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Parallel Selection Protocol for the Maize ATLAS Project

The Maize ATLAS Project[1,2,3,4,5,6,7]

[1]University of Delaware; [2]North Carolina State University; [3]Iowa State University; [4]Texas A&M University; [5]University of Missouri; [6]University of Wisconsin; [7]USDA-ARS

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Randall Wisser
INRAE, University of Delaware

ABSTRACT

As part of the Maize ATLAS project on adaptation and diversification along a latitudinal axis of the USA, this protocol describes procedures used for a parallel selection experiment. A tropical synthetic (TropicS) population was systematically selected for early flowering time at multiple locations managed by a team of research groups (project PIs: N. de Leon; S. Flint-Garcia; J. B. Holland; N. Lauter; S. Murray; W. Xu; R. J. Wisser). The protocol outlines experimental techniques and guidance for planting, sample tracking, tissue sampling, pollination, harvesting and record keeping. The protocol has been used to generate over 20 new populations of maize to investigate the phenotypic and genomic basis of response to selection. This work supported by the Agriculture and Food Research Initiative Grant No. 2011-67003-30342 from the USDA-NIFA (Agriculture and Natural Resources Science for Climate Variability and Change Program).

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Selection Procedure

1 **Seed:** Use a balanced bulk of 15,000 kernels for the experimental plot. The unbalanced bulk of ~3,000 kernels can be used for border rows, if needed (step 3).

2 **Metadata:** If a seed treatment is applied, record the product and dosage used.

3 **Isolation Planting:** Isolate the plot from other corn in the area and/or stagger the timing of planting to minimize the risk of cross contamination. Include border plots of a TropicS seed source on all sides, or plant a hybrid and detassel prior to anthesis.

4 **Planting Density:** Plant the experimental plot with 10,000 seed at 10" spacing (one complete block without alleys), surrounded by at least two border rows of additional plants on each side of the plot. Plant at the designated spacing or overplant and thin accordingly (step 6).

5 **Metadata:** Record the planting date, field information and details about fertilizer and pesticide applications.

6 **Thinning:** If neccessary, thin plants to 10” spacing. Thin plants as early as possible and record the vegetative stage (e.g., V4) when thinning was performed.

7 **Cultivation:** For cultivation, use the recommended rate of fertilizer used for commercial hybrid in your area.

8 **Metadata:** Record details of cultural practices.

9 **Grid selection**: To minimize the impact of field heterogeneity, divide the experimental plot into four blocks. Use a quadrant or define four equally sized blocks that would best control for field variation in the particular field plot. Perform subsequent activities within blocks, until the final steps when seed is harvested and bulked across blocks.

10 **Tissue Collection:** Tissue will be collected for genotyping from three cohorts: (1) random individuals sampled at the juvenile stage (1000 samples; 250 per block); (2) all tagged individuals belonging to the early flowering set (500+ samples; 125+ per block); (3) late flowering individuals (500 samples; 125 per block). ***See the section "Tissue Collection Procedure" for a detailed protocol.***

11 **Stand Counts:** Record the total number of plants per block. Stand counts will be used later for the precise calculation of selection intensities and for determining the timing for sampling the late flowering cohort.

12 **Tagging System:** The following system is designed to aid in setting up and pollinating the earliest plants on a given day. A color-coded tagging system is used to mark and track individual plants selected for pollination and to maintain any genotype-to-phenotype links. Unique tag IDs include a location code, a year code, a unique number, and a color code (e.g. N12001g = Newark, DE 2012 001 green). The tags for male plants have a five-day color sequence. In the fashion of a heat map, the following color sequence is recommended for tagging males across sequential days: w (white), y (yellow), o (orange), s (strawberry), r (red). You may want to hand-mark colored tags into 5-day sets (e.g., A, B, C, etc.) to distinguish repeating instances of the same color separated by 5 days. Tags for female plants are a single color (green) that is different from the male colored tags. On each male and female tag, mark the date it is placed on a plant. When tagging for selection, exclude border rows or ends of rows where plants may show edge effects. ***Note: tags will provide genotype/tissue-phenotype links for the written male and female tag dates (approximate flowering dates) and other traits.***

13 **Pollination Method:** A bulk pollen half-sib chain method will be used for pollinations, crossing a pollen bulk from five selected males to five selected females without selfing and avoiding reciprocals. Therefore, crosses can only be made when there is a set of five males available to cross to a distinct set of five females. ***See the section "Pollination Method" for a detailed protocol.***

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14 **Timing of Tissue Collection for the Late Flowering Cohort:** Using the block stand counts from step 9, monitor the flowering time of random subsamples per block. Commence tissue collection once ~5% are left flowering. Record the collection dates. ***See the section "Tissue Collection Procedure" for further details.***

15 **Harvesting:** Harvest the pollinated ears to make balanced seed bulks: (i) two separate bulks with 3 kernels per ear; (ii) two separate bulks with 30 kernels per ear. The total seed required per ear is 66 kernels. Bulk the remnant seed in an unbalanced bulk. Record the list of female tag IDs used to create the bulks. Use the 30 x 500 bulk to repeat the selection procedure. Save the remaining bulks for other experiments.

Tissue Collection Procedure

16 **Collection Cohorts:**
A) Random plant cohort: Sample the leaf tip during V5-8 stage from a new, but near-fully or fully extended leaf. Fold the leaf tip in half towards the stem to sample a folded piece of tissue that is approximately 3/4-length of the envelope (not less than 1/2-length). Slide the envelope around the folded leaf and tear to collect.
B) Extreme flowering time cohorts: Sample ASAP after flowering or pollination. For these older plants, tissue should be taken from the flag leaf or the leaf below it. If possible, sample tissue from the base of the leaf blade near the collar, excluding the midrib. Similar to the random plant cohort, collect 2-layered tissue samples that are approximately 3/4-length of the envelope. If the tissue looks healthy, the 2 layers could be made by folding a long piece of tissue from one side of the midrib; if not, sample from each side of the midrib.

17 **Collection Techniques and Tips:**
- Always sample tissue from the newest/youngest leaves possible that have little to no signs of damage.
- When collecting tissue from tagged plants, write the tag ID and tagged date of male and female flowering time on the envelope.
- Fold the tip of the leaf over to make a double layer so that nearly double the amount of leaf tissue will be placed into the collection envelope (since there is less DNA per unit area in older tissue more tissue is needed). Slide the envelope over the folded leaf, squeeze the envelope and leaf together with your thumb and tear the leaf (or cut the leaf using scissors) such that it fits into the envelope and the envelope flap can be folded over. Close the envelope flap.
- Collect 10-20 tissue samples at a time and group them together with a rubber band.
- Place each batch in into doubled Ziploc bags, secure and place in a cooler on ice.
- Keep the tissue samples in a cooler on ice shortly after sampling but *DO NOT FREEZE THE TISSUE AT ANY TIME*.

18 **Shipping Tissue:** Immediately ship cold samples overnight in a styrofoam or other cooler with ice or ice packs placed above and below the samples.

Pollination Method

19 **Method:** A bulk pollen half-sib chain method will be used for pollinations, crossing five selected males to five selected females without selfing and avoiding reciprocals. Therefore, crosses can only begin to be made when there is a set of five males available to cross to a distinct set of five females.

20 **Tagging System (described in selection procedure, step 12):** The following system is designed to aid in setting up and pollinating the earliest plants on a given day. A color-coded tagging system is used to mark and track individual plants selected for pollination and to maintain any genotype-to-phenotype links. Unique tag IDs include a location code, a year code, a unique number, and a color code (e.g. N12001g = Newark, DE 2012 001 green). The tags for male plants have a five-day color sequence. In the fashion of a heat map, the following color sequence is recommended for tagging males across sequential days: w (white), y (yellow), o (orange), s (strawberry), r (red). You may want to hand-mark colored tags into 5-day sets (e.g., A, B, C, etc.) to distinguish repeating instances of the same color separated by 5 days. Tags for female plants are a single color (green) that is different from the male colored tags. On each male and female tag, mark the date it is placed on a plant. When tagging for selection, exclude border rows or ends of rows where plants may show edge effects. ***Note: tags will provide genotype/tissue-phenotype links for the written male and female tag dates (approximate flowering dates) and other traits.***

21 **Pollination Setup / Selected Plants:** Use the tag-color sequence to guide selecting the earliest males for pollination. Female tags (green) may be placed while cutting back the silks for pollination. Using color-striped shoot

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bags on females that have been cut back help to easily locate these plants during pollination.

22 **Planning:** To help plan for each day's pollinations and find available plants for pollinating, count the number of tagged males and females per row for each block. With this information, the total number of crosses per block can be pre-planned. This is also helpful for tallying pollinations each day.

23 **Pollinating:** Pollinate plants within blocks, leveraging the tagging system. For each pollination, bulk pollen from five males to pollinate five separate females. For many locations where the plants grow to be very tall and are therefore susceptible to breaking, it is easiest to bulk pollen directly on the fly, without setting up pollination bags on tassels. For each pollination set (10 plants: 5 males x 5 females), use a pollination bag to collect pollen from five male plants. Mark the male tag to know which males were used and to ensure those plants are not used again as a male. Combine the pollen into a single pollination bag (a sieve or other filter mechanism may be used to exclude anthers), then fold and shake to mix. Use the mix to pollinate the 5 separate females, and cover each ear with a new pollination bag marked with the current date.

24 **Mark Pollinated Plants / Tissue for Cohort 2:** Mark used males (e.g., write an "X" on the male tag) and females (automatically marked by pollination bags over the pollinated ear). Tissue will be collected from all plants used for pollination. ***See the section "Tissue Collection Procedure" for details.***

25 **Pollination Techniques and Tips:**
- Use serpentine route along rows for pollinating: try to go through each block from one row to the next until completed.
- Barren plants can be used as males and male sterile plants can be used as females.
- Use males only once and mark the tag or break the tassel to avoid reuse.
- When cutting back, do not cut deep into shoots to find silks—no "digging" for silks; cut only when you see silks emerged.
- Use color-striped shoot bags on females that have been cut back to easily locate these during pollination.
- To help with planning, count the number of males and females per block available for pollinations.
- Use the tag-color sequence to guide selection of the earliest males for pollination. The tags follow a color sequence similar to a heat map from earliest to latest males that repeats across five day intervals.
- Mark each male and female tag once the plant is used, which will also indicate the plants in cohort 2 to collect tissue from.
-Date the pollination bags used to cover each ear post-pollination, which may serve as a backup to damaged tags on information about the dates of pollination (for females) and reference for harvesting.

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