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# Assessment of pre-PCR whole genome amplification of single pollen grains using flowering dogwood (Cornus florida)

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"Assessment of pre-PCR whole genome amplification of single pollen grains using flowering dogwood (*Cornus florida*)"

A thesis submitted in partial fulfillment of the requirements for the degree of Master of Science in Biology at Virginia Commonwealth University.

by

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# Table of Contents

Page

# List of Tables

Page

# List of Figures

Page

# Abstract

ASSESMENT OF PRE-PCR WHOLE GENOME AMPLIFICATION OF SINGLE POLLEN GRAINS USING FLOWERING DOGWOOD (*Cornus florida*)

By Candace Elizabeth Dillon, B.S.

A thesis submitted in partial fulfillment of the requirements for the degree of Master of Science in Biology at Virginia Commonwealth University.

Virginia Commonwealth University, 2009

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Studies of gene flow in natural plant populations often focus on either historical or abiotic dispersal methods (e.g. wind, water, gravity), but there is little information available on contemporary, animal-mediated pollen dispersal patterns. Emerging molecular laboratory techniques allow unprecedented insights into spatial patterns of pollen-mediated gene flow. However, to date, technical challenges have limited their widespread application. The genome of a pollen grain can be amplified via whole genome amplification (WGA) prior to traditional amplification via polymerase chain reaction (PCR) to prevent the stochastic effects associated with low copy number amplification. Even still, WGA can suffer from low success rates or poor repeatability. The present

study examined the extent to which WGA can be used to aid in understanding insect-mediated pollen flow in *Cornus florida* (flowering dogwood) within Virginia Commonwealth University's Inger and Walter Rice Center for Environmental Life Sciences. Initial amplification of DNA isolated from frozen grains was successful, until the pollen had been stored longer than 120 days at -20ºC. After this time point, the PCR targets failed to amplify. The percent success of downstream PCR amplification on fresh pollen grains varied from 20% to 100%, with an average of 62% success. The addition of a common molecular crowder, polyethylene glycol, produced consistent amplification, regardless of input DNA concentration and eliminated the need for triplicate samples. Successful pollination and subsequent reproduction of flowering plants has a substantial ecological and agricultural importance that warrants increased understanding into how insects move pollen across the landscape. Determining the haploid profiles of a single pollen grain will allow scientists to elucidate dispersal patterns of pollen grains and track the movement and efficiency of biotic pollinators.

# INTRODUCTION

*Gene Flow*

The processes that move genes from one individual to another within a plant population can affect both genetic structure and individual fitness. Unrestricted gene flow from one population to another allows for increased allelic diversity at the local scale, potentially increasing the ability to survive changing environmental conditions, including natural catastrophes, disease, and habitat destruction. (e.g. Sork & Smouse, 2006). Plants rely on both pollen and seed dispersal to carry their genes to the next generation. However, the pollen vector is the predominant dispersal mechanism for most species (Ennos, 1993), and has the greatest potential to impact population genetic structure. Although there are currently several approaches to studying how historical gene flow has shaped population structure over many generations, far less work has been done to analyze real-time pollen and seed dispersal (Sork & Smouse, 2006).

By tracking contemporary dispersal of individual pollen grains, the movement of all potential sets of genes that could be passed on to the next generation can be determined, without having to wait for offspring production. Indeed, when analyzing sets of offspring, there has already been a sieve of selection that has changed the set of potential offspring genotypes (e.g. Ellstrand, 1986; Gibson & Hamrick, 1991). Studies of pollen dispersal have primarily focused on abiotic wind-mediated dispersal, wherein pollen is modeled as a

random diffusive process, independent of plant density (Levin & Kerster, 1969; Smouse *et al*., 2001). However, most angiosperms do not disperse pollen passively, but have evolved complex structures that ensure animal-mediated dispersal. It is not clear if the evolution of these biotic vectors produces the same patterns of pollen movement as abiotic dispersal (Chan *et al.*, in review). For most animal-pollinated plants, gene flow is determined by the flight distance of the pollinator, flight frequency, behavior, and direction of consecutive flights (Levin *et al.*, 1971). If pollinating animals do not move genes in a diffusive manner across a landscape in the same way as abiotic agents of dispersal, it is necessary to develop models that can predict animal movements and their responses to ecological heterogeneity to quantify contemporary gene flow within and among natural populations.

*Pollen as a Natural Marker*

Insects participating in pollination are usually studied by using mark-recapture techniques that involve marking with artificial dyes or radioactive/rare elements. Unfortunately, artificial dyes and markers often kill the insect or disrupt normal behavior, and these techniques require marking an immense number of insects since recapture rates are usually very low (Del Socorro & Gregg, 2001; Jones *et al.*, 2001). Due to pollen's durability and easily identifiable outward characteristics unique to each plant species, pollen is an ideal surrogate for studying many aspects of insect behavior, including foraging and migration patterns, as well as if they visit one or many plant species (Jones *et al.*, 2001). When insects feed on the pollen or nectar of a flower, their bodies can become covered in pollen, allowing scientists to track their movement based on the pollen they

have inadvertently acquired on their foraging trips (Jones *et al.*, 2001). Pollen is beneficial because it is a natural marker that removes the human element from the process, allowing the insect to mark itself (Del Socorro & Gregg, 2001; Jones *et al.*, 2001). However, some pollen may be retained only for short periods of time. Although this can help to track recent or local movement, it is not helpful for long-term migration studies (Del Socorro & Gregg, 2001). More recently, Hudson *et al.* (2001) employed the use of pollen tagged with green fluorescent protein to monitor the movement of pollen produced by transgenic plants. Although this technique can potentially distinguish between pollen from engineered versus wild crops, the movement of individual genes via insect-pollinators can not be elucidated because all transgenic individuals carry the same marker (Hudson *et al.*, 2001).

Conversely, with the use of new whole genome amplification technology, insect movement can be tracked by conducting PCR analysis of microsatellite loci or other rapidly evolving marker systems from single pollen grains. If multilocus genotype spectra are available for the reference plant population, the nuclear haplotypes of these single pollen grains can be used to determine the location from which each pollen grain originated, using traditional paternity-type approaches. This is useful for not only ascertaining insect movement, but also the movement of the genes they transfer within a plant population.

*Low Copy Number DNA Samples*

The small amount of DNA available in the three cells comprising a pollen grain (~0.01 ng DNA; Figure 1) poses a dilemma for researchers using traditional PCR because it generally requires at least 1 ng of pure template DNA. Recovering a full STR profile from low copy number DNA samples (<0.1 ng of DNA) is often not possible with the sensitivity limits of routine DNA analysis (Gill *et al.*, 2000; Hanson & Ballantyne, 2005). Some have proposed simply increasing the cycle times of PCR to increase total DNA quantity from limiting samples (Gill, 2001; Kloosterman & Kersbergen, 2003), but this resolution often leads to decreased efficiency of the *Taq* enzyme used for amplification of templates, allelic drop-in and drop-out, stutter, and preferential amplification of one allele over another (Saiki, *et al*., 1988; Gill *et al.*, 2000; Gill, 2001). To overcome this obstacle, the areas of medical diagnostics and forensic biology have explored the use of whole genome amplification (WGA) to analyze limited clinical specimens and irreplaceable forensic samples often encountered in these fields. This technique has been shown to increase the amount of starting DNA template prior to downstream applications like short tandem repeat (STR) marker analysis or sequencing (e.g. Luthra & Medeiros, 2004; Ballantyne *et al*., 2007).

Ideally, a WGA procedure would amplify the genome faithfully and in its entirety, without any amplification bias or loss of alleles (Lasken & Egholm, 2003). This is quite a challenge, especially when other factors that cause preferential amplification like the length and GC content of the sequence are taken into account (Lasken & Egholm, 2003). Other important characteristics to consider when choosing a WGA method is the amount

and size of the products produced, the ability to use the protocol on single-cell starting material, and the fidelity of the DNA polymerase being utilized (Lasken & Egholm, 2003). Several methods of WGA are available, including primer extension pre-amplification (PEP), improved-primer extension pre-amplification (I-PEP), degenerate oligonucleotide primed (DOP) PCR, long products from low DNA quantities degenerate oligonucleotide primed (LL-DOP) PCR, and multiple displacement amplification (MDA). At present, there is not a consensus in the literature regarding the best method, perhaps because each of these methods have different strengths and weaknesses in terms of the amount of input DNA required, quantity and length of amplification products, unbiased and faithful replication of the entire genome, and utility and ease of allelic interpretation in downstream applications. Despite this lack of consensus, the most widely used methods seem to be DOP, I-PEP, and MDA.

*Whole Genome Amplification Methods*

Degenerate oligonucleotide primed (DOP) amplification is a PCR-based method that involves the use of *Taq* DNA polymerase and a proofreading DNA polymerase, partially degenerate oligonucleotide primers, and initial PCR priming cycles with low annealing temperatures, followed by subsequent cycles of more specific priming at higher temperatures (Barbaux *et al.,* 2001). DOP can require as much as 10 ng of DNA (Barbaux *et al.,* 2001) and has been shown to exhibit severe amplification bias and allele drop-out when used on single cell templates (Wells *et al.,*1999; Dean *et al.*, 2002; Paunio *et al.*, 1996; Kittler *et al.*, 2002). This method produces products <2 Kb in length (Barbaux *et*

*al.,* 2001; Hanson & Ballantyne, 2005) and it has been recommended that downstream analysis of DOP products be limited to below 1 Kb (Barbaux *et al.,* 2001).

Improved-primer extension pre-amplification (I-PEP), another PCR-based method, is carried out using a modified version of PEP (Zhang *et al*.,1992), which involves using a different lysis buffer, a mixture of *Taq* and *Tgo* DNA polymerases, and an additional elongation step (Dietmaier *et al*., 1999). I-PEP consistently outperforms DOP (Dietmaier *et al*., 1999; Hawkins *et al.*, 2002), produces even larger fragments than MDA (Hanson & Ballantyne, 2005; Sun *et al*., 2005), and, after optimization, was found to produce full STR profiles from as little as 5 pg of DNA (Hanson & Ballantyne, 2005). This technique has been applied to amplification of single sperm cells (Zhang *et al.*, 1992) because allele drop-out is not as problematic in haploid cells (Lasken & Egholm, 2003).

Multiple displacement amplification (MDA) is an isothermal method that uses of random hexamer primers and *Φ29* polymerase (See Figure 2 for the mechanism of MDA) (Jiang *et al*., 2005). *Φ29* polymerase is especially useful for whole genome amplification because of its processivity, allowing it to bind to the template DNA for long periods of time in order to synthesize long products (up to 70 Kb), until it is displaced from the template by an adjacent newly synthesized strand (Dean *et al*., 2002).

It is generally agreed that MDA produces a higher product yield than I-PEP (Hanson & Ballantyne, 2005; Sun *et al.*, 2005; Barber & Foran, 2006) but it has less specificity (Sun *et al.*, 2005), and often produces extraneous amplicons in post-MDA amplifications (Sun *et al.*, 2005; Barber & Foran, 2006). MDA gives more complete coverage and has less amplification bias due to the large products produced, with an

average of 12 Kb up to 100 Kb (Dean *et al.*, 2002), and elimination of the sequence effects (i.e. GC content of the template) associated with thermal cycling (Lasken & Egholm, 2003). The proofreading activity of *Φ29* produces full genome coverage with little amplification bias (Dean *et al*., 2002). Others have not found great success with MDA, perhaps because it exhibits decreased efficiency with <10 ng of DNA (Dickson *et al*., 2005; Hanson & Ballantyne, 2005).

Sun *et al.* (2005) found that I-PEP and MDA exhibit similar sensitivities when amplifying 10 pg DNA, with both producing allelic drop-out and allelic imbalance with such low quantities of starting material. Both protocols also produce high molecular weight DNA in negative controls containing no template DNA (Dean *et al*., 2002; Sun *et al.*, 2005; Barber & Foran 2006). These exogenous products, presumably from amplification of the random primers, have the potential to inhibit amplification of the target DNA (Barber & Foran, 2006). Based on the aforementioned characteristics of each method, I chose to use MDA in my research, due to the high product yield, most complete genome coverage, and ease of amplification via a commercially available kit. To date, there are no published reports of the use of pre-PCR amplification via the MDA reaction on single pollen grains.

*Amplifying DNA from Single Pollen Grains*

Several successful attempts have been reported that obtained genotypes from single pollen grains. The best technique available, however, is still under debate. The main difficulties involve physical isolation of a single pollen granule, lysing the rigid pollen

exterior to release the DNA, and obtaining accurate genotypes. The lysis method proposed by Matsunaga *et al*. (1999) requires a UV-laser microbeam, which is not available in most laboratories due to the prohibitive cost. Ziegenhagen *et al*. (1996) used pollen germination prior to PCR, circumventing the need for chemical lysis. Germination has been demonstrated with both fresh and frozen pollen, however, frozen samples of *Cornus florida* exhibit germination rates between 50% and 60% of freshly collected pollen (Craddock *et al*., 2000). The chemicals used for germination may introduce inhibitors of PCR (Aziz *et al*., 2005), as well as make it difficult to retrieve single pollen grains from the germination media. Matsuki *et al.* (2007) suggested a protocol better suited for high-throughput analysis, where pipette tips are used to crush pollen grains in a lysis buffer, followed by PCR. Others have used whole genome amplification prior to PCR of single cells to increase the amount of starting template (Zhang *et al*., 1992; Matsunaga *et al*., 1999). Theoretically, this produces reduced levels of stochastic effects that are usually associated with PCR from low quantity DNA templates, resulting in an easier and more accurate interpretation of genotype results (Ballantyne *et al*., 2007).

*Research Goals*

The goal of this project was to use the MDA method of pre-PCR whole genome amplification to produce accurate genotypes from single pollen grains of *Cornus florida*. By removing and genotyping these grains from insect pollinators sampled on a landscape where the multilocus genotype of all potential fathers is known, such genotypes would promote estimation of distance distributions from the locations where the insects were

collected and the donor locations of the collected pollen load, and ultimately contribute to an understanding of the ecological factors that influence insect movement and gene flow via insect pollinators.

Initial PCR reactions were only briefly successful on frozen grains collected in April 2008 and insect pollen loads had been stored too long to be used as template DNA once optimization had been completed. Amplifications performed with fresh pollen samples could not be attempted until the next pollination season, April 2009. This warranted assessment of the MDA reaction on serially diluted DNA that had been extracted from *C. florida* leaf tissue to determine if the reaction was at least theoretically possible with the low copy number levels of DNA found in a single pollen grain.

# METHODS AND MATERIALS

*Study Species*

*Cornus florida*, commonly known as flowering dogwood, is a member of the family Cornaceae, and is one of the most recognized and highly valued ornamental trees in North America (Craddock *et al*., 2000). The natural range of this species covers the eastern United States, extending to Maine in the north, to Florida in the south, and as far west as Texas. Remnant populations exist in Nuevo Leon and Veracruz, Mexico (Little, 1979). *Cornus florida* is associated with the understory of temperate deciduous forests and its flowers are visited by a variety of insects, including bees, beetles, flies, and butterflies (Eyde, 1988; Mayor *et al*., 1999).

Dogwood is among the earliest flowering species in eastern North American forests. As a result, this species, although considered to have a generalist pollinator community, has a reduced number of insect species from which to draw pollinators. Conversely, insects that are active in early spring do not have many other options for pollen and nectar sequestration. Both of these factors make dogwood a particularly good species to use in studying animal-mediated pollen dispersal.

*Sample Collection*

Pollen and insect samples were collected from Virginia Commonwealth University's Inger and Walter Rice Center for Environmental Life Sciences, located in Charles City County, Virginia. This 138-hectare site along James River affords an opportunity for research on a variety of ecological habitats. Since a majority of the site is covered in forest, native *C. florida* can be found throughout the understory of the mixed pine-oak woodlands. Insect visitors of flowering dogwood were captured on inflorescences by hand in plastic vials. All sampled insects were stored on ice (approximately 1 hour) until returning to VCU. For each insect captured, the location was recorded with a GPS unit. Whole inflorescences were also collected for raw pollen samples that had not been harvested by insects. Insects and pollen were stored until further analysis at -20ºC. All collections were made during varying times of the day in April 2008 and 2009, with the goal of capturing a broad sample of the pollinator community.

*Pollen Grain Preparation*

Individual anthers from collected flowers were immersed in 50 µl of a sterile 20% sucrose solution, vortexed vigorously to release grains from the anther, and allowed to germinate for 24 hours. The solution was then pipetted onto a sterile slide, spread into a thin film, and allowed to dry. With the aid of a dissecting microscope, individual pollen grains were removed from the surface of the slide using a pipette tip intended for 1-10 µl volumes. The end of the tips were cut off and transferred to separate 0.2 mL PCR tubes, a method similar to that of Matsuki *et al.* (2007).

*Serially Diluted DNA Samples*

DNA samples were extracted from *C. florida* leaf tissue using a DNeasy® 96-well extraction kit (Qiagen, Valencia, CA). Quantiation of DNA extracts was carried out using a NanoDrop™ 8000 spectrophotometer (Thermo Scientific, Wilmington, DE) and serially diluted with molecular grade $dH_20$ (Invitrogen Corporation, Carlsbad, CA) to final concentrations of 10, 1, 0.5, 0.1 and 0.01 ng/μL.

*Whole Genome Amplification*

Due to the extremely sensitive and non-specific nature of the Multiple Displacement Amplification (MDA) reaction, measures were taken to avoid cross-contamination. Pollen preparation was carried out in a separate pre-amplification dedicated area. Prior to each use, all lab surfaces and pipettes were wiped with a 10% bleach solution, followed by 75% ethanol, and pipettes were sterilized by irradiating under ultra violet light for 15 minutes in a Laminar flow hood. Frequent glove changes were utilized to avoid contamination from lab surfaces or personnel.

Whole genome amplification via MDA was carried out using the GenomiPhi™ DNA Amplification Kit (GE Biosciences, Piscataway, NJ) following the manufacturer's instructions. The amplification profile was as follows: 9 μL of sample buffer and 1 μL of DNA template were heated to 95ºC for 3 minutes then cooled to 4ºC. Once denatured, 9 μL of reaction buffer (containing dNTPs and random hexamer primers) and 1 μL *Φ29* enzyme were added and the mixture was incubated at 30ºC for two hours. The DNA polymerase was inactivated by heating to 65ºC for 10 minutes, and samples were held

indefinitely at 4 ºC. Both positive (1 ng/ µL template included with kit) and negative controls ($dH_20$ in place of template DNA) were carried out with each set of reactions. Omission of the initial denaturation step of the MDA reaction was also tested on long-term stored pollen grains as suggested by Dean *et al.* (2002).

The effectiveness of the addition of a common molecular crowding agent (PEG 400; USB Corporation, Cleveland, OH) to the GenomiPhi™ kit was assessed. PEG was added to a 2.5% (v/v) final concentration in the MDA reaction as suggested by Ballantyne *et al.* (2006) following empirical optimization. Following WGA, amplified products were purified via ethanol precipitation as recommended by the manufacturer. Quantiation of WGA products was carried out using a NanoDrop™ 8000 spectrophotometer (Thermo Scientific, Wilmington, DE). Visual confirmation of all post-amplification reactions was performed on a 1% agarose gel run at 80 V (55 mA) for 120 minutes, followed by visualization by staining with ethidium bromide and photo illuminated under ultra violet light.

*PCR Amplification*

Dinucleotide microsatellite repeat loci were amplified via PCR using the WGA product as template DNA on an MJ Research PTC-100 Peltier Thermal Cycler (GMI, Ramsey, MN). The reaction components were as follows: 3 µL $dH_20$, 2 µL 5X Colorless GoTaq® Flexi Buffer (Promega, Madison, WI), 1.6 µlL dNTP mix diluted to 1.25mM final concentration (Bioline, Taunton, MA), 0.8 µL $MgCl_2$ at 25mM (Promega Madison, WI), 0.5 µL 100X Purified BSA at 10 mg/mL (New England BioLabs, Ipswich, MA), 0.5

μL of Cf-N10 (Cabe & Liles, 2002; Table 1) primer mix (10 μM each), 0.1 μL GoTaq® Flexi DNA Polymerase (Promega, Madison, WI), and 1 μL of WGA product, as suggested by Ballantyne *et al.* (2007). Both positive (50 ng of extracted leaf DNA) and negative controls ($dH_20$ in place of input DNA) were included with each set of reactions.

The PCR cycle parameters were as follows: 94ºC for 2 minutes, followed by 40 cycles of 94ºC for 30 seconds, 57ºC (primer specific) for 30 seconds, 72ºC for 30 seconds, followed by a final extension step ay 72ºC for 5 minutes, and then held indefinitely at 4ºC. All PCR amplification treatments were performed in triplicate to ensure consistent amplification. Visual confirmation of all products was carried out on a 1 % agarose gel, run at 80 V (55 mA) for 180 minutes, followed by visualization by staining with ethidium bromide and photo illuminated under ultra violet light.

*Statistics*

Statistical tests were conducted in SAS® version 9.1 (SAS Institute Inc.) and evaluated at the 0.05 significance level. The mean WGA product yield for the 10, 1, 0.1, 0.05, and 0.01 ng/μL diluted DNA samples were analyzed using a one-way Analysis of Variance (ANOVA). The mean WGA product yield for non-PEG and PEG treatments and were compared using a one-way ANOVA.

# RESULTS

*Frozen Single Pollen Grain Amplification*

The MDA reaction yielded WGA product from frozen single grain templates and initial tests of downstream STR amplification was successful, albeit with extraneous amplification products (Figure 3). However, after the pollen had been stored longer than 120 days at -20ºC, the MDA reaction yielded reduced WGA product (Figure 4), and subsequent PCR reactions were unsuccessful (Figure 5).

*Fresh Single Pollen Grain Amplification*

The total MDA product yield (reported as the mean ± standard deviation) from fresh single pollen grains was 25.7 µg (± 12.3), with the lowest observation of 12.5 µg and the highest observation of 82.9 µg (N=50). The percent success of downstream PCR amplification on fresh grains varied from 20% to 100%, with an average of 62% success (Table 2, Figure 6).

*Effectiveness of Molecular Crowders on Serially Diluted Leaf DNA*

The total MDA product yield (reported as the mean ± standard deviation) for each MDA without PEG treatment was 18.8 µg (± 5.3), 27.2 µg (± 5.7), 30.7 µg (±13.0), 19.7 µg (± 4.15), and 21.8 µg (± 5.3) from an input of 10, 1, 0.5, 0.1. and 0.01 ng, respectively

(N=4 per treatment; Figure 8). No statistically significant differences existed between input treatments ($p$ = 0.2700). MDA reactions performed without the addition of PEG yielded variable results (i.e. only one or two of the triplicate samples successfully amplified the allele) in downstream amplification via PCR (Figure 9).

The total MDA product yield (reported as the mean ± standard deviation) for each MDA with PEG treatment was 19.7 µg (± 2.7), 17.1 µg (± 2.8), 18.1 µg (± 1.7),17.5 µg (± 3.0), and 18.2 µg (± 3.8) from an input of 10, 1, 0.5, 0.1. and 0.01 ng, respectively (N=4 per treatment; Figure 8). No statistically significant differences were observed between input treatments ($p$ = 0.8348). MDA reactions performed with the addition of PEG yielded consistent results in each triplicate sample of downstream amplification via PCR (Figure 9).

The only statistically significant differences in mean product yield of MDA versus MDA with PEG existed in the 0.5 ng treatment group ($p$=0.0226). The overall mean amount of product yield for MDA on all leaf DNA samples was 23.6 µg (± 8.9) and for MDA with PEG was 18.1 µg (± 0.9). The differences between the overall means were found to be statistically significant ($p$=0.0237). Even though the average amount of product was lower than non-PEG samples, the addition of PEG to the MDA reaction produced more consistent amplification across all sample treatments, regardless of input DNA concentration.

*Serially Diluted Leaf DNA Amplification*

The MDA reaction was successful on all serially diluted leaf DNA, even at concentrations corresponding to the equivalent amount of DNA in a single pollen grain (0.01 ng). The overall mean product yield for MDA of leaf tissue, regardless of input DNA concentration was 19.5 µg (± 2.8) [N=100]. The total MDA product yield (reported as the mean ± standard deviation) from serially diluted leaf DNA samples was 18.7 µg (± 8.6), 25.1 µg (± 31.3), 18.5 µg (± 12.6), 18.0 µg (± 6.9), and 17.3 µg (± 4.1) from an input of 10, 1, 0.5, 0.1 and 0.01 ng template DNA, respectively (N=20 per treatment; Table 3, Figure 7). The total MDA product yield from the negative control (reported as the mean ± standard deviation was 22.2 µg (± 4.4), There was no statistically significant difference ($p$=0.5404) among the serially diluted template groups, including the negative control (0 ng template).

# DISCUSSION

*Amplification of Single Pollen Grains*

Although MDA and subsequent successful PCR genotyping has been shown to work on single cells (Zhang *et al*., 1992; Dietmaier *et al*., 1999; Wells *et al*., 1999; Hanson & Ballantyne, 2005; Jiang *et al.*, 2005), it had yet to be reported for single pollen grains. Initial WGA and subsequent PCR of single pollen grains was successful up to 120 days storage at -20ºC. Craddock *et al*. (2000) found that C*ornus florida* pollen remains viable for germination for up to 108 days in either -20ºC or -196ºC storage conditions; however, no reports are available on the effect of long-term storage (longer than 108 days) on viability in *C. florida*. Although MDA reactions from long term-stored single pollen grains produced WGA product, this product was not successful when used for PCR. This protocol is known to produce high molecular weight DNA in negative controls containing no template DNA (Dean *et al*., 2002; Sun *et al.*, 2005; Barber & Foran 2006; Dillon, personal observation). These exogenous products, presumably from amplification of the random primers, produce quantitative results post WGA reaction, but are unsuccessful templates for PCR amplification of STR loci. In high concentrations, they also have the potential to inhibit amplification of the target DNA in subsequent assays (Barber & Foran, 2006).

These long-term stored grains may exhibit the same characteristics of degraded DNA templates; therefore tests for the effectiveness of the omission of the initial

denaturation step of the MDA reaction were conducted. This step can degrade DNA and cause locus misrepresentation (Dickson *et al.*, 2005) and its omission improves binding specificity, and produces more successful amplification on degraded templates (Dean *et al.*, 2002). However, after omission of this step, successful PCR amplification was not successful with long term-stored pollen grains. Ballantyne *et al*. (2007) found the loss of alleles on degraded samples to be dependent on concentration. Therefore, there is a high possibility for loss of alleles in a low copy number DNA single pollen grain sample, especially one that has been degraded.

The variation of success of fresh single pollen grain amplification may be due to two factors. First, when a locus fails to amplify in PCR, it can not be confirmed if that specific PCR reaction simply failed, if the STR locus was not amplified during the MDA reaction, or if a pollen grain was not transferred to the PCR tube for WGA. The last scenario is especially difficult to determine since negative controls produce quantifiable post-MDA results and a positive reaction does not necessarily indicate the presence of a pollen grain. Second, the MDA reaction produces highly variable results with single-cell templates. Kuman *et al.*, (2008) found that the genotyping accuracy of the MDA reaction followed by PCR on single human cells varied from 37% to 100%, with an average of 80%, results that are very similar to those reported here. Success can also vary due to the quality of the template DNA utilized for the WGA reaction and should only be used to enhance the quantity of template DNA, not the quality. (Gunn *et al.*, 2007)

Due to the fact that viability of frozen pollen grains can fall below 50% of that of freshly collected (Craddock *et al.* 2000), these long term-stored pollen grains may have

lost the ability to germinate, rendering them useless for WGA and subsequent PCR reactions. Omission of the initial denaturation step of the MDA reaction did not recover a successful profile from long-term stored grains; it is therefore recommended that all MDA reactions be performed with freshly collected pollen or short-term stored grains to achieve the best results.

*Effectiveness of Molecular Crowders*

Several publications support the use of PEG and similar substances as molecular crowders capable of increasing the success of DNA amplification reactions (Minton, 2001; Karimata *et al.*, 2004; Ballantyne *et al.*, 2006) by increasing the binding of DNA polymerases to DNA templates (Zimmerman & Harrison, 1987). Specifically for *Φ29* DNA polymerase, the addition of a molecular crowder increases both polymerase and primer binding by reducing the effective reaction volume (Ballantyne *et al.* 2006). Ballantyne *et al.* (2006) were the first to report that the addition of a molecular crowder produced more consistent amplification when using MDA template DNA for multiplexing PCR amplification of STR loci, which increased the number of detectable alleles and therefore the amount of genetic information available.

Here, the addition of 2.5% PEG to the MDA reaction significantly increased the consistency of the downstream PCR amplification of the MDA products when compared to PCR amplification of MDA products without the addition of PEG. Ballantyne *et al.* (2007) suggested the use of replicate samples to obtain a full genetic profile, due to the high frequency of stochastic effects during the MDA reaction, which can cause a loss of

alleles in some samples. Therefore, triplicate replicates were conducted on each PCR reaction for both non-PEG and PEG samples. The non-PEG reactions did not produce consist results (i.e. only one or two of the triplicate samples successfully amplified the allele), but the PEG samples were consistent, with all three replicates producing PCR product. To reduce the need for the time-consuming and expensive process of conducting multiple replicates, the use of 2.5% PEG 400 is recommended during the MDA reaction.

*Amplification of Serially Diluted Leaf DNA*

The serially diluted leaf DNA served as an indicator of the threshold limits of input DNA for the MDA reaction. This kit works best at 10 ng input DNA, and the manufacturer does not recommend its use with input templates less than 1 ng. The 0.1 and 0.01 ng of input DNA tested were both representatives of low copy number samples, whereas the 0.01 ng dilution was intended to simulate a single pollen grain input. WGA was successful on all diluted samples, regardless of input DNA. They also all produced relatively equal concentrations of amplified DNA, allowing the WGA product to be used in downstream applications without measuring concentrations or diluting samples, even if different amounts of starting template were utilized. Dean *et al.* (2002) reports a consistent yield between 20-30 μg, regardless of input, which is very similar to the amount of product reported here (between 17-30 μg), regardless of the starting template concentration. Although the average product yield for the negative control did not differ from any of the template groups, none of these samples were successful templates for subsequent PCR reactions, indicating an inherent level of background amplification.

*Future Investigations*

The results from this study can be built upon to carry out the original intent of this work to the fullest extent. Insects can be collected directly from inflorescences and their pollen grains can be utilized for genetic analysis. Further optimization will be needed to multiplex the PCR reaction, so that a broader analysis of the success and accuracy of the MDA reaction on multiple alleles can be conducted, in turn yielding a more accurate representation of percent allele drop out for single pollen grains. Also, it should be determined if the pollen grain haplotype matches the genotype of the tree from which it originated. From this, concordance rates can be calculated because the STR profiles of the original sources already have been established in the Rice Center population. This will allow for the establishment of average distance distributions for pollen transport based on the genotypes of the trees that insects visited, elucidated from their pollen load.

*Broad Significance*

Successful pollination and subsequent reproduction of flowering plants have both ecological and agricultural importance. Insect diversity across many taxa appears to be declining, which could have drastic effects on interconnected biological communities that rely on insect pollinators for reproduction (Biesmeijer *et al.*, 2006). With growing dependence on plants as a platform for industrial and biomedical protein synthesis, it becomes increasingly important to understand how genes move across landscapes. The output of many commercial crops also depends upon frequent visitation by pollinating

insects, and a better understanding of insect movement allows for maximization of pollination efficiency and protection of migration corridors (Jones *et al.*, 2001). The growing use of transgenic crops also warrants increased understanding of how insects can move these altered genes across the landscape to prevent invasion into natural populations (Del Socorro & Gregg, 2001; Hudson *et al.*, 2001). By determining the haploid DNA sequences of a single pollen grain, scientists can elucidate pollen dispersal patterns and track the movement and efficiency of animal pollinators, as well as facilitate a new methodology for quantifying how animals move through heterogeneous environments.

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## Literature Cited


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# APPENDIX A

## Tables and Figures

**Table 1:** Dinucleotide microsatellite primer pairs developed by Cabe and Liles (2002) from flowering dogwood to be used for DNA amplification.

| Locus | Primer Sequence | Motif | Annealing Temperature | Fragment Size Range (b.p.) |
|---|---|---|---|---|
| **Cf-N10** | F:*TGATTGAATAACCTTTTGATGC<br>R: GGATGCTTCAAATGTCAACG | $(GT)_{17}$ | 57° C | 178-206 |

* Fluorescently labeled primer

**Table 2:** Variation of percent success of PCR on WGA product from fresh pollen grain amplification.

| Trial | Proportion of Successful PCR Amplifications |
|---|---|
| 1 | 0.6 |
| 2 | 0.4 |
| 3 | 0.9 |
| 4 | 1.0 |
| 5 | 0.2 |
| Average | 0.62 |

**Table 3:** Total MDA product yield (reported as the mean ± standard deviation) from serially diluted leaf DNA. (N=20 per treatment).

| Input DNA (ng) | Total MDA Product Yield (µg) |
|---|---|
| 10 | 18.7 ± 8.6 |
| 1 | 25.1 ± 31.3 |
| 0.5 | 18.5 ± 12.6 |
| 0.1 | 18.0 ± 6.9 |
| 0.01 | 17.3 ± 4.1 |
| 0 | 22.2 ± 4.4 |

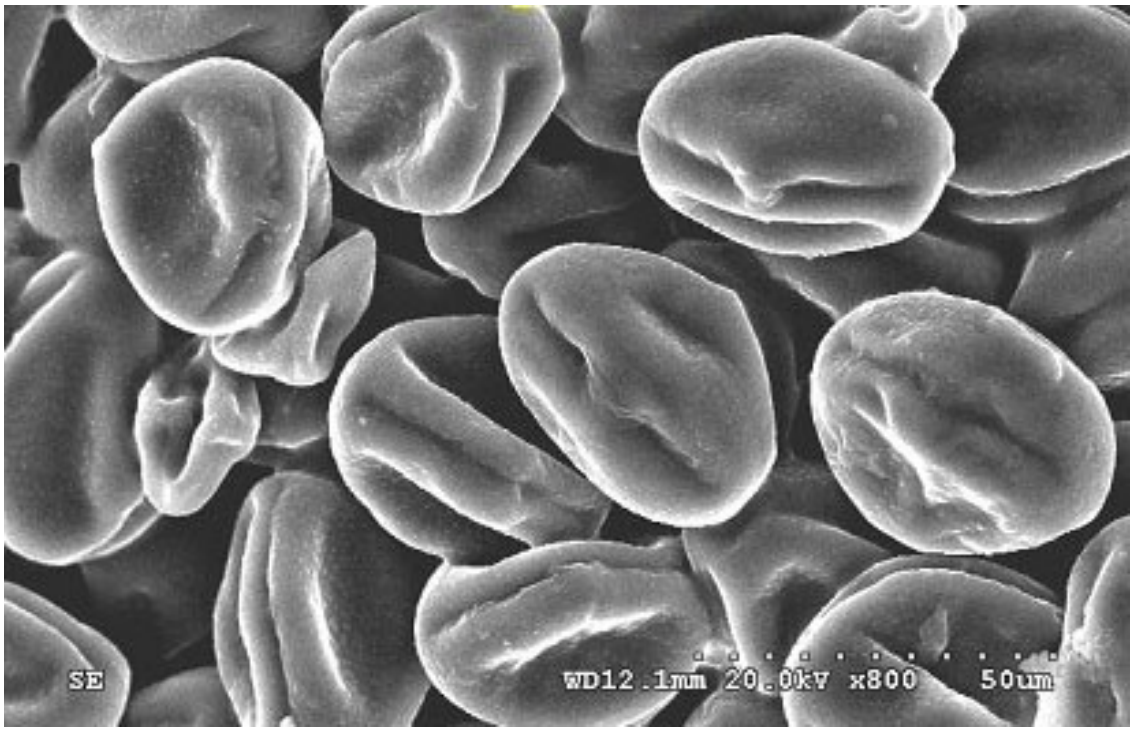


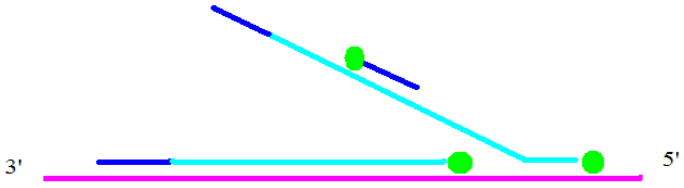

**Figure 1.** Scanning electron micrograph of *Cornus florida* pollen grains.


Template DNA (denatured). 3' and 5' ends of DNA are labeled.

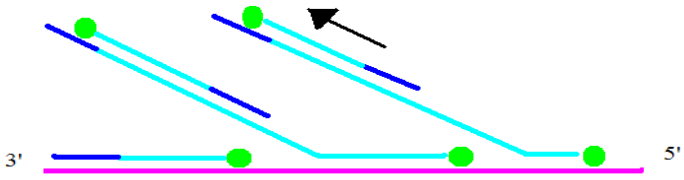

Binding of random hexamers on template DNA.


*Φ29* binds and begins polymerization.


Synthesis of new strand by *Φ29*. Black arrows indicate direction of synthesis (5' to 3').




Newly synthesized strand displaces downstream strand. Primer binds to single strand that has been displaced.




*Φ29* binds and begins polymerization of strands complimentary to displaced strands. Black arrows indicate direction of synthesis (5' to 3').

**Figure 2.** Multiple Displacement Amplification reaction, as described by GE Healthcare (manufacturers of GenomiPhi™ DNA Amplification Kit [25-6600-01]).

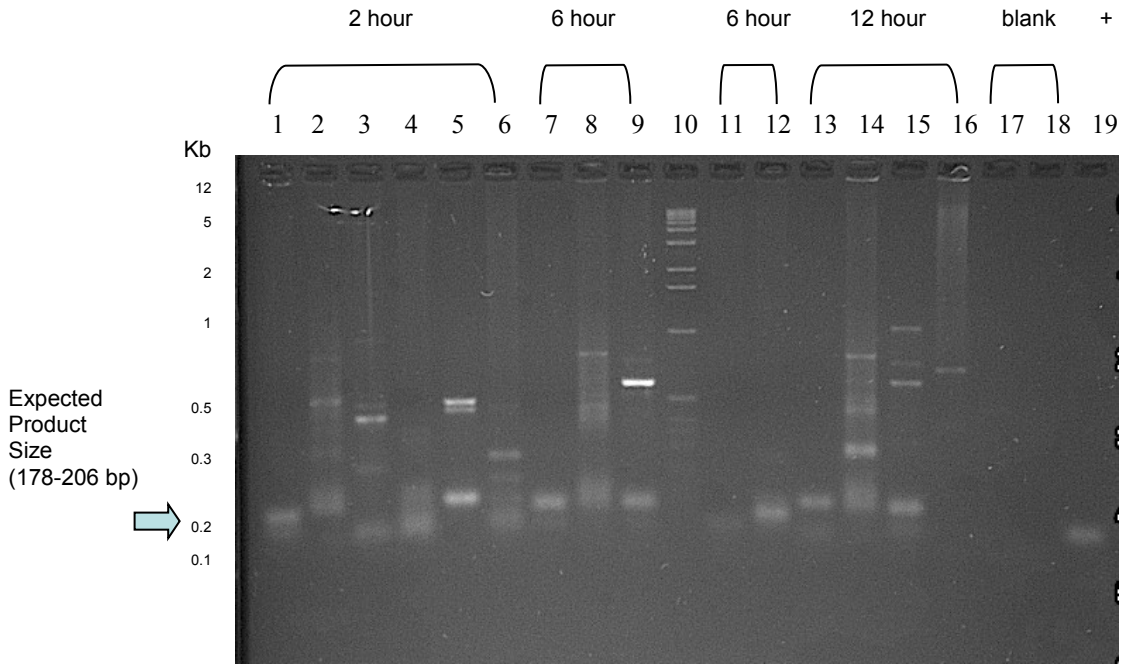



**Figure 3.** Initial PCR results from short term-stored pollen grains. Lanes 1-6: Single pollen grain template with 2 hour MDA reaction time. Lanes 7-9 and 11-12: Single pollen grain template with 6 hour MDA reaction time. Lane 10: 1 Kb ladder (Invitrogen Corporation, Carlsbad, CA). Lanes 13-16: Single pollen grain template with 12 hour MDA reaction time. Lanes 17-18: blank. Lane 19: Positive dogwood control (50 ng/µL).

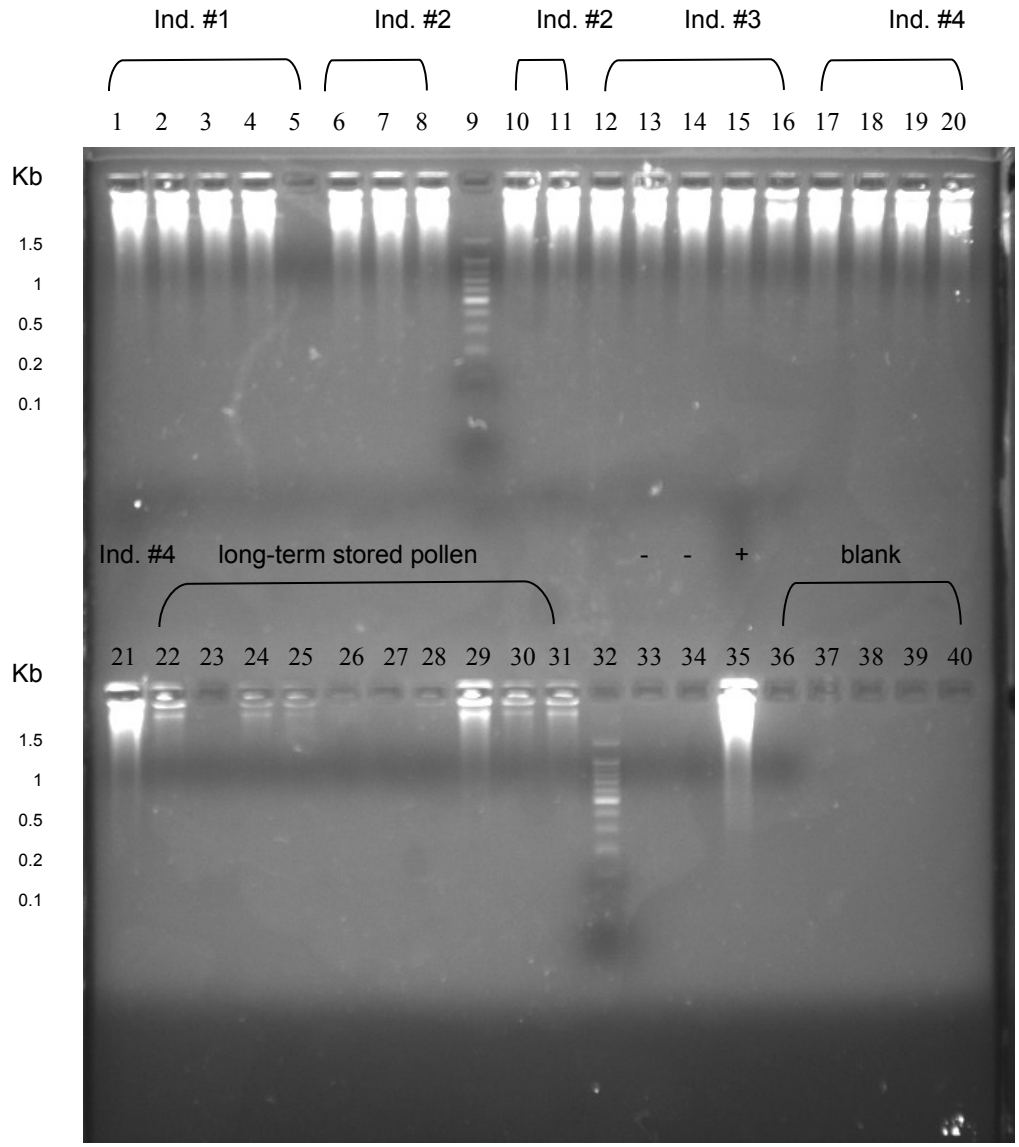



**Figure 4.** Whole genome amplified product with serially diluted leaf DNA. Lanes 1-5: Individual #1 - 10, 1, 0.5, 0.1 and 0.01 ng/µL template. Lanes 6-8 and 10-11: Individual #2 - 10, 1, 0.5, 0.1 and 0.01 ng/µL template. Lane 9: 100 bp ladder (New England BioLabs, Ipswich, MA). Lanes 12-16: Individual #3 - 10, 1, 0.5, 0.1 and 0.01 ng/µL template. Lanes 17-21: Individual #4 - 10, 1, 0.5, 0.1 and 0.01 ng/µL template. Lanes 22-31: Long-term stored single pollen grain templates. Lane 32: 100 bp ladder (New England BioLabs, Ipswich, MA). Lanes 33-34: Negative controls. Lane 35: Positive control (1 ng DNA template). Lanes 36-40: blank.

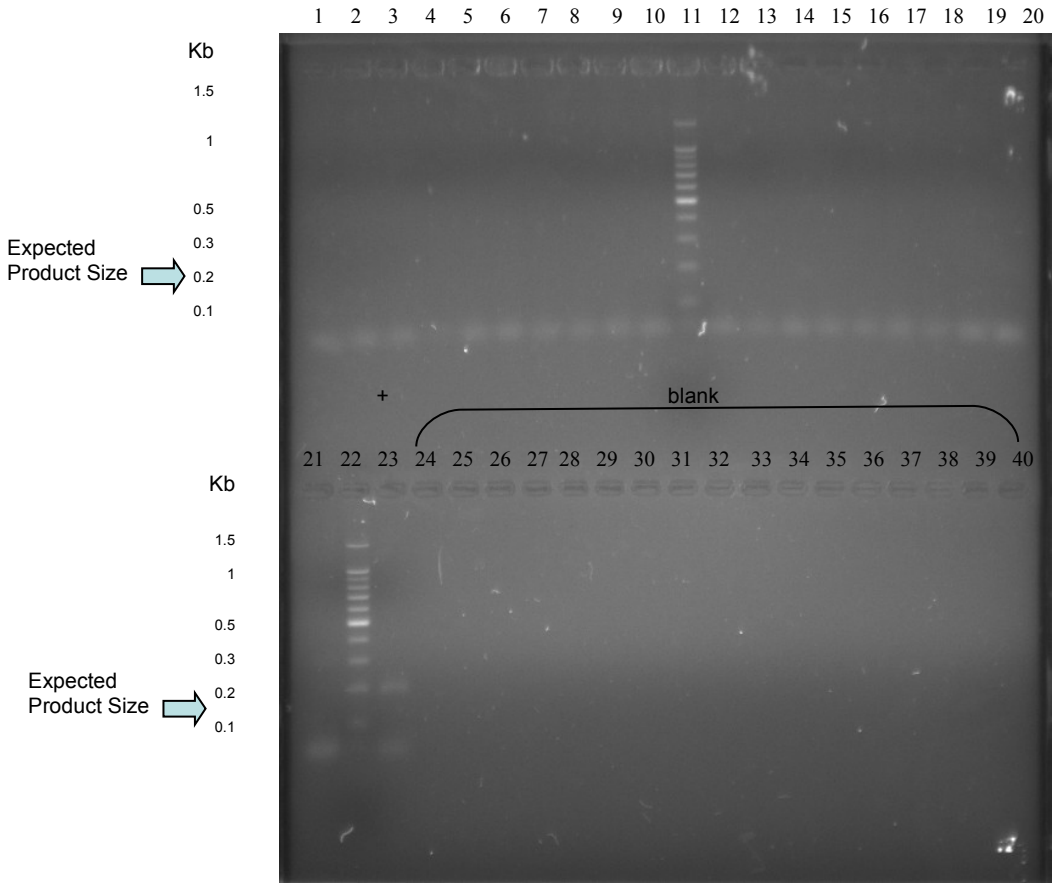



**Figure 5.** PCR product using WGA product with omission of denaturation step during WGA on single pollen grains. Lanes 1-10: Single long-term stored pollen grain PCR product. Lane 11: 100 bp ladder (New England BioLabs, Ipswich, MA). Lanes 12-21: Single long-term stored pollen grain PCR product. Lane 22: 100 bp ladder (New England BioLabs, Ipswich, MA). Lanes 23: Positive dogwood control (50 ng/µL). Lanes 24-30: blank.

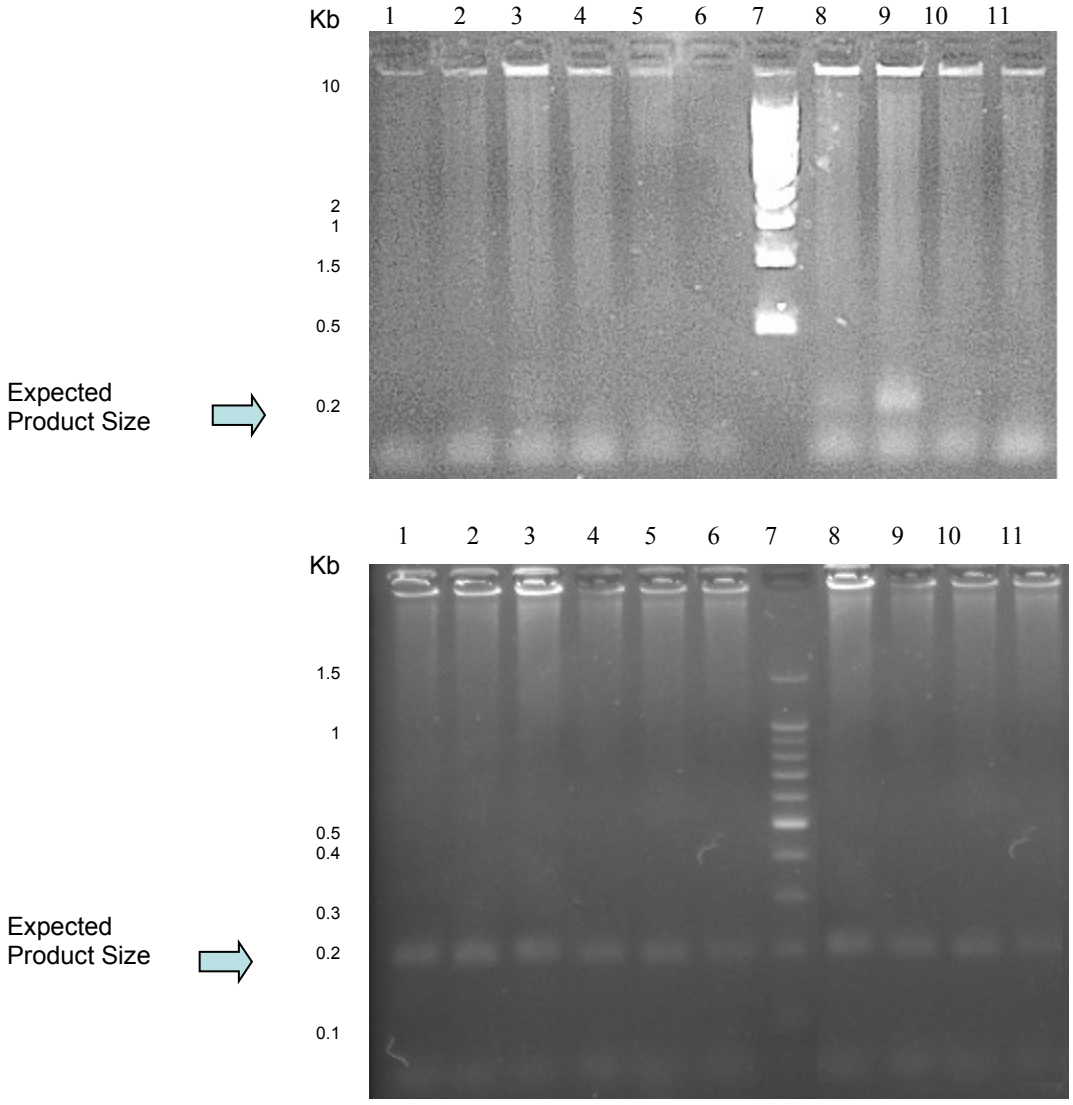



**Figure 6.** Variability of PCR success on MDA product from fresh single pollen grains. Top Gel - Lanes 1-6 and 8-11: PCR product using a single fresh pollen grain as a template for the MDA reaction. Lane 7: 100 bp ladder (New England BioLabs, Ipswich, MA). Bottom Gel - Lanes 1-6 and 8-11: PCR product using a single fresh pollen grain as a template for the MDA reaction. Lane 7: 1 Kb ladder (Promega, Madison, WI).

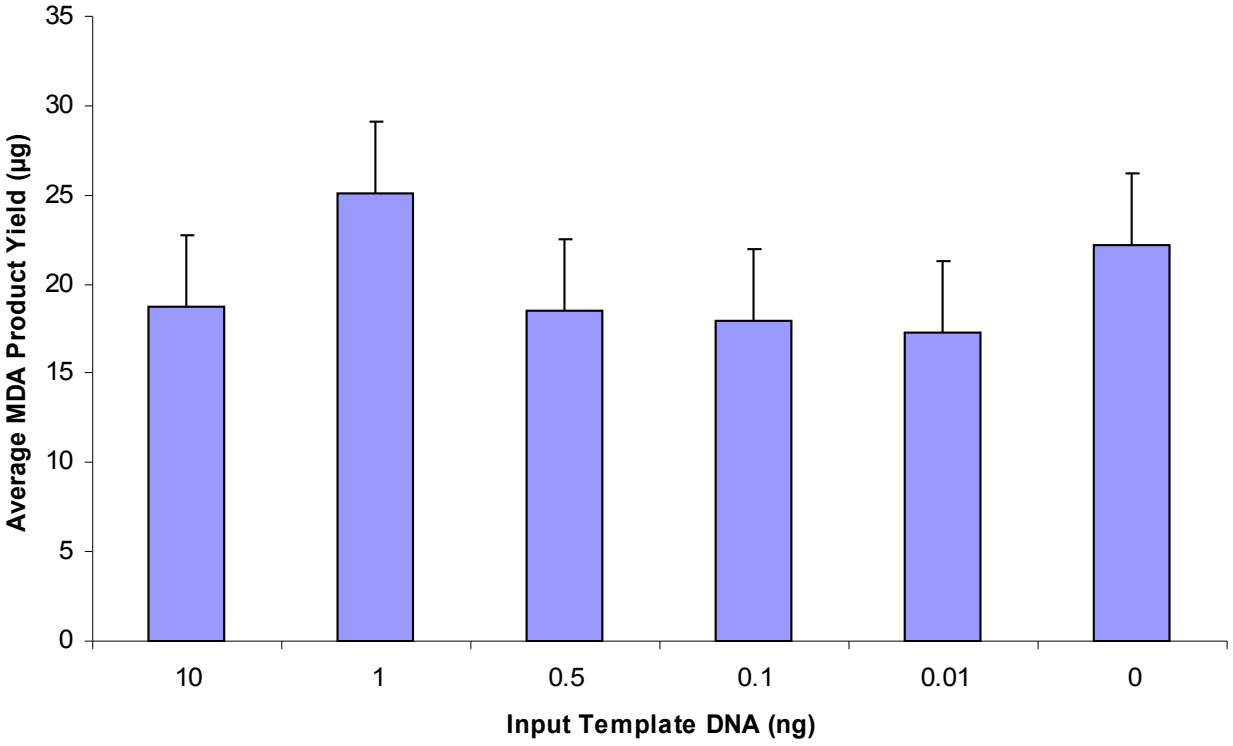



**Figure 7.** Average MDA product yield from serially diluted leaf DNA. (N=20 per treatment). Error bars represent standard deviation.

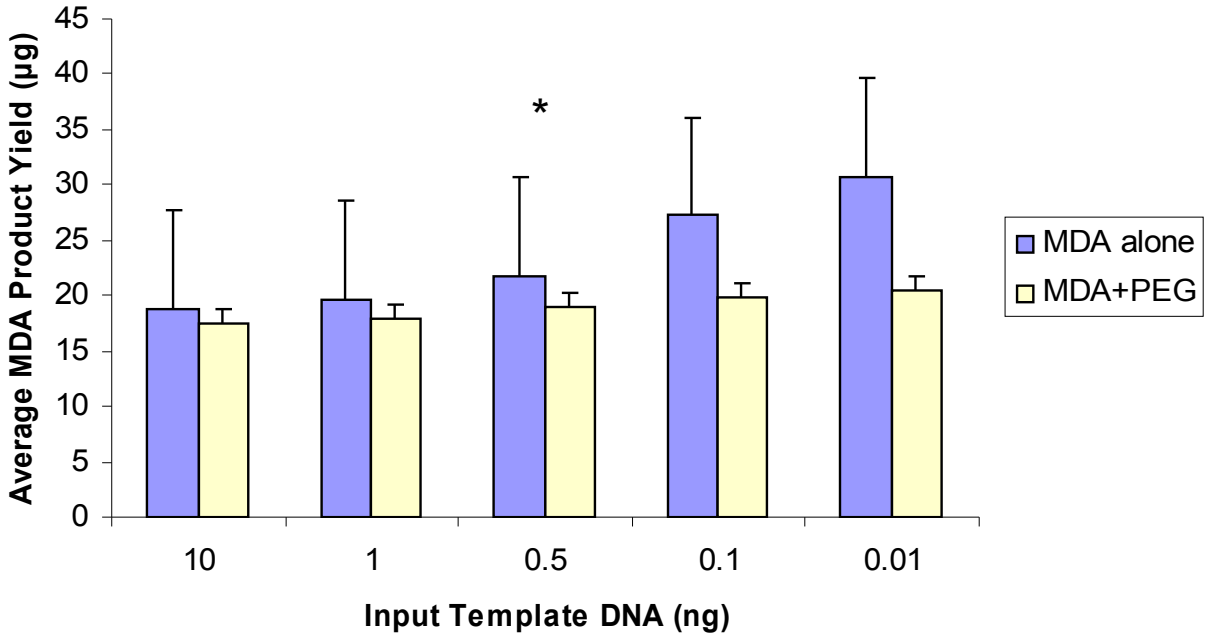



**Figure 8.** Average WGA product yield from serially diluted leaf DNA in the presence or absence of 2.5% PEG in the MDA reaction. (N=4 per dilution treatment). Error bars represent standard deviation. * Indicates statistically different treatment groups.

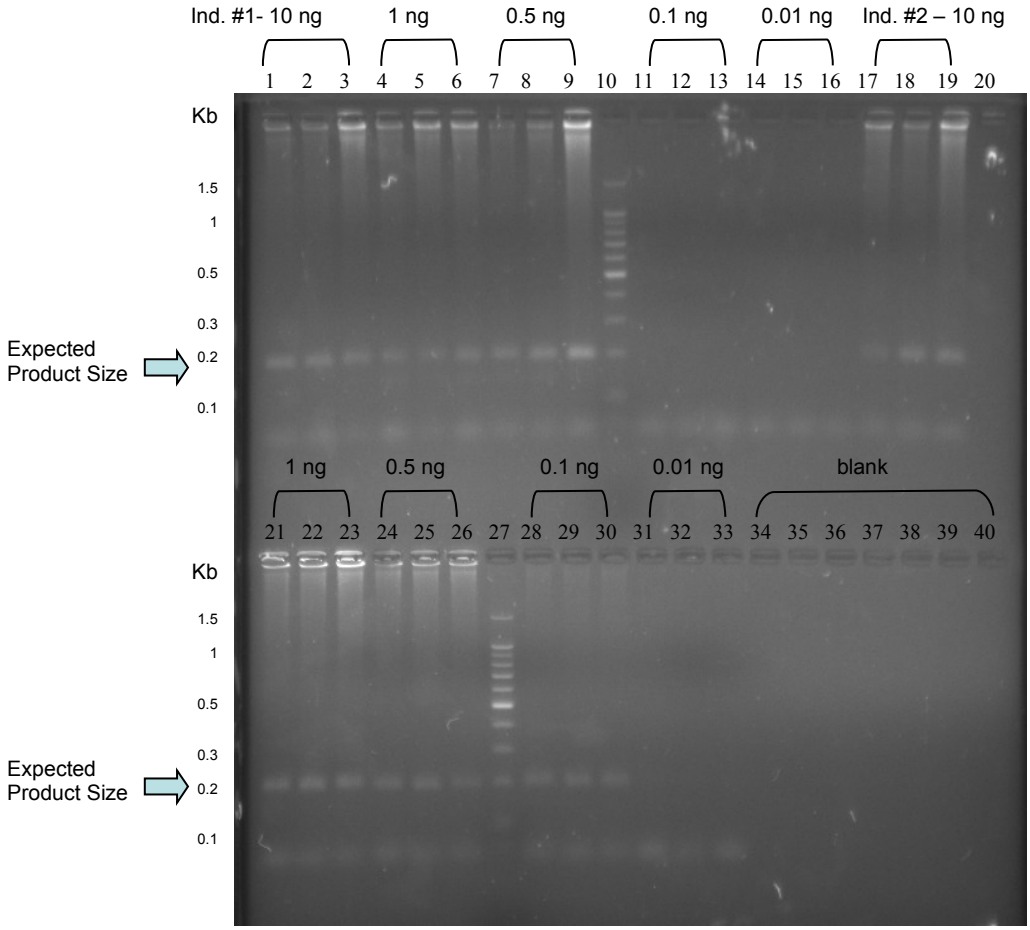



**Figure 9.** Triplicate samples of PCR product from whole genome amplified leaf DNA from 10, 1, 0.1, and 0.01 ng input DNA and 2.5% (v/v) PEG. (Left to Right): Lanes 1-3: Individual #1 – 10 ng. Lanes 4-6: 1 ng. Lanes 7-9: 0.5 ng. Lane 10: 1 kb ladder. Lanes 11-13: 0.1 ng. Lanes 14-16: 0.010 ng. Lanes 17-19: Individual #2 - 10 ng. Lanes 20: blank. Lanes 21-23: 1 ng. Lanes 24-26: 0.5 ng. Lane 27: 1 kb ladder 100 bp ladder (New England BioLabs, Ipswich, MA). Lanes 28-30: 0.1 ng. Lanes 31-33: 0.01 ng. Lanes 34-40: blank.

# VITA

Candace Dillon was born on June 12th, 1985 in Enid, Oklahoma. She received her Bachelor of Science in Forensic Science from Virginia Commonwealth University in 2007. Candace received a Graduate Teaching Assistantship during her graduate study. She taught a 300 level Biotechniques course and served as the Lead Teaching Assistant for the 2008-09 school year. She also received a Graduate Research Assistantship from the Graduate School at VCU for the summer of 2009. After graduation, Candace plans to pursue a career as a Forensic DNA analyst or as a Biology teacher.