

DEVELOPING A MOLECULAR-BASED SEX DETERMINATION ASSAY FOR HEMP

COLORADO STATE UNIVERSITY AND COLORADO SEED LAB AT ARDEC

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PROJECT INTRODUCTION

Due to the competitive and rapidly growing nature of the hemp industry, it is imperative to develop tools and methods to assist in breeding and growing strains to fit specific industry needs. Most *Cannabis* varieties are dioecious, meaning that male and female reproductive organs occur on separate plants. Most uses of hemp, especially CBD production, require exclusively female plants because their flowers produce the desired cannabinoids. A non-pollinated female can produce up to two times the amount of CBD as a pollinated plant. One male plant left unnoticed amongst females can pollinate up to an acre of land, which is approximately 1000-3000 plants depending on what the hemp product being produced is.

Current solutions to avoid male plants and subsequent pollination, has been by using feminized hemp seeds. These seeds are created by crossing a normal female plant (XX) with a hermaphroditic female plant (XX), of which can produce pollen. The lack of Y chromosomes results in seeds that will all be genetically female. This method of crossing hermaphroditic plants with normal female plants has worked for decades to eliminate the need to scour male plants from fields.

When buyers purchase feminized hemp seed, they need to ensure that the increased price is worth the investment. According to the U.S. Wholesale Hemp Price Benchmarks published in May 2020, one pound of non-feminized CBD hemp seed (1 lb averages to 25,000 seeds) costs approximately \$328.00, while the average price of a single feminized CBD hemp seed is \$0.48. This pricing reflects that one pound of feminized CBD hemp seed costs around \$12,000, indicating a price increase for feminized CBD seeds of 3,558.5%.

In implementing this sex-determination assay alongside the germination and contamination tests already available, the hemp industry can continue to develop into a reliable and trustworthy network of breeders, growers and processors.

HOW DOES THIS APPLY TO YOUR EDUCATION

I am studying Botany with an interest in climate adaptation, genomics, and biotechnology. I am very passionate about the potential of hemp in our society and having the opportunity to develop my skills in researching molecular biology and genomics, while working with a plant so dear to me, has been an exceptional opportunity.

This internship allowed me to practice the necessary laboratory skills that I will use in higher degrees and careers following my graduation at Colorado State. The skills that were essential for me to learn and develop this assay include molecular techniques such as:

- DNA extraction
- KASP qPCR
- data collection and interpretation
- spectrophotometry
- general lab work and maintenance
- proposing and redesigning hypotheses and experiments
- communicating academic ideas with my mentors, peers, and the public



Above: Example of my lab station while preparing for DNA extractions

Because of my experiences in this internship, I am much more aware of my strengths and weaknesses as I move forward in my career and future endeavors.

INTERNSHIP GOALS

1. Develop a sex determination assay for hemp that will be offered by the Colorado Seed Lab at ARDEC as a paid service to nationally located hemp growers and breeders.
2. Develop the sex determination assay to be as throughput as possible to reduce time, labor, and costs while still ensuring accurate determination results.
3. Gain experience in hemp cultivation, molecular assay development, data management and interpretation, protocol development, and experimental design.
4. Learn more about the hemp industry and its needs regarding seed safety, distribution, testing, and commerce.



Left: an example of a female hemp plant showing the buds that develop the CBD/ THC



Right: shows an example of a male plant with numerous pollen sacs

Credit: Both plants grown by Megan at ARDEC

WHAT YOU LEARNED

- Hemp industry needs
 - Standardization of quality including germination, feminization, and processing
 - Communication improvements between breeders, growers, processors, and the public
 - Education and information sharing between the industry, the public, and lawmakers
 - Transportation, logistic, and policy improvements when working with businesses across state lines
- Genomics
 - Practiced KASP genotyping, DNA extraction, spectrophotometry, experimental design, and data collection and analysis
 - Researching publications for information on:
 - Genes that may influence a desired genotypic or phenotypic trait of interest
 - Different experimental protocols and how to incorporate them to achieve a desired procedure
 - Ways to analyze data and form conclusions based on the evidence provided
 - Good lab practices including PPE, lab maintenance, and chemical safety

NEXT STEPS

- Look for other markers/genes that may be more definitive for sex determination
- Test other methods than KASP genotyping that may offer more insight into sex determination
- Look for markers/ genes that can indicate if a feminized crop is likely to have a hermaphroditic progeny
- Work with growers and breeders to create a standard of quality for germination, feminization, and processing

WHAT YOU DID

In order to determine whether a seed is male or female, we developed a KASP (competitive allele-specific PCR) genotyping assay that uses a fluorescence based marking mechanism to identify nucleotide variants at specific loci. The nucleotide variants utilized by KASP include SNPs (single nucleotide polymorphisms) and indels (insertions/ deletions).

When a breeder submits a new strain testing, we begin by extracting DNA from ~80 individual seeds. Then, we mix each DNA sample with the KASP master mix and the SNP-specific primer mix, which we designed based on a SNP we identified, through previous research, as a strong indicator of sex. One of the primers in the SNP-specific mix anneals to the female SNP (FAM oligomer) and the other that anneals to the male SNP (HEX oligomer). Each of these primers include a unique tail sequence that is initially quenched; however, as PCR runs, the attached primer's tail unquenches allowing the tail sequence to fluoresce the associated color indicating the SNP's concentration within each DNA sample.



Above: Megan testing DNA concentrations before proceeding to KASP qPCR

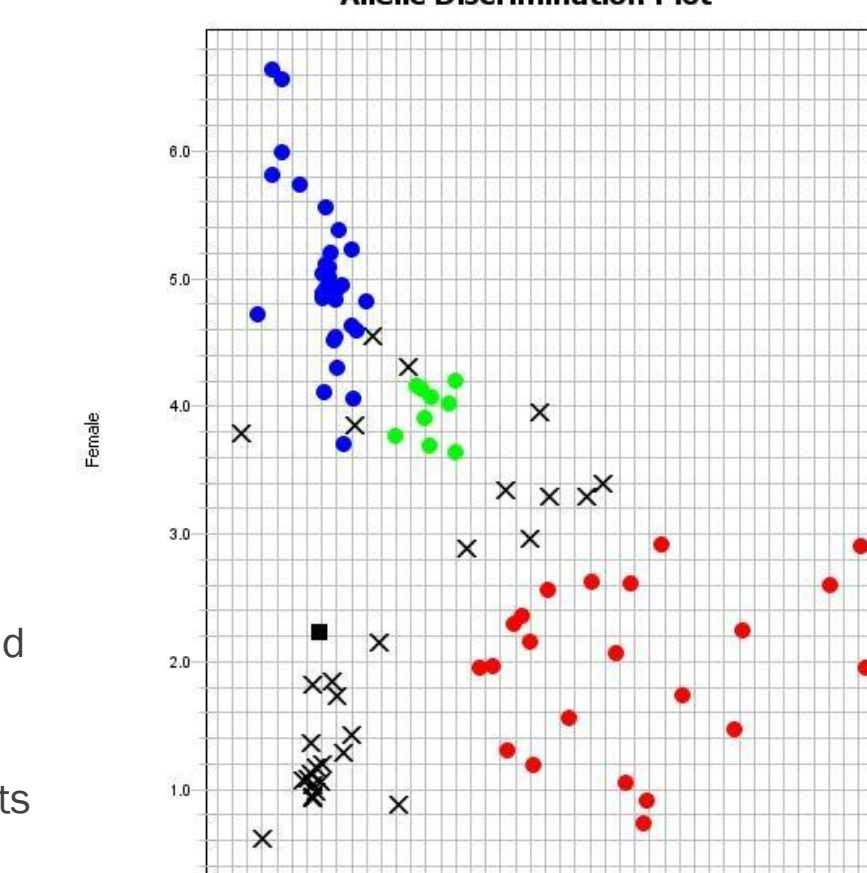
After the PCR process finishes, the fluorescent signal of each sample is recorded and plotted on a cartesian plot along the associated axis. We then can use this data to create a ratio (% confirmed female) of each sample's sex. This method is especially useful when looking at feminized hemp seed crops. Any given feminized seed crop should theoretically have a 100% female fluorescence recorded, however taking error into account, the ideal value is closer to 95% or higher for female seeds. If a given crop tests below this 95%, we can assume that there may have been pollination contamination from a missed male, neighboring fields or possibly a mislabeled batch of seeds; however, the industry has yet to set a standard of quality for feminized seeds. Once a standard is decided upon, breeders and growers of hemp can then use this data to determine an appropriate price for their seeds alongside the germination and noxious- weed contamination tests.

If the sample is homozygous for a given SNP it will record as only one of the two fluorescent colors. If the sample is heterozygous for the SNP then it will record as a third color in the mid range of the plot. After the plot is created it can be analyzed to find the genotype for each sample.



Left: example of growth chambers used for germination and previously used to grow plants for sex- determination test

Above: example of plate data of allelic discrimination results after running KASP



Right: example of cartesian plot of allelic discrimination results after running KASP

Legend: #Heterozygous Male/Male, #Heterozygous Female/Female, #Heterozygous Male/Female, XUndetermined