

QUAKING ASPEN BARK CHLOROPHYLL GENETICS

Group 4 (2013)

Abstract

Quaking aspen (*Populus tremuloides*) carries a unique adaptation—the ability to photosynthesize in winter due to chloroplasts and chlorophyll in their bark. Also, aspens reproduce asexually via an extensive root system capable of cloning itself by having multiple offshoots from the same root system. Here this study looks into whether the tree's color caused by the chlorophyll occurs as a response to the tree's external environment or by the tree regulating itself via genetic circadian rhythms. By looking for consistencies within the coloration of clones, a correlation may appear, suggesting genetic control, or a lack of it, suggesting trees within the clone react independently as a response to stresses in its environment. The DNA from many trees within an aspen grove was sequenced and analyzed to determine clones and digital photography was used to pull quantitative color values from the trees. The two variables were compared using an analysis of variance (ANOVA), yielding a lack of correlation between a particular clone and the color value associated with its trees. This suggests the trees' bark chlorophyll concentrations vary independently of clones as a response to changing environmental stresses.

This study was performed as a collaborative effort led by Dr. Jon Seger (University of Utah) in which several research groups conducted studies concerning *Populus tremuloides* variation. The groups performed several independent studies, each looking at a different aspect of aspen variation. All groups shared some data with the other groups, contributing to each other's information.

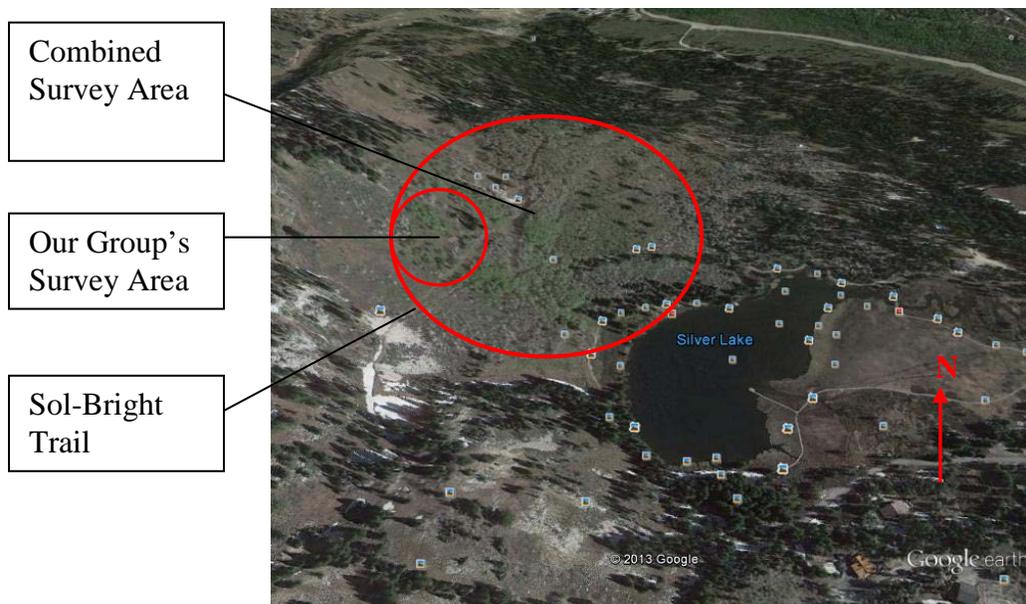
Introduction:

Owing to an extensive range across the often extreme environments of northern North America (Mock, 2013), quaking aspens (*Populus tremuloides*) carry an array of adaptations to facilitate survival. Among them include mass cloning, causing many offshoots from the same root system (Mock, 2013). As a result of this, aspens make almost ideal organisms to study genetic variation because large variation can occur within a clone, allowing easy analysis of heritable variance compared to acquired variance (Seger, 2013). Another unique adaptation in aspens involves an ability to photosynthesize in their bark as a result of chlorophyll in the outermost layers of their bark. Unusually, aspen bark chlorophyll concentrations vary depending on the time of year. In summer, when leaves perform most of the photosynthesis, the bark chlorophyll's concentration decreases. The inverse phenomenon occurs in winter. In the absence of leaves, the bark chlorophyll's concentration increases (Pearson and Lawrence, 1958). The seasonal fluctuations could result from either changing seasons causing environmental stresses or the trees' genes controlling them through circadian rhythms (Ibáñez et al, 2010). By analyzing color discrepancies within clones, one could expect a correlation within colors within all trees in a clone cluster should the trees' genes cause the variation, while on the contrary, a lack of correlation would suggest color and chlorophyll change as a result of seasons and other environmental stresses.

Methods:

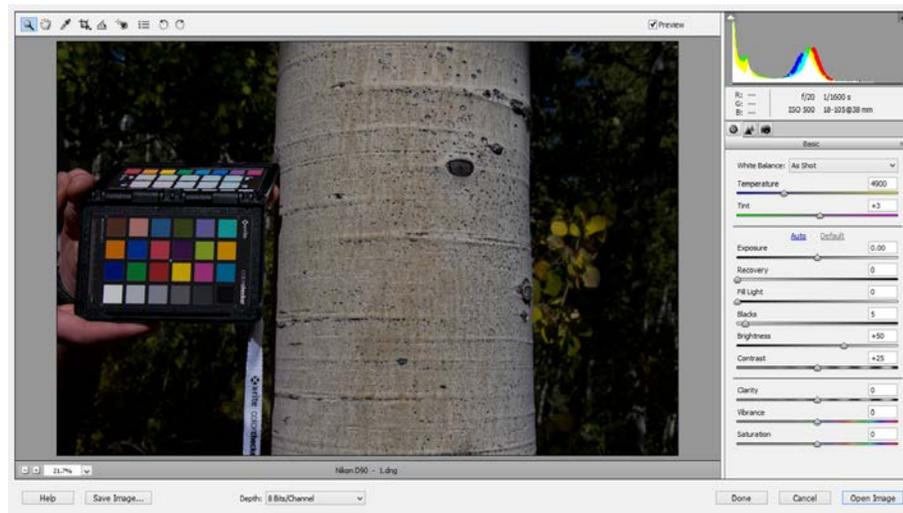
We performed our experiment in two main phases: a field phase followed by a lab phase. The field phase involved traveling to a study area and collecting physical specimens, while the lab phase involved both further qualitative and quantitative analysis using some high-tech methods in comparing quantitative color values to the clone to which the tree belonged to.

This study's specimens came from a grove of *Populus tremuloides* (quaking aspen) on the west slope overlooking Silver Lake in Big Cottonwood Canyon, Utah. Before collecting specimens, we looked at photographs of the study area from amateur photographers and Google Earth to predict clones within the grove. Looking in the pictures for consistencies in the picture such as canopy "texture" and color patterns in autumn (i.e. Different clones change colors at different times, resulting in uneven leaf coloration across the grove in autumn.), we chose a area on the south side of the grove above the Sol-Bright ski trail that bisects the grove.



Within the study area, we selected nine random trees in the grove as our test subjects. Over a course of three visits, usually one visit occurring every 1-2 weeks, leaf samples were collected from each tree, dislodged by either throwing rocks or fallen branches at the trees, firing a giant slingshot, or using pruning shears mounted on a long pole. We stored the samples in Ziploc® bags, pressed the leaves under a stack of several heavy books, and refrigerated them. Also when visiting the sites, we photographed all nine of our study trees with a digital SLR (single-lens reflex) camera. While photographing the tree, one group member held a ColorChecker® Passport next to each tree, while the photographer ensured the entire Passport

appeared in the frame unobstructed by uneven light across its surface. An actual photograph from this study demonstrating this technique is shown below. In addition to our own trees, we also photographed several trees outside our study area to expand our survey into trees unlikely to be clones to our own. As previously mentioned in the abstract, this study formed a part of a much larger group effort, so each group in its own respective area collected the leaf samples from their trees for the genotyping of the whole study area.



The photography ended the field phase of the procedure. The lab phase immediately followed. It began with extracting DNA from the collected leaves and running a polymerase chain reaction (PCR) on the extracted DNA. After the PCR, the DNA was sequenced on a computer, using PeakScanner® software, which read the DNA and used it to organize trees into clones based on eight microsatellite loci. For a more detailed protocol for this genotyping (reagents, DNA loci, etc.), see Callahan et al. (2013).

The second part of the lab phase involved editing the photographs to compensate and neutralize the pictures, filtering out lighting abnormalities by using the ColorChecker® Passport as the color standard. The passport's corresponding software read the passport and adjusted the pictures' color using the passport as the standard. Doing so created a legible template, which

enabled Adobe Photoshop® to pull quantitative color values on the image. We assumed varying chlorophyll concentrations contributed to the color variety (Pearson and Lawrence, 1958). On Photoshop, we analyzed the picture we perceived as the best based on ColorChecker Passport software editing, overall image quality, and absence of factors such as extreme lighting (both bright and dark), shadows, foreign objects blocking the passport, etc.

On Photoshop, we opened our chosen pictures and extracted color values for the aspen bark. To compensate for shadows creating uneven lighting conditions on the bark in the pictures, we cropped the largest maximum area bark in the same lighting conditions of the ColorChecker Passport. Before determining the average color value of the whole crop, we analyzed ten random loci (individual pixels) for their RGB (red, green, and blue intensity) values. Photoshop reveals the RGB values of a particular pixel by simply clicking on a location within the image. This step was done to display the color variation within one tree. After analyzing the ten pixels, we pulled the average RGB values for the entire cropping. We performed the same procedure photo editing procedure for all trees.

Although Photoshop accurately pulled RGB values for all trees, the RGB values varied considerably owing to diverse lighting conditions when we snapped the pictures. Some trees yielded abnormally high RGB values because of direct sunlight, but others produced much lower values due to complete shadow. To compensate and obtain a more average color spread for all the trees, we added an extra step to the procedure here. Here, we reasoned if colors were identical across trees due to the trees' genetically-controlled circadian rhythms, the ratio of the R to G to B values would be entirely consistent across clones. To test this assumption, we obtained the clone data and ran an analysis of variance (ANOVA) using the statistical computer program R to relate the individual RGB values to the clones determined by eight microsatellite loci to

determine the probability of those RGB values being connected to a particular aspen clone.

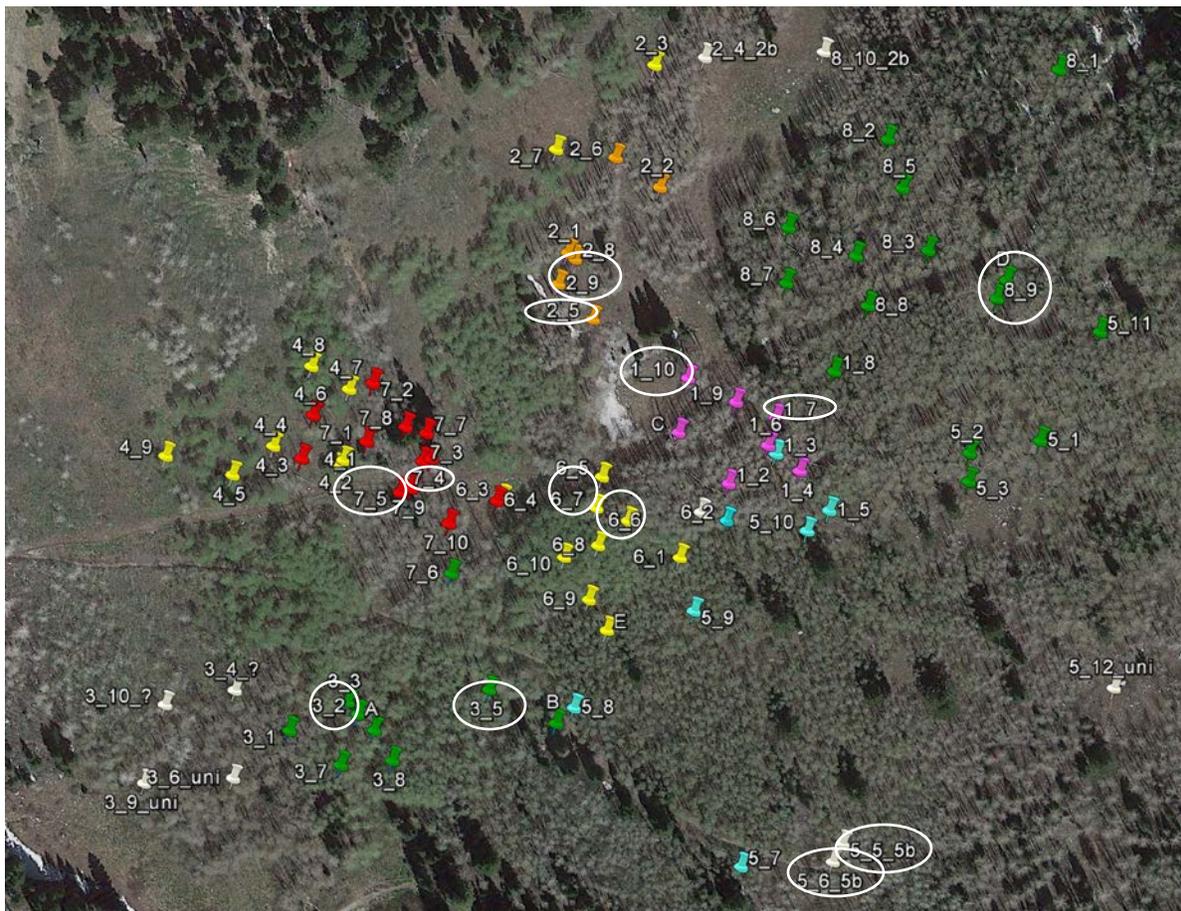
Also, we ran an ANOVA on the R: G, G: B, and R: B ratios also correlating them with the genes.

The ANOVAs were done on both the ten average pixels and the overall average RGB values.

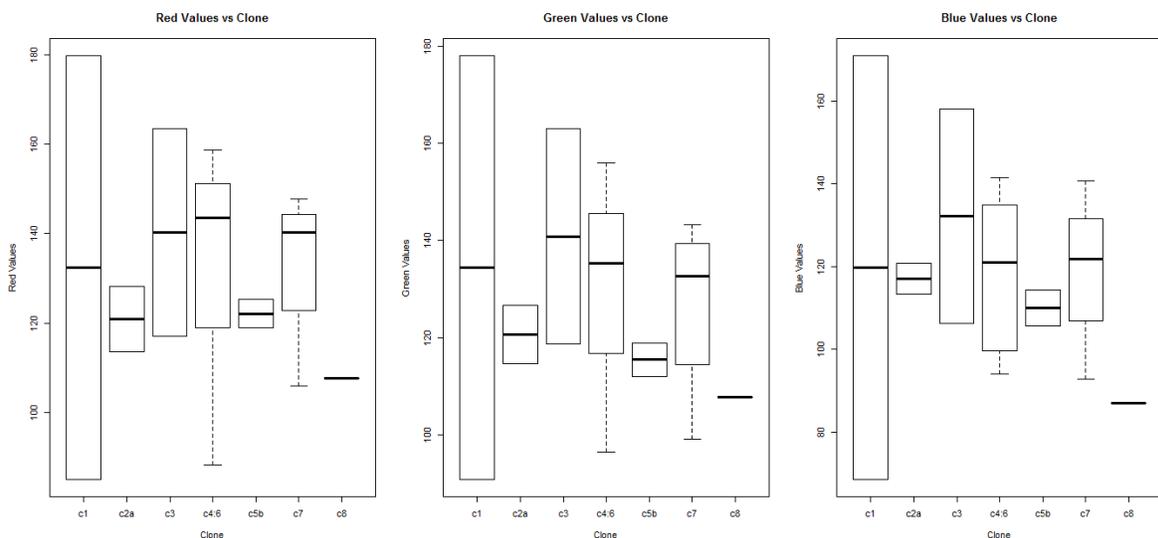
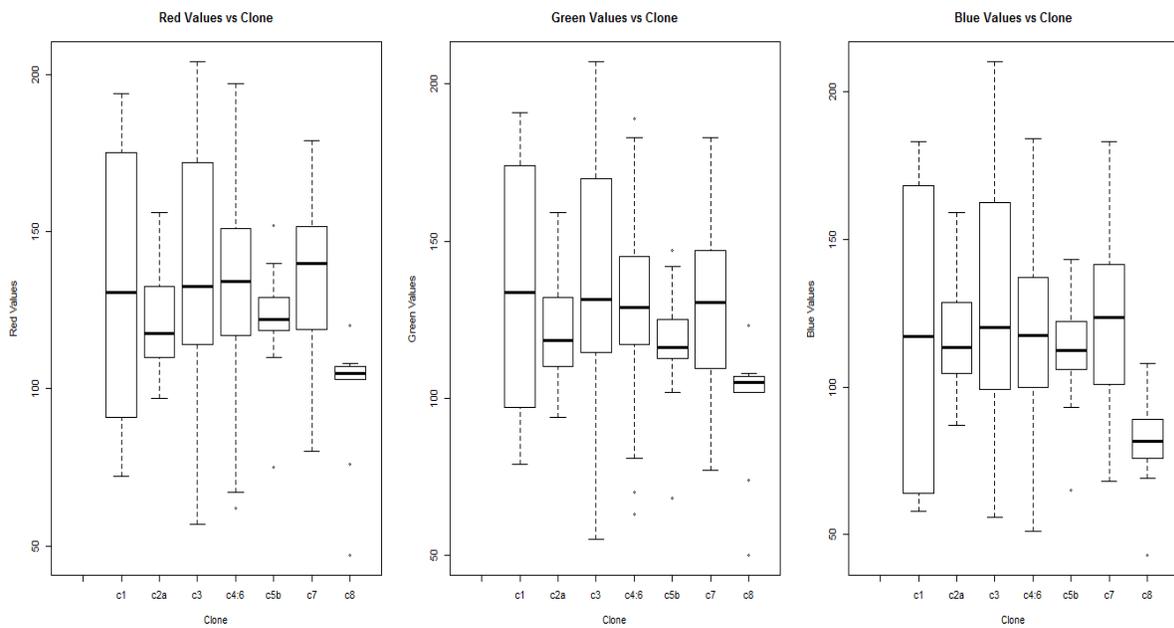
The variances and associated values revealed the plausibility of color's connection to the trees' clone. These values are discussed in further detail in the results section of this report.

Results:

The PCR of the DNA, the DNA's subsequent sequencing, and the combined group data revealed a set of at least seven aspen clones in the survey area. The map below shows the survey area from a different angle, with each color denoting a different clone. The numbers next to each marker are simply tree ID numbers. Trees outside our study area have their ID circled in white, and although not labeled, all trees in are 4 were surveyed and photographed.



In the statistical analysis, R enabled us to see the full spread of the data. The very large variance with respect to color existing in one clone surprised us. Even the ratios, which we believed would reduce the variation, still showed large deviations within the same clone. R organized the spread of this data into box-and-whisker plots, displayed below. The first chart shows the distribution of color variance for the ten random pixels per picture. The second shows the same, but with the average ratios rather than the ten randomly selected pixels.



As we initially expected, some ratio box-and-whisker plots show much less variance than the ratios simply because of less data to work with, but some actually expand the inner-quartile range effectively increasing the variance. Since we photographed all the trees in our area and only two trees each from all the other groups' research sites, the genotyping revealed a huge underrepresentation of some clones, evidenced by some plots only showing a mean or the inner-quartile range.

When we had R perform ANOVAs on the random RGB values and the RGB ratios compared to clone identity for both the random pixels' RGB values and the average image's RGB values, two very contrasting data sets emerged. The ANOVAs for both the random ratios and the random values compared to the clone gave a miniscule P-Value (shown on the charts as $Pr(>F)$), inferring a high probability of connection the trees' genes. However, the same ANOVA test on the trees' average RGB values showed the opposite effect. A much higher P-value appeared, indicating any correlation as random and occurring independently. The various sets told us the color discrepancies occur as either a result of random deviations as a response to environmental factors or controlled genetically.

The chart to the right displays the ANOVA results for the random values compared to clones. The asterisks next to the P-values serve as a marker telling the high likelihood of the observed trait being connected to the clone the tree grew in.

```
> a <- aov(redvalues~Clone, data=colors)
> summary(a)
          Df Sum Sq Mean Sq F value Pr(>F)
Clone      6  15612    2602   3.04 0.00714 **
Residuals 213 182337     856
---
Signif. codes:  0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1
19 observations deleted due to missingness
>
> b <- aov(greenvalues~Clone, data=colors)
> summary(b)
          Df Sum Sq Mean Sq F value Pr(>F)
Clone      6  15090    2515.0   3.161 0.00545 **
Residuals 213 169484     795.7
---
Signif. codes:  0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1
19 observations deleted due to missingness
>
> c <- aov(bluevalues~Clone, data=colors)
> summary(c)
          Df Sum Sq Mean Sq F value Pr(>F)
Clone      6  17388    2898.1   3.064 0.00676 **
Residuals 213 201448     945.8
---
Signif. codes:  0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1
19 observations deleted due to missingness
```

This chart shows the RGB values

of the random pixels, but in this

case shows (from top to bottom)

the R: G, G: B, and R: B ratios.

The high probability of genetic

involvement appears again

evidenced by the asterisks next to

the P-Values.

```
> f <- aov(redvalues/greenvalues~Clone, data=colors)
> summary(f)
              Df Sum Sq Mean Sq F value    Pr(>F)
Clone          6  0.1382  0.023040    3.964 0.000883 ***
Residuals     213  1.2381  0.005813
---
Signif. codes:  0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1
19 observations deleted due to missingness
>
> g <- aov(greenvalues/bluevalues~Clone, data=colors)
> summary(g)
              Df Sum Sq Mean Sq F value    Pr(>F)
Clone          6  0.5378  0.08963    8.905 1.12e-08 ***
Residuals     213  2.1439  0.01007
---
Signif. codes:  0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1
19 observations deleted due to missingness
>
> h <- aov(redvalues/bluevalues~Clone, data=colors)
> summary(h)
              Df Sum Sq Mean Sq F value    Pr(>F)
Clone          6  0.274  0.04560    2.844  0.011 *
Residuals     213  3.415  0.01603
---
Signif. codes:  0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1
19 observations deleted due to missingness
```

Here, the chart shows the same test as the first one,

but with the average values. Notice the asterisks

next to the P-values disappear here, suggesting a

negligible possibility of the color trait having a

connection the trees' genetics, opposite of what the

random values ANOVA suggests.

```
> a <- aov(redvalues~Clone, data=colors)
> summary(a)
              Df Sum Sq Mean Sq F value    Pr(>F)
Clone          6   1090   181.7    0.232  0.959
Residuals     15  11727   781.8
>
> b <- aov(greenvalues~Clone, data=colors)
> summary(b)
              Df Sum Sq Mean Sq F value    Pr(>F)
Clone          6   1271   211.8    0.325  0.913
Residuals     15  9762   650.8
>
> c <- aov(bluevalues~Clone, data=colors)
> summary(c)
              Df Sum Sq Mean Sq F value    Pr(>F)
Clone          6   1498   249.7    0.348  0.9
Residuals     15 10757   717.1
```

The ANOVA for the (from top to bottom)

R: G, G: B, and R: B ratios of the average

coloration of the whole image shows the

same trend as did the other average. The

correlation with clones disappears again.

```
> f <- aov(redvalues/greenvalues~Clone, data=colors)
> summary(f)
              Df Sum Sq Mean Sq F value    Pr(>F)
Clone          6  0.01493  0.002488    1.59  0.218
Residuals     15  0.02347  0.001564
>
> g <- aov(greenvalues/bluevalues~Clone, data=colors)
> summary(g)
              Df Sum Sq Mean Sq F value    Pr(>F)
Clone          6  0.05238  0.00873    1.335  0.302
Residuals     15  0.09810  0.00654
>
> h <- aov(redvalues/bluevalues~Clone, data=colors)
> summary(h)
              Df Sum Sq Mean Sq F value    Pr(>F)
Clone          6  0.0354  0.005899    0.704  0.652
Residuals     15  0.1258  0.008385
```

The schism over whether the tree's genetics correlate with the observed color variations astounded us at first. According to Seger (the research leader), the schisms occur as a result of differences in degrees of freedom (abbreviated Df in the ANOVA charts). Because a vastly

larger set of data constituted the random pixel colors, the number set had greatly larger degrees of freedom, facilitating the ability to draw a correlation between the clones and the colors. The average values, therefore, lacked this large data set and resulted in much fewer degrees of freedom, hindering the ability to draw a reasonable correlation between clones and bark color intensity. Because we perceived the ten random pixels as inadequate in describing the overall color in the bark, we decided to eliminate them in drawing a conclusion. Although the ten random pixels did show significant color variation within a tree, we decided to analyze the over picture average in drawing a conclusion. Therefore, using the relatively large P-values obtained from the ANOVAs, color variations in aspens appear the result of a response to the trees' external environment rather than regulated by genetically-controlled circadian rhythms. If genes do have any effect, their influence seems almost entirely negligible.

Discussion:

From analyzing the average color intensity on an area of aspen bark and comparing those values within clones, this study suggests color variation, as a result of varying chlorophyll concentration, occurs as a tree within a clone's response to its changing environment rather than occurring as a result of genetically induced circadian rhythms. These results support and give insights to past studies on the topic of bark chlorophyll. Pearson and Lawrence (1958) first demonstrated the bark chlorophyll concentrations fluctuating as the seasons changed, with the peaks occurring in winter with the absence of leaves and the troughs occurring in summer with the presence of leaves. Here, our study adds to their findings by suggesting how the fluctuation varies by arguing some change occurring with the seasons contributes to increasing quantities of chlorophyll in aspen bark. Possibly, the absence of leaves allows more sunlight to reach the

aspen bark, so the tree may respond by increasing bark chlorophyll quantities. However, validating or disproving that hypothesis requires a further investigation.

The greatest margin for error in this study comes from not having standards when taking the pictures. We simply took the pictures in broad sunlight as much as possible, ignoring factors such as the specific side of the tree (angle of sunlight could result in uneven RGB values around the tree, assuming sunlight intensity affects chlorophyll production), the camera's distance from the tree, and camera height. A method of correcting this factor could involve taking the pictures at night, free of risk of sunlight interference, and using the camera flash to create a constant lighting across the whole shot. Also potentially causing error, we analyzed nine trees from our study area, but only two trees from all the other study areas, possibly creating underrepresentation of some clones, reducing the certainty of our results for those trees. Smaller contributing factors to error include the uneven color scheme on aspen bark caused by dark gashes accenting the white and some bumps in otherwise smooth bark. Certain camera settings (f-stop, shutter speed, ISO, etc.) likely do not affect the RGB values because the ColorChecker software uses the Passport to correct for lighting conditions, allowing for accurate RGB value extraction. Machines performed almost the genetic and statistical analysis, greatly reducing the risk of human error. But regardless of any design flaw or human/technical error, this data seems very valid. Workable, data did come out of the RGV values evidenced by how R pulled a strong correlation between clones and the random values. Therefore, we see this as viable data. However if one desires to replicate this experiment, we recommend taking the listed measures to achieve maximum accuracy and precision in the numerical data.

As one study often yields several others, this project raises several other questions. On a small, more molecular scale, determining the seasonal factor(s) resulting in fluctuating

chlorophyll concentrations could lead to discovering how chemical processes alter to both produce and reduce bark chlorophyll. Also, this study showed a large variance within clones, small scale geographical analysis using similar methods to what Pearson and Lawrence (1958) did could show patterns in bark chlorophyll concentrations. For example, if light stimulates chlorophyll production, a dense grove would show more bark chlorophyll in peripheral trees and less in central trees. Because aspens have such an extensive range through a variety of environments, bark chlorophyll could vary not only on a small scale within clones, but also on large scale owing to the type of ecosystem the aspen grows in, whether in temperate mountains or subarctic. Extreme northern latitudes experience sunless winter days, while more southern latitudes experience sunny winter days, which could affect bark chlorophyll, production cause pending. The biogeography of aspens could greatly affect the variation within bark chlorophyll and other quaking aspen adaptations, so a potentially diverse genome and a variety of environments (Mock, 2013) could contribute to a remarkable variance all in the same species.

Acknowledgements:

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