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Growing on a Scar: Genetic Variation of Achillea millefolium near Meeker, Colorado

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Growing on a Scar: Genetic Variation of *Achilla Millefolium* near Meeker, Colorado

The National Mining Association (2016) reported that metal ore mining contributed almost 1.8 billion dollars towards Colorado's gross domestic product (GDP) in 2015. At the beginning of 2017 there were 38 applications for mineral extraction across the state of Colorado (Colorado Division of Reclamation, Mining and Safety, 2016). Human mining activity can impact an environment by introducing high concentrations of heavy metals into soils and waterways. Heavy metals and hydrocarbons deposited by industry and mining can alter ecosystem interactions and are sources of environmental selection pressures (Atibu et al., 2018). Organisms have shown adaptations to anthropogenic environments, such as changing their generational period in the case of earthworms in a copper-saturated forest (Fisker et al., 2011). The xenobiotic compounds that are a byproduct of mining interfere with plant metabolic pathways, requiring plants to direct energy towards sequestering them away from vital tissues. Consequently, novel anthropogenic conditions in an environment can select for genotypes that alter the genetic structure of plant populations to their benefit or ill.

Populations' adaptation to environmental stress informs our understanding of adaptation during natural and artificial disturbance events and can aid in balancing economic activity with conservation (Carpenter, 1976). For example, in a scenario where populations are differentially affected by mining and subsequent reclamation procedures, focus and money can be precisely allocated to sites that require more intensive mitigation. The goal of this project was to determine the impact of mining on western yarrow (*Achillea millefolium)*, a perennial wildflower in the Colorado Rocky Mountains. We focus on identifying whether mining activity is changing the genetic makeup of populations. This study tested the general hypothesis that human-induced

soil pollution is a significant selection pressure and impacts genetic variation in local flora. Specifically, we tested the null hypothesis that there would be no genetic difference between plants growing on a mine and plants growing away from a mine.

Review of Related Literature

Environmental Change and Adaptation

Soil chemistry is a fundamental variable that defines an ecosystem. Soil qualities such as acidity and nutrient content determine what plants and soil invertebrates are most likely to grow in a specific location. Mining results in extreme changes to acidity and deposition of toxins such as arsenic that disrupt enzyme activity in soil (Bhattacharyya et al., 2008). These novel changes can profoundly impact plant-community dynamics. The ability for many species to grow is greatly reduced by the presence of various heavy metals such as zinc, cadmium, and lead. Species richness in a community can decrease simply by existing adjacent to a mine or smelter, leading to the danger of an ecosystem collapsing or undergoing permanent dynamic change (Hernandez & Pastor, 2008; Vidic et al., 2006). Soil organisms such as bacteria and micro-arthropods tolerate moderate pollution, but high enough concentrations, such as those normally detected at mines, cull populations before adaptation can occur (Salminen et al., 2001). Heavy metal concentrations are, thus, a significant selective force that can change community relationships and organisms genotype frequencies.

In fertilization experiments on two flowering species, McNeilly and Antonovics (1968) showed that the ability for the plants growing on a mine to mate with an isolated population was greatly reduced. This evidence supported barriers to migration because of the mine. Furthermore, additional data indicated that the barrier was genetic due to changes in flowering

times between mine plants and non-mine plants (McNeilly & Antonivics, 1968). This isolation of populations could lead to reduced genetic variation if only a minority of alleles are capable of existing on toxic soils or by genetic drift.

Some exhausted mines and polluting industrial centers are found close to human populations or are situated in or adjacent to recreational and public lands. Public concern associated with the impact of industrial sites is often focused on the immediate health effects on iconic fauna rather than the whole community, prompting studies of mammals and birds at polluted sites. At the Rocky Mountain National Arsenal wildlife refuge, mule deer have grazed around the toxic waste site since before the refuge's inception in 1992 and long-term impacts of the effects of grazing is a cause for investigation. Despite significant concerns of negative impacts from pollution at this site, Creekmore et al. (1999) found no evidence to support that grazing at the site was harming the deer.

Organisms naturally evolve mechanisms for tolerating extremely toxic environments. Sulfur-compounds are a lasting threat to freshwater ecosystems and a common waste product from industrial operations and mining. Sulfides compete with oxygen in metabolic respiration in organisms, disrupting ATP production. However, sulfides occur naturally in rivers adjacent to volcanic activity, causing naturally acidic rivers and streams. In Mexico, the Atlantic molly (*Poecilia mexicana)* exists in rivers affected by volcanic sulfide run-off. This is due to a novel mutation in the mitochondrial genome but populations of mollys had been geographically isolated for some time. An investigation into the mitochondrial lineages suggested that this adaptation evolved in independent incidences across distinct molly populations (Pfenninger et al., 2014). Extreme environments exist on earth, but adaptation can result in novel genotypes for these extreme conditions and diversify the genetic makeup of species.

Plants have also evolved physiological tolerance mechanisms to deal with extreme environments. Their roots passively absorb ions and other nutrients as part of their growth and unintentionally absorb heavy metals and other toxins. Physiological pathways have evolved in some species to sequester biologically harmful substances into nodules or vacuoles in the stems and leaves and away from areas vital to growth and reproduction (Dubey et al., 2014). The roots uptake heavy metals, where they are then moved to nodules in the roots or vacuoles in the cell. Some species deal with metals more effectively and will have greater success in polluted soils, while other plants experience severe damage to their roots and reproductive systems, exhibiting stunted growth (Kazemeini et al., 2013). Species that do not adapt as well to managing toxic intake must divert more energy from growth and reproduction, thereby affecting their fitness. (Fomina & Gadd, 2014). The efficiency of their metabolism is influenced by a number of physiological pathways that are ultimately regulated by genetics.

Discourse on the Impacts of Mining Pollution

The genetic diversity of a species is a commonly used in tracking evolutionary divergence and in assessing how likely a population will tolerate sudden changes to its environment. Populations can be quantitatively analyzed by examining differences in multiple single strand repeat (microsatellite) loci in a species (Avise, 2004). These regions are subject to natural mutation and are commonly used as a measure of population health by studying the quantity of genetic variation in the form of private alleles and heterozygosity (Kashi et al., 1997). Heterozygosity is a strong measure of a population's robustness against novel environmental change. A greater level of heterozygosity in a species or population generally allows it to have a more robust response in the presence of a new pressure in the environment (Leberg & Vrijenhoek, 1994). Additionally, because microsatellite alleles can have variable sizes, the

diversity and range of alleles can be an indicator of quantitative traits that can show graded adaptation to an environment (Vilas et al., 2015).

Environmental contaminants are traditionally viewed as detrimental with populations overall being unable to tolerate them. Using genetic markers, gene flow has been shown to be maintained between populations at contaminated sites in Canadian deciduous forests (Makela et al., 2016). While heavy metals affected tree growth and health, heterozygosity and effective number of alleles appeared to be maintained by gene flow from beyond the contaminated sites, due to high mobility of the plant's pollen. Additional genetic studies in Canada found no association between genetic variation and a population's vicinity to mining activity even over a generation (Dobrzeniecka et al., 2011; Vandeligt et al., 2011). Genetic variation in both Poaceae, Caryophyllaceae, and Crassulaceae were even found to be higher in populations existing on industrial and contaminated sites than in unimpacted populations (Deng et al., 2007; Mengoni et al., 2000; Xie et al., 2016). This suggests that the contamination selected for higher rates of polymorphisms and represented adaptation to heterogenic environments.

Methodology

Study Site

The Butterfly-Burrell Mine (Figure 1) is located northeast of Meeker, CO, in the White River National Forest. This uranium/vanadium mine has been closed since the early 2000s and reclamation of the area by a private company started in 2005 and ended in 2006. Prior to reclamation, concentrations of vanadium and arsenic at the mine were detected up to 100 times the background concentration, and arsenic was detected at high levels downslope in the local watershed (AAK, 2005). Radiation contamination was also detected, especially gamma

radiation, which is a mutagen that profoundly effects biological processes. This mine (see Figure 1) was selected for this study because of the high abundance of our study organism in the region and for its accessibility.

Study Organism

Western yarrow (*Achillea millefolium* L., Asteraceae, Figure 2) is a long-lived perennial herbaceous plant with varying flower color. It does not self-fertilize, and its seeds are highly mobile; the species is distributed across the northern hemisphere with only minor differences in its morphology (Warwick & Black, 1982). Western yarrow grows to a maximum height of one meter and has compound, pubescent leaves (Weatherby, 2002). In North America, western yarrow is used medicinally in most indigenous tribes by steeping its leaves in water to make tea or by using leaf pulp to treat cuts and sores. Western yarrow is reported to contain over 200 secondary compounds with various physiological effects and is studied as a potential source for novel treatments to ulcers, cancer, and diabetes. Syso et al. (2016) demonstrated that western yarrow sequesters pollutants such as lead, cadmium, and zinc in its leaves and stems along roads and industrial parks where disturbances to the soil are high. Western yarrow is currently under review as a phytoremediator in restoring damaged habitat in the Rocky Mountains (Winslow, 2014).

Data Collection and Analysis

On June 30, 2017, we collected leaf tissue from 88 individuals at both mines and systematically along the forest access road at 100 meters, 1,000 meters, and 2,000 meters from the mines (Table 1). Each individual's location was recorded using a GPS positioner and later uploaded into ArcMap (Esri 10.5) (Figure 3). Leaf tissue was ground in liquid nitrogen with a mortar and pestle, and genomic DNA was extracted using Plant DNEasy Kits.

Microsatellite Amplification

Two microsatellite loci were selected from Rahimmalek et al. (2011) and adapted for a florescent tag technique (Table 1). The technique involves adding a "tag" sequence to the five-foot end of either the forward or reverse primer. This modification allows for a three-primer PCR reaction with the forward and reverse primers, plus a fluorescent labeled primer that is complementary to the known sequence (Boutin-Ganache et al., 2001). Additional optimization of the primers was done by modifying anneal temperatures and magnesium concentrations during PCR on Mastercycler proS Thermal Cyclers(Table 1). Successful fragment amplification was confirmed with gel electrophoresis and samples were sent to Arizona State University Core DNA Lab for fragment analysis with an Applied Biosystems 3730 Genetic Analyzer.

Data Analysis

Microsatellite loci were scored in Geneious^{TM} version 8.0.4. Two measures of genetic variation were examined using GenAlEx (Peakall & Smouse, 2012): Observed heterozygosity ($H_{o.}$) and average number of alleles. Measures of genetic diversity were averaged across both loci and reported with a standard error. We compared the mean heterozygosity between the mine and off-site plants using an unpaired t-test. Allelic frequency and range of each locus was compared to see if the populations had distinctive alleles.

Results

Primer Success and Allelic Frequency

Primers for the loci Amk54 and Amk439 amplified reliably and were used in this study. The Amk439 locus varied in size from 255–267 bp, with the 261 bp and 263 bp alleles being most common at all populations (Figure 4). The Amk54 locus varied in size from 220–242 bp,

with the 236 bp allele being most common at all populations. Amk54 and Amk439 have a mean number of alleles of 4.8 and 4.2, respectively (Table 2). The 2k population had two private alleles, one in each locus. The Burrell population had one private allele in Amk54 while Butterfly and 1k shared an allele in Amk439 (Figure 4).

Heterozygosity

Heterozygosity varied slightly between the mines, likely due to their proximity to each other. The highest measured heterozygosity was recorded at 1 km population ($H_0 = 0.688 \pm 0.250$) and the lowest was measured at the 2 km population ($H_0 = 0.473 \pm 0.340$) (Figure 5, Table 2). Overall, heterozygosity is high across all populations (Table 1). No statistically significant difference in heterozygosity was detected between the mine and off-site populations which supports our null hypothesis (Table 3).

Discussion

Adaptive Responses to Metal Stress

This research asked if the contaminated soil of a uranium/vanadium mine in the Rocky Mountains impacted local genotypes in a way that would impact the long-term viability of populations. The general concern of conservationists and environmentalists is that intense pollution selects for lower heterozygosity by removing no adapted genotypes. Mining in general serves as a proxy for primary succession because the habitat is destroyed to access underground ore and organisms must recolonize once mining is completed.

The evidence produced in this study did not detect a difference between western yarrow growing on and off the mine. While private alleles were detected, they did not occur at a frequency to suggest selection (Figure 4). Western yarrow as a generalist species could naturally

have more allelic diversity which would allow for variable tolerance mechanisms to deal with the variety of habitats it encounters. Generalist plants can thrive in varying light availability and soil quality and may have more genetic variation, allowing them to tolerate disturbances (Theriault et al., 2013). Another possibility is that the reclamation activity in the mid-2000s effectively reduced the contamination present at the mine, reducing the presence of selective pressures on colonization and growth. Theriault et al. (2014) found that genetic variation was not impacted at a reclaimed site in northern Canada over several generations and suggest that a threshold of contamination needs to exist to produce selection. Remediation activities at Butterfly-Burrell could have removed enough contamination to reduce any selective pressure on yarrow.

Extreme soil conditions from mining activities can persist for millennia (Kelepertsis & Bibou, 1991) and the dynamic nature of populations allows them to respond to rapid and persistent disruptions in the environment. A beneficial mutation may survive initial population disruption, then thrive and propagate throughout the population. High genetic variation in a population allows for more opportunities for a population to persist through rapid change (Thorsten et al., 2005). The western yarrow in this study was not lacking in heterozygosity, lending support to its robustness of its genome. As a cosmopolitan species with high migration, western yarrow may be less impacted by drift and ecological isolation, allowing it to adapt more easily and colonize heterogeneous environments (Blanquart et al., 2012) Given the species' global distribution, it is likely adapted to a wide variety of soil types and levels of contamination.

High heterozygosity after exposure to various heavy metals has been reported in invertebrate communities and represents natural adaptation (Hochmuth et al., 2015). Tolerance and high heterozygosity in an experimental population of *Daphnia magna* occurred after only eight generations. The authors also noted that while their populations recovered, the dynamics of

population size and reproductive output changed as a response to the metal it was exposed to and never returned to the conditions of the control group (Hochmuth et al., 2015). Bergmann and Hosius (1996) grew a successive generation of seedlings in metal-contaminated soils and found that survivorship was low in their off-spring, yet the survivors had greater heterozygosity. These previous studies suggest that heavy metal tolerance could be a mechanism for rapid adaptation and isolation among populations. Genetic variation was not recorded in the study western yarrow population before mine closure; therefore, we are unable to determine if heterozygosity and allelic diversity were different before remediation efforts. This was addressed by comparing western yarrow populations that are upslope and distant from the populations growing on the mine. If there was a selective force at work, variability would indicate so in the results.

Studies of this kind generally focus on genetic variation to determine a population's tolerance to polluted soils. However, plant responses to metal stress can involve changes in a plant's growth (Kazemeini et al., 2013) which are due to the siphoning of energy to upregulate pathways responsible for producing anti-oxidant metabolites and removing metals from vital tissues (Dubey et al., 2014). These traits may be more accurately represented in markers by allelic diversity because they relate to quantitative traits in coding genes (Vilas et al., 2015). Allelic diversity may also be a more accurate diversity measurement than heterozygosity in situations where the number of available loci for a species is limited. Allelic diversity in the yarrow does not indicate any pattern or exception in the population which indicates that the populations did not differ from each other.

An additional issue that arose during this investigation came from the microsatellite primers. Two primers for genetic analysis represents a small sample size. A minimum of six microsatellites is a general baseline with studies utilizing as many as 20 loci for analyzing gene

flow and genetic structure. The low rate of successful optimization of the primers could be due to the tagging technique used by our lab or to divergence of western yarrow lineages between America and Eurasia, with markers being derived from Eurasian plants. Ultimately, the small genetic sample size means the results presented must be considered with caution and treated as a preliminary study. Further studies of western yarrow in America will require developing a robust database of loci to increase sampling.

This study focused on microsatellite variation in the genome of yarrow. While analyses of population genetic diversity provide an understanding of a species' adaptation to novel selection pressures, the value of a comprehensive approach should not be ignored. The relationship between yarrow and its pollinators and herbivores was not considered in this study and could provide a community wide understanding of how mine tailings will impact the larger ecology of a habitat.

In conclusion, soil pollution was explored as a selective pressure that impacts genetic diversity and therefore the likelihood of local extinction in plants. Our species, western yarrow did not show evidence that its frequency or diversity of alleles was affected. Metal contamination of the soil may not present an important selective factor in these populations.

Acknowledgements

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Figures

Figure 1. Burrell Mine, White River National Forest, June 30, 2017. The fencing in the background is where imported plant seeds were sowed.

Figure 2. Western Yarrow from the field site. This individual is of a less common white-purple phenotype.

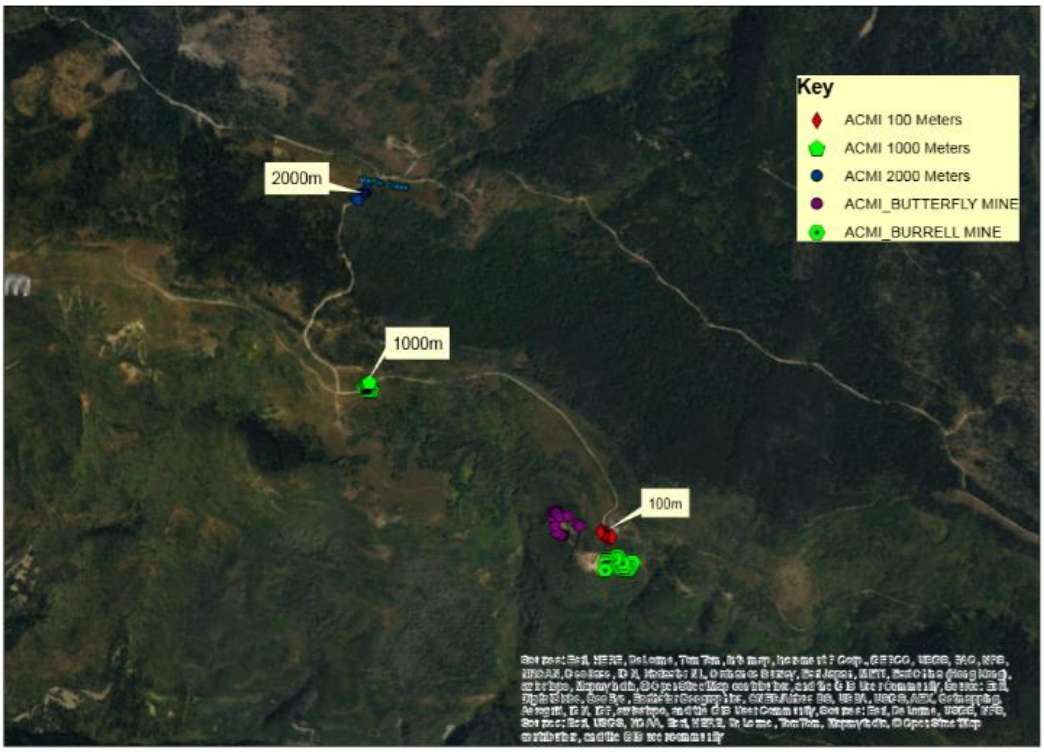

Figure 3. Individual GPS position of plants sampled for this study.

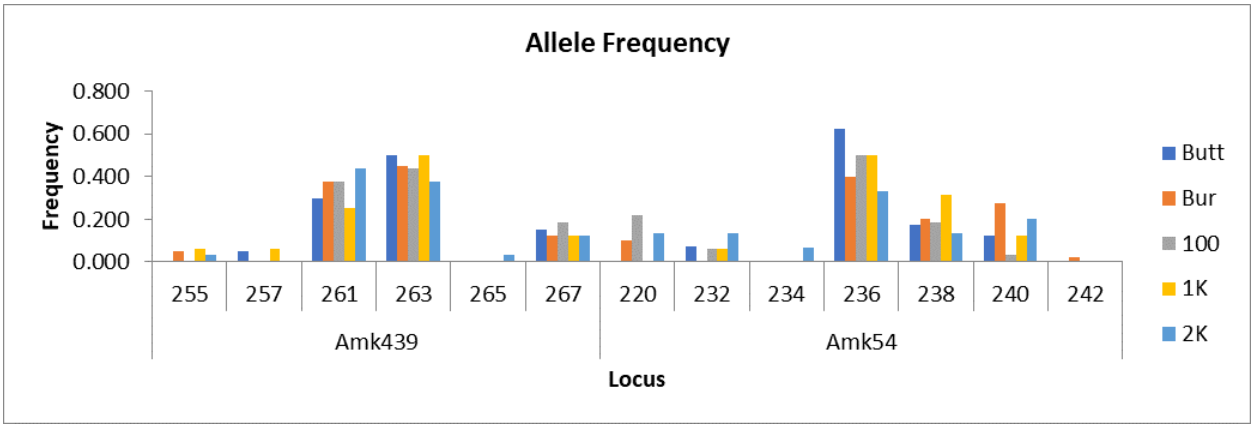

Figure 4. The frequency of alleles at each locus. *Butt* refers to the Butterfly mine population (n=20). *Bur* refers to Burrell (n=20). The additional populations are *100* = 100 meters (n=16), *1k* = 1 kilometers (n=16), and *2k* = 2 kilometers (n=16).

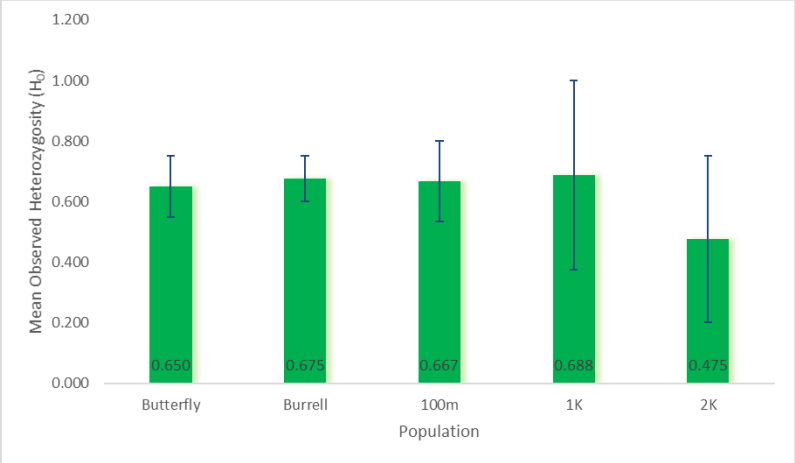

Figure 5. Mean observed heterozygosity of yarrow using two loci with standard error.

Tables

Table 1

Primer Characteristics and Amplification Conditions Based on Loci Adapted from Rahimmalek et al. (2011)

Locus ID	Repeat Motif	Anneal Temperature (˚C)	25 nM $MgCl_2$ (μL)
Amk54	TA	61.7	1
Amk439	(ATG)TG(ATG)	65.1	3

Table 2

Population Genetic Diversity Characteristics Including Number of Sampled Individuals, Mean Heterozygosity (Ho), and Mean Number of Alleles, ± the Standard Error

Population	*Number of individuals*	*Mean Ho*	*Average Number of Alleles*
Butterfly	20	0.650 ± 0.100	4.0 ± 0.0
Burrell	20	0.675 ± 0.075	4.5 ± 0.5
100 meters	16	0.667 ± 0.125	4.0 ± 1.0
1 Kilometer	16	0.688 ± 0.250	4.5 ± 0.5
2 Kilometers	16	0.475 ± 0.340	5.5 ± 0.5

Table 3

Comparison of Observed Heterozygosity Between Each Mine and all Non-Mine Populations Using an Unpaired T-test

Mine	*Non-mine*	*df*	*T-statistic*	*P-value*
Butterfly	100m	34	0.2404	0.8114
Burrell	100m	34	0.0848	0.9332
Butterfly	1k	34	0.1002	0.9207
Burrell	1k	34	0.21	0.8349
Butterfly	2k	34	0.7097	0.4837
Butterfly	2k	34	0.6424	0.5249