filter size selection should be strategically based on the size of both the targeted pollen and any unwanted particulates. Employing additional filters with pore sizes larger than the pollen grains is effective for removing larger particles, while smaller debris may necessitate the opposite approach. Furthermore, it is important to perform a microscopic examination of the used filters after isolation. This step is important for evaluating the isolation's efficacy and observing how the grains react to the vacuum pressure applied. Adjusting the vacuum pressure with a gauge can enhance the efficiency of the isolation process. Finally, while the centrifugation speed and time reported in this study (15,000 g for 10 minutes) did not damage the morphology of pollen grains, it is suggested that a preliminary test with a subsample of pollen grains be conducted to determine the optimal centrifugation speed and duration before processing the entire sample. This could be achieved by mounting a subsample of pollen grains pre- and post-centrifugation on a slide for visualization with a microscope, to discern whether the centrifugation conditions tested are appropriate.

Manual counting to determine the number of isolated grains with a hemocytometer was selected over automated techniques (e.g. cell counters and/or image analysis with ImageJ/FIJI Schindelin et al., 2012) in this study, despite their advantages in reducing human intervention and saving time. This choice was made because of the small sample size and the low concentration of pollen grains in most samples. If the user has sufficient pollen, it is recommended to use larger volumes for subsampling the isolated pollen and conduct multiple counts as technical replicates to diminish potential random and systematic errors. It should be noted that the hematocytometer method may be unsuitable for certain types of pollen due to clumping and uneven distribution in a solution (Ali et al., 2022). However, these issues were not encountered with the

hydrated pollen grains from the plant species isolated in this study.

Researchers using this method to isolate pollen for downstream DNA analysis should be aware that studies have reported an approximate 1% annual decrease in the amount of extractable DNA in herbarium specimens (Erkens et al., 2008). While the preservation of DNA in herbarium material (including pollen grains) is primarily influenced by the methods used during sample preparation, such as drying and chemical treatment, preference should be given to more recently collected material for isolation if possible (Erkens et al., 2008; Shepherd, 2017). For both short-term and long-term storage of isolated pollen grains, the growth of fungi and mold in the sucrose storage solution can be inhibited by using an antimycotic, such as Thiabendazole. However, if subsequent DNA analysis is to be performed, the potential interference of the antimycotic should be assessed beforehand.

CONCLUSION

In this study, a practical and cost-effective method was developed to isolate pollen grains that were sourced from herbarium specimens, commercially purchased and fresh hand-picked samples. The method does not involve the use of harsh chemicals or expensive equipment, which not only preserves the morphological features and genetic material of the grains, but is also an environmentally friendly approach. Isolated pollen grains using this method from desired species could be combined to create a mock standard for inclusion in either morphological (e.g. analyst proficiency testing (Sikoparija et al., 2017)) or DNA-based identification experiments. In an ongoing study utilizing mock standards prepared with this pollen isolation method, we successfully sequenced the pollen species within these standards to validate experimental functionality, efficacy and operational performance. This approach enabled us to determine the species-specific minimum pollen count required for detection, assess quantification differences between nuclear and plastid markers, and identify biases introduced by soil addition. In addition to positive controls, contamination can occur at any stage of the protocol, particularly in DNA sequencing, including specimen collection, pollen isolation using this method, and downstream processing. To identify contamination sources and ensure reliable results, researchers should incorporate negative controls at each step and adjust their analyses accordingly.

Future studies could explore the use of various filter sizes to increase isolation efficiency, investigating practical methods for separating pollen grains from similarly sized debris, and differentiating between pollen grains of identical size from distinct species. Furthermore, the creation of automated isolation processes to minimize time and human intervention presents a promising research direction.

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ADDITIONAL INFORMATION

CONFLICT OF INTEREST. The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this article.

AUTHOR CONTRIBUTIONS STATEMENT AND ETHICS. OSK performed the formal analysis, curated the data and visualized the results. Both OSK and KAM conceptualized the study, conducted the investigation and wrote the manuscript. OSK and CB developed the methodology. KAM and BJC provided supervision. KAM, BJC and CB administered the project and acquired the funding. All authors reviewed, edited and approved the manuscript.

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DATA AVAILABILITY STATEMENT. No genetic data or programming code was generated for this study. All sample data and information are available with this paper.

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