Introduction:

Rapid adaptation, such as tolerance to new, and often time harsh environments, is a phenomenon that has become extremely relevant as the Earth increasingly faces rapid climate change and pollution that affect many plants and animals (Brown, 2015). It is predicted that 3,000-30,000 of all species will go extinct annually and 250,000 species have already gone extinct in the last century because they lack the capabilities to adapt quickly enough (Woodruff, 2001). Some well-known examples of organisms that have experienced rapid adaptation include bacteria accumulating antibiotic resistance (Davies et al., 2010), *Drosophila* adapting and tolerating to heat stress due to high expression levels of heat-shock proteins and/or other metabolic gene products (Fallis et al., 2011; Uy et al., 2015), and certain tilapia developing a tolerance to cold water through thermoregulation (Cnaani et al., 2000). Plants have also been shown to develop tolerance to many factors including damage caused by herbivores, droughts, and high salinity (Craine et al., 2013; Rosenthal et al., 1994; Wu et al., 2012).

Metal Contaminated Soil in Context

Metal contaminated soil is another stress factor that has also led to rapid adaptation and tolerance in plants (Wong, 2003). This event was often a consequence from mining which researchers took advantage of to observe plant tolerance (Wong, 2003). They have looked at tolerant plants subjected to varying degrees of metal contamination to determine whether co-tolerance with other metals is possible (Baker, 1987) The same authors attempted inducible tolerance by placing non tolerant plants in metal contaminated soil, and compared different physical components of plants including root length. It was observed that some plants may be co-tolerant, found a certain extent to

inducible tolerance, and that root length tended to be longer for those plants that are more tolerant to metal contaminated soils. (Baker, 1987).

Throughout studies on tolerant plants, researchers were conflicted on their understanding of the underlying genetic basis or mechanism regarding how rapid adaptations occur on the genetic level (Baker, 1987). For this reason, investigators sought out a plant which undergoes rapid adaptation to serve as an ideal candidate for genetic analysis. *Mimulus guttatus*, commonly known as the monkeyflower, was such a plant as it has variable populations, is widespread in large numbers, and is not difficult to manipulate in terms of crossing and seed collection (Allen et al., 1971; Hall et al., 2006; M. R. MacNair, 1983). This plant has developed tolerance to copper and is most centrally located in California by old mines (Mark R. MacNair et al., 1993).

The Role of Copper & M. guttatus Tolerance

Copper is an essential metal for plants that plays an important role in photosynthesis, the electron transport chain, and other key cellular processes (Yruela, 2009). However, excess of this metal is toxic to most plants because it leads to the creation of reactive oxygen radicals that damage cells and can potentially damage protein structures (Yruela, 2009). Certain plants such as *M. guttatus* and those of the *Brassica* genus are able to tolerate elevated copper levels by sustaining high concentrations within roots and tissues, pumping out excess metal from the cells, and executing other internal detoxification metal tolerance mechanisms (Deng et al., 2004; Yruela, 2009).

Research has been conducted on *M. guttatus* leading to the discovery of tolerance attributed to a multi-local trait involving multiple genes (Antonovics, 1975; M. R. Macnair, 1977). More specifically, the use of backcrossing led Wright and others to

locate and name the locus, *Tol1*, most associated with copper tolerance (K. M. Wright et al., 2013). While we know which locus contributes to tolerance, it is still unclear how populations of *M. guttatus* managed to acquire it (Antonovics, 1975; M. R. MacNair, 1983). It has been hypothesized that rapid evolution of tolerant plants occured either as a result of a low frequency of tolerant genotypes in the ancestral population or from new mutations soon after the development of mines (Gregory et al., 1965). Further analysis of a candidate gene within the *Tol1* region on more on- and off-mine (any population on uncontaminated soil) populations pertaining to metal tolerance in *M. guttatus* may help clarify which hypothesis is valid.

Experimental Objectives

The goal of this project is to distinguish if rapid adaptation and tolerance is the result of standing variation or more recent mutations in populations of *M. guttatus*. The four populations chosen for this study are located at varying distances from a copper mine field in California and the samples were collected by fellow researchers in the Willis laboratory of Duke University. I evaluated these populations both phenotypically and genotypically through population assays and DNA analyses, respectively. Within the survival assay, if all populations are found to withstand the copper contaminated soil, the hypothesis of standing variation is supported. Researchers have found that a survival of 4.5% within the populations would directly corroborate the hypothesis of standing variation (Kevin M. Wright et al., 2015). I have taken the percentage survivors for each population, performed comparisons to other populations, then genotyped them to determine if all survivors have the same genetic basis.

There are approximately 40 genes within the *Tol1* region which we could explore but only one multicopper oxidase gene was chosen for analysis in this study (Kevin M. Wright et al., 2015). I used a marker for this gene to run polymerase chain reactions (PCR) on different individuals from the four parental line populations to investigate whether the marker would make a good choice for further study on survivors from the survival assays. Additionally, we hoped to detect divergence, if any, between populations and therefore any occurrence of gene flow. If only known tolerant, closer to on-mine individuals have the copper oxidase gene, I would infer that the marker could be used to distinguish populations by level of distance. However, it would be necessary to confirm the results by testing other on-mine and off-mine populations.

The results of this project helps in understanding evolution in the context of how much variation needs to be in a population for an adaptive allele to be fixed in a short period and the significance of distance in levels of tolerance. The knowledge gained may be applied to organisms other than *M. guttatus* to understand how other species evolve quickly to new and harsh environments.

Methods:

Model Organism

Mimulus guttatus, of the Scrophulariaceae family, can be either an annual or perennial herbaceous plant that occurs grows predominantly throughout western North America in wet meadows or stream banks . These plants are known for their tubular, yellow flowers (Ritland, 1989).

M. guttatus Seeds

Researchers previously visited and obtained different *M. guttatus* populations surrounding a mine in Copperopolis, California. Once the plants were collected, researchers also collected and labeled seeds from varying populations. I selected four of these populations which were located 0.4 miles, 2.4 miles, 4.96 miles, and 100+ miles away from the mine for analysis. They are respectively labeled CTS, BLK, CVS, (Calaveras County, California) and IM (Oregon), and are depicted below in **Figure 1**. The soil collected from the mine has been labeled RTP, which was also used in this study.

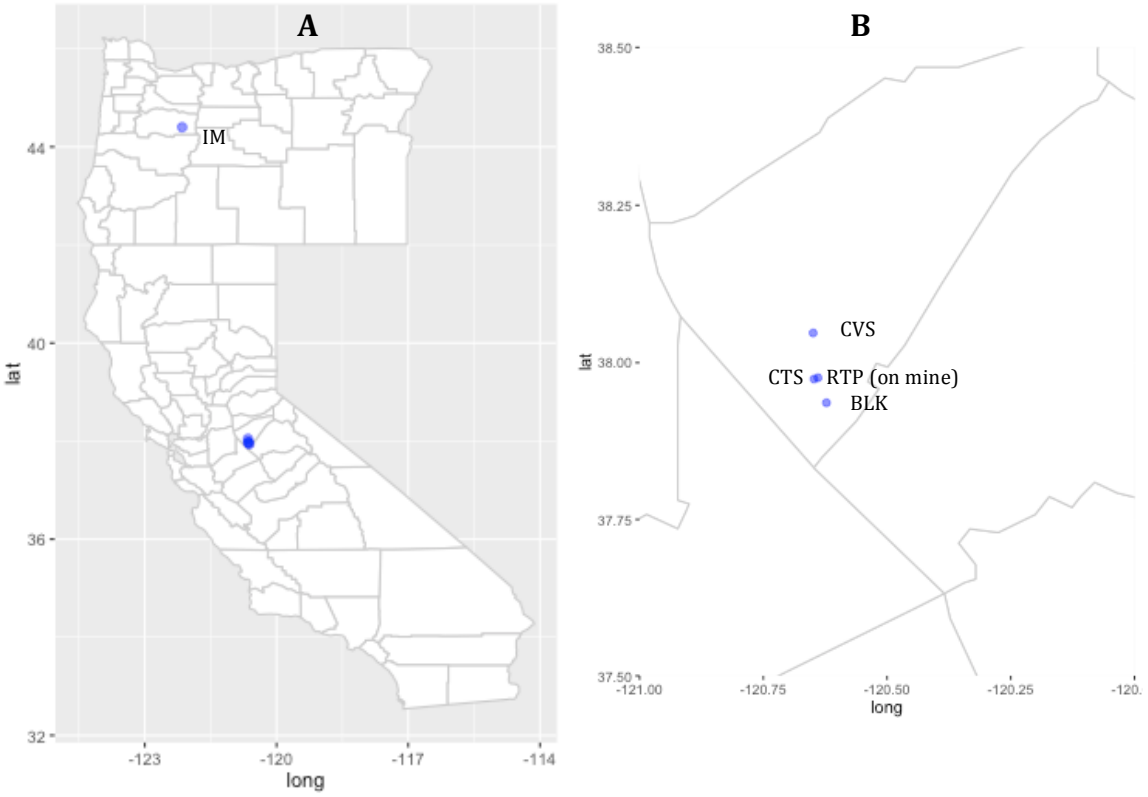

Figure 1- A and B: Maps of the varying locations (CVS, CTS, RTP, BLK, IM) of studied *M. guttatus* populations,
Figure A shows all populations and figure B zooms onto Calaveras County, California to show the three populations located there in more detail.

Survival Assay

Each population was grown in flats that would either contain RTP soil or normal potting soil for comparison. Specifically, a total of six flats were used, in which four flats contained RTP soil and each population separately and two flats had normal potting soil (control experiments) that would have two of the populations. For the flats containing RTP soil, rockwool, a growing media that retains water as the soil dries quickly (Jeong, 2016), was first placed about halfway in each slot. Then, the flats were filled with soil that had been sifted and autoclaved to remove any seed contaminants. Each flat contained 288 slots where 1-2 seeds were placed in each such that the number of seeds planted varied from 144-288. The flats were incubated in a cold room set at 4°C for one week to stimulate germination and, thereafter, were then placed in the greenhouse. Ultrapure water was used approximately every other day to maintain moisture levels within the soil. It was imperative to use ultrapure water so as not to introduce additional variables (bacterial and/or chemical contaminants) into the experiment. Once the seeds began to germinate, the flats were censused approximately twice a week for a month and the number of germinations and deaths were recorded. A plant was not considered a survivor if the tissue was brown, dry, and brittle and if growth stopped at the point of initial germination. On the other hand, survivors typically had some root growth as well as brightly colored leaves.

DNA Analysis

Once seeds that were planted grew big enough to have buds, two bud samples of each individual plant were placed in a tube, which was inserted into a 96-well plate. These samples were then stored at -80°C freezer for later use of DNA extraction later. A modified cetyltrimethyl ammonium bromide (CTAB) protocol was used for the DNA extraction (Kelly et al., 1998). The DNA samples were then used for 10μL PCR amplification reactions (Kelly et al., 1998). The resulting PCR products were then analyzed using gel electrophoresis where a 1% agarose solution was used with TAE buffer. Gels were ran for approximately 45 minutes, prior to visualization under UV light, using the program Quantity One® (Bio-Rad Laboratories).

Results:

Survival Assay

Total percentages of germinations from varying *M. Guttatus* populations were recorded after planted seeds were censused in both control and RTP (on-mine) soils (**Figure 2**). The number of survivors from the point of germination was used as the observed number in calculating the χ^2 value for each population. This was done to evaluate the goodness of fit of the data in regards to the 4.5% of survivors expected to follow the hypothesis of standing variation, as shown in **Table 1.**

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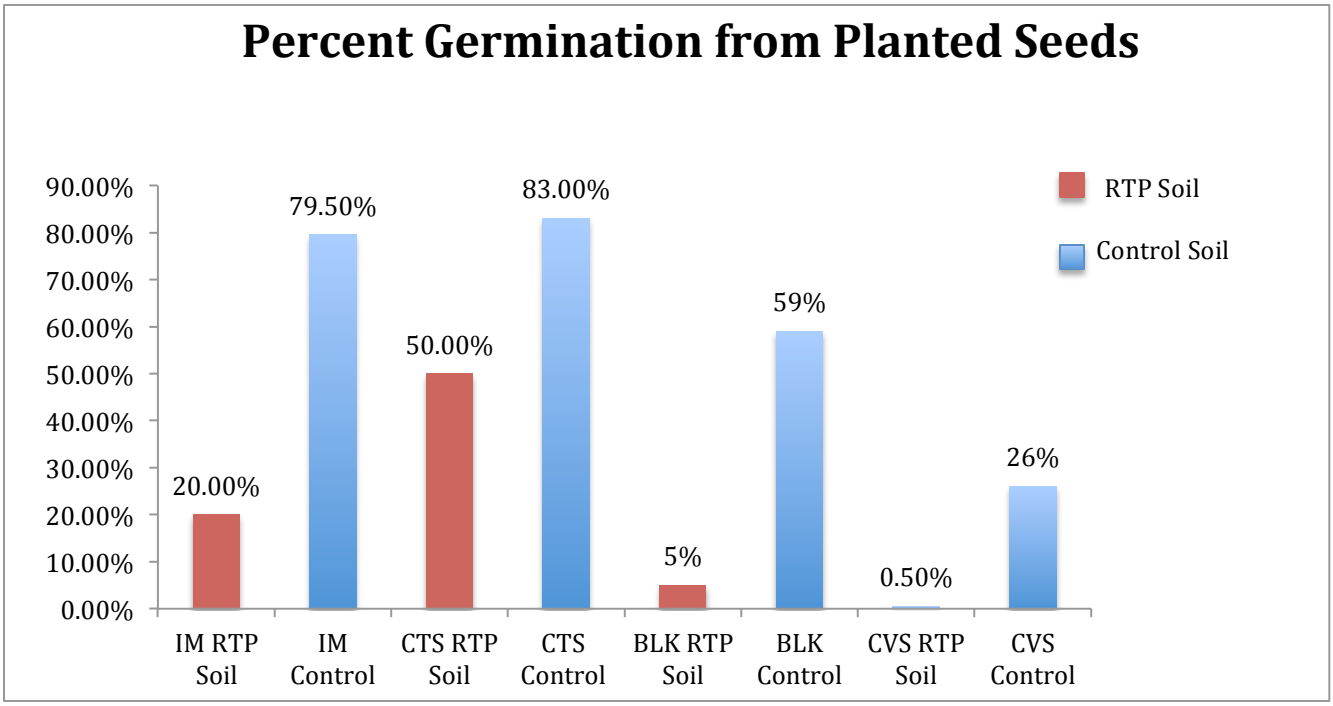

Figure 2: Percent germination of populations in RTP soil and control, Planted populations include IM(N = 278 in RTP soil, N = 252 in control), CTS (N = 144 in RTP soil, N = 186 in control), CVS (N = 288 in RTP soil, N = 144 in control), and BLK (N = 237 in RTP soil, N = 124 in control).

Table 1: Calculation of the χ^2 value from observed and expected survivors

Population	Observed	Expected	(O-E)^2/E (χ2 value)[a]	*p*-value
CTS	10	7	1.28571	0.256
BLK	7	13	2.7692	0.0961
CVS	1	13	11.0769	0.000874[b]
IM	12	13	0.0769	0.781
Total	30	46	5.565	0.0183[b]

[a] Chi-squared calculated using $\chi^2 = \frac{(\text{Observed}-\text{Expected})^2}{\text{Expected}}$ of each population. This was then used in RStudio with the code: pchisq(q = calculated *χ2 value* , df = 1, lower.tail = FALSE).

[b] *p*-values significant at the 0.05 level.

DNA Analysis

The gel results corresponding to the percentage of PCR products from extracted DNA of parental line bud samples using a marker for a copper oxidase gene are presented in **Table 2.** The presence of both raw DNA, for positive control, and PCR product were recorded. 46 samples of from the IM parental line population (100+ miles) and 30 samples from the RTP (on mine) population were taken. A 100 bp band size was in accord with this gene and a representative of a gel of the PCR products is presented in **Figure 5**.

Table 2: Percent of DNA samples and PCR product that expressed bands

Population	% Raw DNA and PCR Product Present
IM	61.9%
RTP	70%

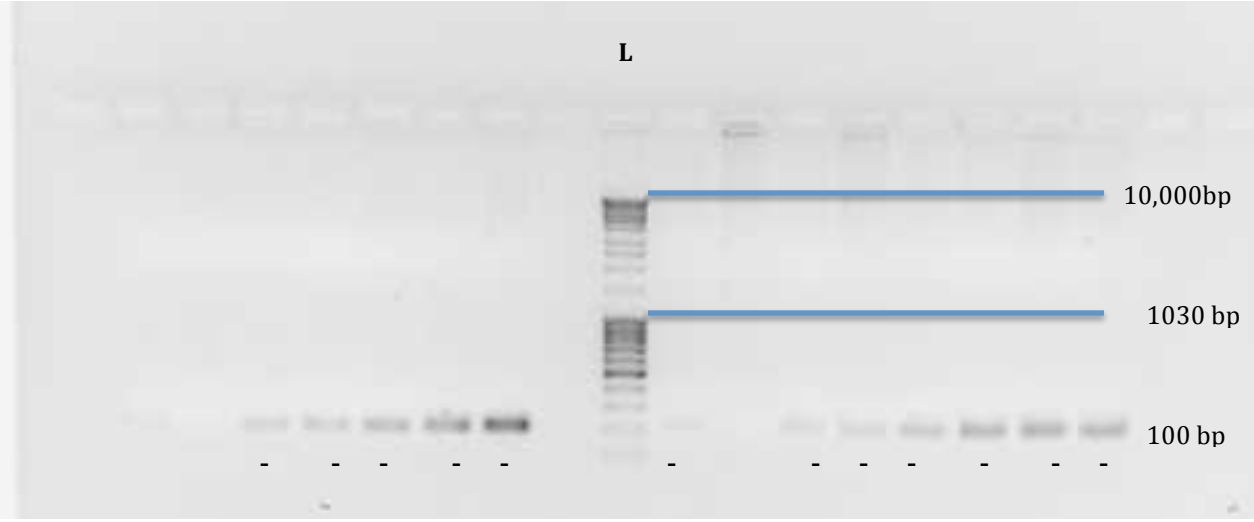

Figure 5: Gel electrophoresis results from PCR products,
This gel was done on the PCR products from individuals of the RTP parental line. On the right hand side are corresponding base pair sizes with 100 bp signifying a band for the copper oxidase gene. The ladder has a designated **L** above it. Recorded bands have a horizontal ticks below them.

Discussion:

I completed both a survival assay and DNA analysis of varying populations of *M. guttatus* to provide distinct and meaningful phenotypic and genotypic results. These experiments were necessary to unequivocally determine whether rapid adaptation evolutionary occurs due to standing variation or more recent mutations of genes. Previous researchers who studied plants tolerant to metal contaminated soil could not fully address these hypotheses mostly because they were confined to almost exclusively using phenotypic analysis (Antonovics, 1975; M. R. MacNair, 1983). Now, with the rise of simple, efficient, and affordable DNA analysis techniques, I could expand research that was previously performed. *M. guttatus* was the best option for this purpose because it was easy to manipulate and quick to grow (Ritland, 1989). The locus, named *Tol1,* for tolerance had also been identified and studied (Antonovics, 1975; M. R. Macnair, 1977; K. M. Wright et al., 2013). With that, I was able to choose and use a marker for a specific gene within this locus coding for copper oxidase which I inferred would illustrate differences in the varying distances of the populations that could be used for further study (Kevin M. Wright et al., 2015).

I completed the survival assays in two separate stages where IM and CTS were grown first in the fall of 2015 and then I planted the BLK and CVS populations afterwards in the spring of 2016. In the first phase assay, we observed more than 100% germinations from planted seeds of both IM and CTS populations. The most plausible cause for these results is that the RTP soil, which was collected directly from the copper mine, was contaminated with tolerant seeds. These seeds then

germinated and appeared to be survivors, contributing to false positives. Therefore, I decided to repeat this segment of the experiment with autoclaved soil in the spring of 2016. This resulted in germinations as shown in **Figure 2**.

I used the results from the assays to complete a chi-square goodness of fit model with the expected values deriving from the calculated expected frequency of survivors being 4.5%. This value was specifically estimated for the expected survival percentage of the multi copper oxidase gene (Kevin M. Wright et al., 2015). *P*-values were calculated for each value and also for the total. Three out of the four populations had *p*-values above the 0.05 significant level, indicating that the observed survivors did not deviate from the expected significantly. Only the CVS population and the combined populations had statistically significant *p*-values. I believe that these findings provide support that there is standing variation within these populations. However, other possible explanations for the results cannot be excluded. There may have been gene flow between populations along with standing variation, skewing the level of tolerance populations experience. Also, there were only four populations used out of the 1000+ and the survival assays were only conducted once for each population, with less than 300 seeds for each. It should also be noted that researchers believe the 4.5% of tolerance may be an inflated number, as this gene has a small scaffold and few single nucleotide polymorphisms (SNPs). In actuality, it may be closer to 0.4%-0.6% (Kevin M. Wright et al., 2015). These limitations are reasons this research should be expanded further.

In terms of the DNA analysis, the percentage of bands from PCR products was comparable between both IM and RTP populations (**Table 2)**. This suggests that the

marker is not useful in distinguishing the distances of the populations from the site of copper soil contamination. However, the marker proves useful for investigating, as it is within the *Tol1* region and contributes to the tolerance of *M. guttatus.*

Future Directions

While standing variation is supported in the context of this study, *M. guttatus* should be studied in greater depth to fully understand and predict tolerance based on distance more accurately as well as the relevancy of different DNA markers outside the *Tol1* region. The survival assay can be improved by using a larger sample size from more populations, repeating the assay for each population to prevent skewing, and including populations that extend more in distance. For example, there are samples of *M. guttatus* which researchers have taken from as far as British Columbia while the IM population I used was the furthest from the site of copper soil contamination, located in Oregon. The combination of these precautions would provide more accurate and conclusive results.

There is also a considerable breadth of research that should be conducted with DNA analysis. With the discovery of many markers in the *Tol1* region, each one needs to be studied to truly comprehend how tolerance functions in *M. guttatus* at the allelic level (Kevin M. Wright et al., 2015). Some of these studies may include quantitative trait locus (QTL) mapping and running more PCR reactions on varying *Mimulus* populations. Identifying how each marker may affect the varying populations would clarify the impact of gene flow and standing variation. This provides a broader impact on understanding not only metal tolerance but also the

overall patterns of rapid adaptation and tolerance, which is becoming more and more relevant as the environment continues to change rapidly.

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