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Developing Genetic Tools for Sochan (*Rudbeckia laciniata* L., Asteraceae) to Ensure Sustainable Harvest within the Great Smoky Mountains National Park

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Abstract

The native flora of the Great Smoky Mountains have been traditionally utilized by local communities for their nutritional content or ceremonial value. Sochan (*Rudbeckia laciniata* L., Asteraceae) is one such plant that has been harvested by the Eastern Band of Cherokee Indians (EBCI) for thousands of years, eaten in spring ceremonies. Since the National Park Service (NPS) was created in 1934, multiple sets of rules and regulations have been enacted for the collection of native plants. Most recently, under 36 CFR Part 2, tribal governments such as the EBCI can collect and harvest native plants if they hold traditional value. However, the effects of such harvests on population size, structure, and genetic diversity of plants including *R. laciniata* is unknown, and means to measure sochan's genetic diversity have yet to be developed. The purpose of this research was to develop genetic markers in order to examine the effects of harvesting on *R. laciniata* populations in order to recommend sustainable harvesting practices. First, we collected 30 leaf samples from each of three *R. laciniata* populations in western North Carolina; each site had more than 100 sochan individuals. Thus far, 32 leaf samples have had their DNA extracted and amplified at 24 microsatellite loci. Of these loci, 15 have yielded amplicons, and 5 of those showed polymorphisms. These loci and data collected from them will be given to NPS as a baseline measure of genetic diversity, and compared to plants from harvested and unharvested park populations. This research intends to preserve not only the species, but also traditional practices of the EBCI.

1. Introduction

The Great Smoky Mountains National Park (GSMNP) is home to a diverse array of native plant species, including the cutleaf coneflower (*Rudbeckia laciniata* L. var. *laciniata*). *R. laciniata* is a perennial herb belonging to the Asteraceae family and endemic to eastern North America which can grow up to three meters high during spring months. Known as sochan to the Eastern Band of Cherokee Indians (EBCI), and referred to hereafter as sochan, the shoot and leaves have been harvested as a food and ceremonial resource by the EBCI for roughly ten thousand years[1]. In 1983, the National Park Service (NPS) placed regulations on harvesting and foraging of *R. laciniata* within park boundaries[2]. These regulations attempted to sustain *R. laciniata* and other native flora in national parks by prohibiting actions that could cause disturbances or extirpations (local extinctions). *R. laciniata* was considered for exemption because it not only serves as a nutritive resource for the Eastern Band, but is also an important component of their heritage. In 2016, the NPS finalized the Gathering of Certain Plants or Plant Parts by Federally Recognized Indian Tribes for Traditional Purposes[2]. This ruling authorized the propagation and gathering of plant species for use by tribal members in limited park locations.

Both the NPS and EBCI have expressed interest in maintaining the natural flora of park resources[2]. However, it is possible that harvesting *R. laciniata* could have a detrimental impact on the species' survival, at least within park

boundaries. Previous research on ramps (*Allium tricoccum*), a perennial plant in the GSMNP that is also a target of ECBI harvest, determined that 10% of harvest could only take place every ten years to maintain sustainable harvests [3]. However, methods used by Rock et al. (2004) were not representative of EBCI traditional harvests. Tissue collection in this study was completely destructive due to removal of *A. tricoccum* bulbs, but the EBCI traditionally collect *A. tricoccum* above the root. These experimental methods were acknowledged in 2009, but not before the results of the study influenced prohibition of ramp collection within GSMNP in 2002[1]. With this in mind, it would be beneficial to study populations of *R. laciniata* both before and after harvest, with harvest done in a way that upholds tribal traditions.

Populations with low genetic diversity have decreased intraspecific fitness and survival due to loss of habitat, pathogens, harvest pressures, climate change and inbreeding[4,6]. Traits related to fitness found in previous research, such as seed production and germination success, are negatively affected by low genetic diversity[5]. Diminished allelic variances and heterozygosity within a population, can, in turn, reduce population size further. This negative feedback loop, known as an extinction vortex, could occur within sochan populations if they are overharvested[6]. In order to analyze the conservation efforts made by both the EBCI and GSMNP, allelic variances among sochan individuals and populations must be understood.

Research in the 1980s demonstrated the presence of microsatellites across multiple eukaryotic genomes. Microsatellites consist of 1-6 base pairs (bp) of repeated regions in tandem within mostly intergenic regions of the genome[7]. Mutation rate is directly correlated with repeat number; extension or contraction of repeats occur during DNA replication when DNA polymerase slips from the template strand. Closely related individuals and populations are often distinguished by differences in microsatellite loci[8]. Difference in the number of tandem repeats within a population (polymorphisms) can be detected with polymerase chain reaction (PCR). Therefore, microsatellite repeats offer an effective way to study population genetics due to their reproducibility, low-cost, variability, and abundance within genomes. Genetic diversity analyses using microsatellites have been conducted on populations of groundnuts (*Arachis hypogaea*), citrus (*Citrus* sp.), soybeans (*Glycine max*), peaches (*Prunus persica*), almonds (P. amygdalus), and European raspberry (*Rubus idaeus*), among many other plant species[9].

The intended goal of this research was to use demographic monitoring and genetic assessment to determine the status of *R. laciniata* populations within the GSMNP. We will use our information to provide recommendations for sustainable harvest based on traditional methods that are sustainable to conserve the plant's genetic diversity. We also expect that this research will provide GSMNP staff with the appropriate knowledge to make informed decisions about sites that are most suitable for harvest.

2. Methods

Potential microsatellites for sochan were developed in 2018. First, DNA was extracted from a representative *R. laciniata* individual using a modified CTAB method developed by Doyle and Doyle (1987). The extract was mailed to West Virginia University (WVU) for Illumina sequencing (Illumina 2018). Electronic data provided by WVU core facility was forwarded to Drs. Matt Estep and Jennifer Rhode Ward. Low quality data was removed, and microsatellite regions were identified by Dr. Estep and research students with msatcommander[11,12]. Based on msat commander results, primers were ordered for *R. laciniata* genetic analyses.

In conjunction with GSMNP personnel, three large western North Carolina populations of sochan were chosen in July 2019. Populations were picked based on increased perceived diversity (populations greater than 100 individuals) and harvest potential. The three locations in North Carolina were separated by >10 km to avoid metapopulation connectivity and dynamics. In order to obtain accurate ranges of diversity, thirty plants from each site were selected at random for genetic characterization[7]. In order to avoid destruction, one leaf from each plant was collected, stored in silica to dehydrate the tissue, then moved to a -80°C freezer until DNA extraction. Tissues were ground with a pellet pestle and Pellet Pestle Cordless Motor (Kimble®/Kontes, Rockwood, TN) and autoclaved sand in a 1.5 mL Eppendorf tube and extracted using a DNEasy Plant Mini-Kit™ (Qiagen, Hilden, Germany). In order to ensure the quality and quantity of extracted DNA, 1% agarose gel electrophoresis and Nano Drop ND-1000™ spectrophotometer were utilized.

Each PCR reaction contained 5.5 µL of PCR H_2O, 0.5 µL of 10 µM forward and reverse primers, and 12.5 µL of Master Mix (DNA polymerase, dNTPs and reaction buffer) in a 0.2 mL PCR microtube. 6 µL of DNA were transferred to a PCR microtube, making it a total reaction volume of 25 µL. Amplification of DNA was performed on a T100™ Thermal Cycler for 35 cycles. The PCR protocol was an initial 2 min at 94 °C followed by 40 sec at 94°C, 40 sec at T_A, and a final 1 min at 72 °C. Annealing temperatures were chosen based on GC content predictions from msat commander CITE. Samples were then held at 12 °C until taken out of the thermocycler. DNA amplification success

was analyzed on 1% agarose gel in TAE buffer (40 mM Tris, 20 mM acetic acid, 1 mM EDTA). Gels were imaged using a BioDoc-It®2. Loci screened are listed below (Table 1).

Table 1. Characteristics of loci screened.

Repeat Pattern	Locus ID	Microsatellite
Dinucleotide	3668, 6256, 6988, 7389, 494083, 507198, 712442, 915159, 924167, 1958302, 2266431, 2372717	AG, AT, AT, AT, AT, AT, AT, AG, AT, AT, AG, AA
Trinucleotide	819404, 914246, 1157684, 1373052, 1447346, 2562754, 2681589, 3506761	ATC, ATC, AAT, AAC, ATC, AAG, AAG, AAG
Tetranucleotide	226501, 415218, 2192491, 2742773	AAAG, AATG, AAAC, ACAT

3. Results

A total of 52 leaf tissue samples have had DNA extracted. Of the 52 DNA extracts, 32 have been screened. All of the primers containing tetranucleotide repeats successfully produced at least 50% PCR products. Six of the twelve dinucleotide repeats and five of the eight trinucleotides were successful (Table 2). Of the primer pairs screened, two trinucleotide repeats and three tetranucleotide repeats showed polymorphisms.

Table 2. Percentage and total number of nucleotide repeats amplified at 24 different *R. laciniata* loci.

Repeat Pattern	Number Loci Screened	% Amplified	% Polymorphic
Dinucleotide	12	50%	0%
Trinucleotide	8	75%	25%
Tetranucleotide	4	100%	75%

4. Discussion

This research developed 15 genetic markers of sochan populations in order to conserve this species. Neutral genetic markers, ones that do not affect fitness, provide researchers a way to analyze genetic variation without environmental influences[11]. Collecting genetic information through microsatellite markers over time can give additional insight to molecular diversity fluctuation within and among populations. Thus far, a total of 24 microsatellite loci have been screened on 32 *R. laciniata* DNA extracts.

Further research can now use these approaches to screen more loci, and use these tested markers to determine the diversity and relatedness of multiple *R. laciniata* populations. Ideally, analyses would use at least 10 working primer pairs. I propose that in order to continue this research and develop genetic markers, 40 more *R. laciniata* collected samples need to be extracted and amplified at different microsatellite loci. After locus identification, the R Core Team 2018 package *polysat* will be used to estimate diversity by assigning alleles to isoloci using microsatellite data. In

order to analyze removal risks, we can use the R package *demography* on each population. We will also perform spatial analyses of *R. laciniata* by using methods similar to Van Zonnefeld et al. (2013).

Spatial analyses would provide insight on sochan demographic structure, evolutionary processes and conservation efforts over a period of time[13]. In order to map both local and regional genetic variances, *alpha* and *beta* diversity will be determined. Populations of significant size and high diversity will be recommended for protection from harvest and possible species extinction. We intend to uphold both the collection rights of indigenous peoples and the preservation of the GSMNP's natural resources with the research.

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