MICROSATELLITE MARKER DEVELOPMENT IN *ALLIUM TRICOCCUM*
POPULATIONS FROM THE SOUTHERN APPALACHIAN MOUNTAINS

A Thesis
by
DAVID S.K. CAMP

Submitted to the School of Graduate Studies
at Appalachian State University
in partial fulfillment of the requirements for the degree of
MASTER OF SCIENCE

August 2021
Department of Biology

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APPROVED BY:

_____
Howard S. Neufeld
Chairperson, Thesis Committee

_____
Ray Williams
Member, Thesis Committee

_____
Jennifer Rhode Ward
Member, Thesis Committee

_____
Zack E. Murrell
Chairperson, Department of Biology

_____
Mike McKenzie, Ph.D.
Dean, Cratis D. Williams School of Graduate Studies

Abstract

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David S.K. Camp
A.S., Nashville State Community College
B.S., Appalachian State University

Chairperson: Howard S. Neufeld

Allium tricoccum Aiton is a widely foraged spring ephemeral that has become

threatened by overharvesting in recent decades. The life cycle of *A. tricoccum* is

unconducive to the intensive harvests experienced by populations in the southern

Appalachian Mountains and steps have been taken by officials from the National

Parks Service to reduce illegal harvesting within their borders. Now, park officials

hope to restore foraging rights to the Eastern Band of Cherokee Indians who have

traditionally foraged for *A. tricoccum* on their land and that within Great Smoky

Mountains National Park, which was once Cherokee territory. However, to ensure

populations of *A. triccocum* can withstand renewed foraging, the National Park

Service requires an assessment of possible further impacts to the species. To

investigate genetic diversity of *A. tricoccum* populations in Great Smoky Mountains

National Park, this study focused on the development of microsatellite markers

specific to this taxon. Marker development was largely unsuccessful for reasons as

yet unknown, but possible explanations are explored here as well as the implications of a possible loss of genetic diversity in analyzed populations.

Acknowledgments

Dedication

This work is dedicated to all those who came before, those who still hold ancient knowledge that has been passed down generation to generation and has survived countless threats to its existence. May the world recognize the importance of this unsevered thread before it is too late.

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Introduction

Each year as winter gives way to spring and the snow begins to melt, one of the first spring ephemerals to emerge in eastern North America is *Allium tricoccum* Ait., more commonly known as ramps. Shaking off a season of dormancy these herbaceous plants send out their leaves, seeking beams of sunlight still filtering down through the naked canopy. Each year for millennia, these early greens have been enjoyed by people who endured long winters without such fresh food. Before being formally described in 1789 by William Aiton, ramps were known by a variety of names, but were called '*uwasti*' by the indigenous Cherokee people in what is now Southern Appalachia and the surrounding areas (Perry 1974). It has even been suggested that the name of Chicago, IL came from the Menominee name for a place around Lake Michigan where ramps grew so abundantly that the tribe gave it the name "Shik'Ko" or "skunk place" (Calvey et al. 1998). The Cherokee traditionally used ramps as a treatment for colds, croup, and earaches but also consumed them as food (Calvey et al. 1998).

Once colonial Americans settled in the Southern Appalachian region, some Europeans of Scotch-Irish heritage recognized ramps as being close to the European native *Allium ursinum*, or bear leek, and subsequently also began regularly consuming *A. tricoccum*. Even in the twenty-first century, elders in Southern Appalachian communities report growing up consuming ramps whole to treat colds and to "clean blood," a remedy based on the idea that over a winter without fresh food, the blood became "dirty" (Cavender 2006). Around this time the name "ramps" was adopted, most likely rooted in the European "ramson," used to describe *A. ursinum* (Core 1945).

Ramps can be found in mesic deciduous forests where soil moisture and nutrients are the most important factor in their growth (Vasseur and Gagnon 1994). Their range extends from Quebec, Canada south to northern Georgia in the United States and from there west as far as the Dakotas. As they break through the snow, ramps are not difficult to spot with their oblong to elliptic leaves and distinctive petiole which is typically reddish pink due to anthocyanins but sometimes lacks this pigmentation (Bell 2007; personal observation). The life cycle of ramps is broken up into stages such that only once the leaves begin to senesce after the canopy above has filled in will a scape emerge, finally splitting open to reveal an umbel of 30-55 white flowers in fall (Weakley 2015). Not long after, ~2 g bluish black fruits (Sitepu 2018) are produced before the last signs of the plant wither, waiting for spring to return when they may leaf out again. The pungent flavor of their leaves, somewhere between garlic and scallions, is the result of a host of thiosulfinate compounds which have made them a delicacy throughout their range (Calvey et al. 1998).

However, in spite of intense contemporary collection practices, ramps have a life history that is not conducive to regular harvest. A single individual of *A. tricoccum* takes between five to ten years to reach maturity and begin to reproduce, primarily through asexual reproduction (Nault and Gagnon 1993). Leading up to this, ramps photosynthesize until the forest canopy fills in, storing this energy in an underground bulb. It has been demonstrated that the amount of photosynthesis an individual is able to undergo is directly linked to better bulb growth and seed production as well as time of senescence, which is delayed for those plants with higher photosynthetic capacity (Dion et al. 2017).

Allium is a monophyletic clade located in the subgenus *Anguinum* within the family Amaryllidaceae, and this genus contains nearly one thousand species (Herden et al. 2016).

Previously, this economically significant genus *Allium*, which contains onions, garlic, shallots, and more, *Allium* had been placed in the family Alliaceae, but thorough analysis of internal transcribed spacer (ITS) sequences sorted species into the more biologically realistic subgenera in which they now reside (Friesen et al. 2006). Multiple studies have since confirmed Friesen's taxonomic revision through the use of ITS, Erthyroblast Transformation Specific (ETS), and chloroplast markers (Nguyen et al. 2008; Choi et al. 2012; Li et al. 2016).

Additionally, it is now known that *A. tricoccum* is not sister taxa to *A. ursinum* and is actually more closely related to *A. ochotense* (Siberian onion) and *A. victorialis* (victory onion) (Li et al. 2016). Interestingly, it has been discovered that *A. tricoccum* is likely a hybrid between species from the Eurasian and East Asian clades (Herden et al. 2016). These results make sense especially when looking at karyotypes of North American *Allium* species. Here, most North American species fall in with those considered New World with a chromosome number of n = 7. However, *A. tricoccum* conspicuously deviates from this pattern with n = 8, a commonality between it and other Old World *Allium* species which carry either 8 or 16 chromosomes (Herden et al. 2016). Speciation occurred approximately 2.5 mya when the lineage migrated across Beringia (Herden et al. 2016) and established itself in North America (Li et al. 2010).

A relationship that has not been so cleanly resolved is that between *A. tricoccum* and *A. burdickii*. The latter was first described in 1946 based on differences in size and petiole pigmentation from *A. tricoccum*; however it has still not been determined whether the two should be considered separate taxonomic entities or if *A. burdickii* is a variety of *A. tricoccum*. *A. burdickii* has been said to flower at different times than *A. tricoccum*; however,

at the northern extent of their range, the two are found to grow sympatrically and flower simultaneously (Vasseur et al. 1990). As the debate has continued with proponents for splitting (Jones 1979) and against (Bell 2007), comparisons at a genetic level should offer the clearest resolution to this issue.

Recently, *A. tricoccum* has come under the culinary spotlight and has been featured in dishes by prominent restauranteurs in cities such as New York City and Chicago (Nordahl 2015; Bookwalter 2015). As their use in folk medicine fades and culinary-based ramp festivals such as those found around southern and central Appalachia gain more attention, those who have a longer history with the plant have grown concerned due to the delicate care that must be taken when harvesting ramp populations. In the 1980s, local extirpations were already being reported in Quebec, Canada where eager foragers harvested patches until they disappeared entirely (Dagenais 1985). A current estimate of the number of ramps harvested each year in the United States is at least two million (Davis-Hollander 2011), however it is difficult to track the sale and consumption of non-timber forest products such as ramps due to their popularity with roadside vendors and farmers' markets (Chamberlain et al. 1998). *Allium tricoccum* is currently a species of special concern in Maine, Rhode Island, and Tennessee, and *A. burdickii* is listed as endangered in New York and threatened in Tennessee (USDA 2021a; USDA 2021b).

This study focused on the development of species-specific microsatellite markers for *Allium tricoccum*, as few currently exist. Microsatellite markers are co-dominant markers that target hypervariable regions of the genome that allow for the comparison and contrast of genetic diversity both within and among populations (Kalia et al. 2011). First described in 1982, microsatellites were found to be present in both animal and plant genomes, the former

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containing more AC rich regions and the latter more AT (Hamada et al. 1982; Powell et al. 1996). Microsatellite regions are found in nuclear (Kalia et al. 2011), mitochondrial (Chung et al. 2006), and chloroplast DNA (Rajendrakumar et al. 2007), but are more common in the plant nucleus. Microsatellite markers can be used for functional genomics, association mapping, diversity analyses, genome mapping, transferability and comparative mapping, and genome tagging and quantitative trait loci analysis (Varshney et al. 2005).

Microsatellite diversity has been linked to a number of potential causes including single-strand DNA slippage, double-stranded DNA recombination, mismatch or double-strand break repair, and retrotransposition (Kalia et al. 2011). Microsatellite markers have been successfully used in *Arabidopsis* (rockcress), *Brassica* (cabbage and mustards), *Quercus* (oak), and *Castanea* (chestnut) among many others (Kalia et al. 2011; Table 1). They have also been effectively used within the *Allium* genus and cross-amplification to other *Allium* species has had its successes (Khar et al. 2011) and failures (Sitepu 2018). The markers found in my study will allow for future work that focuses on the population genetics of *A. tricoccum*.

Name of Species	Species Rarity	Number of Loci Screened	Number of plants screened	Motif	Na
Allium cepa L.	GNR	60	46 *A. cepa*, 12 wild species, 2 *A. roylei*, 2 *A. fistulosum*, 1 *A. chinense*, 1 *A. x proliferum*, 1 *A. hookeri*, and 1 *A. caroliniamum*	Dinucleotide = 2 Trinucleotide = 9 Tetranucleotide = 3 Pentanucleotide = 1 Combination of Motifs = 4	Onion = 2.84 Wild Species = 4.26
Allium sativum L.	GNR	222	21 *A. sativum*	Dinucleotide = 33 Trinucleotide = 91 Tetranucleotide = 62 Pentanucleotide = 20 Hexanucleotide = 15 Heptanucleotide = 3	2.60
A. sativum L.	GNR	50	20 accessions from 5 *Allium* species	Dinucleotide = 33 Trinucleotide = 3 Tetranucleotide = 2 Combination of Motifs = 6	1.45 - 1.91 (varies by locus)
Gastrodia flavilabella	GNR	28	20 *G. flavilabella*, *G. elata*, *G. javanica*, *G. confusoides* (genotyping and population genetics analysis) 2 individuals from 13 *Gastrodia* taxa (for cross-amplification analysis)	Dinucleotide = 13 Trinucleotide = 11 Pentanucleotide = 1 Hexanucleotide = 1 Combinations of Motifs = 2	1.90
Herbertia zebrina Deble.	Critically Endangered	15	50 *H. zebrina*, unknown numbers of *H. darwinii* and *Calydorea crocoides*	Dinucleotide = 4 Trinucleotide = 4 Pentanucleotide = 1 Hexanucleotide = 1 Combinations of Motifs = 5	8.30, 6.83, and 6.50 for 3 populations
Acrocomia aculeata Jacq.	N/A	34 (from *Astrocaryum aculeatum* and *Elaeis oleifera*)	192 *A. aculeata*	Dinucleotide = 10 Trinucleotide = 1 Tetranucleotide = 2 Pentanucleotide = 2 Combinations of Motifs = 3	2.40 - 6.80 (varies by locus)
Petunia exserta Stehm.	N/A	34	126 wild individuals and 13 hybrids	Dinucleotide = 4 Trinucleotide = 9 Tetranucleotide = 1	10.20
Hemerocallis middendorffii Trautv. & C.A. Mey	N/A	12	68 *H. middendorffii*, 4 *H. lilioasphodelus* var. *thunbergii*, 10 *H. hakuunensis*	Dinucleotide = 5 Trinucleotide = 7	*H. middendorffii* var. *esculenta* = 8.75 *H. lilioasphodelus* var. *thunbergii* = 3.67 *H. hakuunensis* = 4.58
Dracaena cambodiana Pierre ex Gagnep.	N/A	26	22 *D. cambodiana*	Dinucleotide = 13 Combinations of Motifs = 13	9.18
Yucca brevifolia Engelm	G3: Vulnerable	21	52 *Yucca brevifolia* var. *brevifolia* 61 *Yucca brevifolia* var. *jaegeriana*	Dinucleotide = 4 Tetranucleotide = 1 Combinations of Motifs = 7	var. *brevifolia* = 13.25, 10.25 var. *jaegeriana* = 11.08, 14.58

Table 1. Application of microsatellite markers across the order Asparagales.

Similar studies focusing on microsatellite development have encountered difficulties with their design. Complications arising during the development process may be attributed to either the chosen methodology or complexities at a biological level (Marquez et al. 2003). While microsatellite markers have been developed for other species in the genus *Allium*, it has been demonstrated that microsatellite abundance is correlated with genome size

(Hancock 1996). Unfortunately, the genome size of *A. tricoccum* is yet unknown, but it has been shown that species in the genus have undergone a significant increase or decrease in genome size throughout their evolution (Ohri et al. 1998).

Genomic analyses of mollusks have also encountered hurdles in the development of microsatellite markers. In a 2010 study, researchers determined that the biological complexities specific to their taxa could have resulted in the problems that arose (McInerney et al. 2011). The complications were attributed to the presence of transposable elements and the fact that the activity of such elements could affect rates of DNA multiplication and recombination. Interestingly, nested transposable elements containing microsatellites have been found in *A. cepa*, something that could conceivably also occur in *A. tricoccum* (Vitte et al. 2013) and may lead to similar difficulties as those encountered in the development of microsatellite markers for molluscs.

One of the primary concerns when dealing with the conservation of imperiled species is the connectivity of populations. Human development and agriculture have led to the fragmentation of once contiguous wilderness that would normally facilitate gene flow through the migration of individuals or their genetic information by pollinators (Nantel et al. 1996; Whitney 1996). *Allium tricoccum* may be of particular concern because of its specific life history and reproductive methods. Additionally, the effects of genetic drift, in which certain alleles become fixed in a population, may be particularly exacerbated by isolation caused by fragmentation. While this is not always the case (Ezard and Travis 2006), it should be stressed that the effects of genetic drift are stronger in populations with a decreased effective population size (Vasseur 2001).

The amount of mutation that populations of *A. triccocum* experience may be above average as well. A 2019 study looked at the complete chloroplast genome of six Chinese *Allium* species and compared them to another 35 non-Allioideae species (Xie et al. 2019). These researchers found that in a majority of *Allium* species tested, Ka/Ks ratios, which determine the ratio of nonsynonymous to synonymous substitutions, were above average. This makes biological sense as previous studies have demonstrated that *Allium* is an ancient clade that has undergone massive amounts of diversification at a genetic level, but which has not necessarily translated to observable morphological variation (Li et al. 2010).

Perhaps the most relevant selective pressure currently experienced by *A. tricoccum* is that brought on by intensive harvesting of wild populations (Edgar et al. 2012). The particular life history of *A. tricoccum* with its drawn-out stage of maturation is obviously not conducive to yearly harvests on a mass scale. Estimates of harvests in Gatineau Park, Quebec total 25,000 ramets in a single season (Nantel et al. 1996), and those in New York between 18,000 to 20,000 pounds each year by a single foraging operation (Sen 2011). Considering the five to ten years it takes for an individual to mature and begin to reproduce, it is clear what this level of harvesting can do to a population.

One benefit *A. triccocum* may have in this instance is the variety of reproductive methods it employs. The species reproduces primarily by vegetative recruitment via bulb division but is also self-compatible, though seedling recruitment from sexual reproduction has been found to be low and *A. tricoccum* exhibits biennial reproductive patterns (Nault and Gagnon 1993). Non-random asexual reproduction is the process by which *A. tricoccum* produces clones of itself and diploid species often create clones that are likely identical by descent (Glémin et al 2019). Although mutations can occur, they have been shown to be

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insignificant in terms of generating genetic diversity at the population level in some species (Van der Hulst et al. 2003).

In *Arabidopsis thaliana*, progeny born from selfing had depressed rates of outcrossing, heterozygosity, and genetic diversity (Mable and Adam 2007). Additional studies on the genetic diversity of selfing populations have reported mixed findings though the effects are particularly pronounced in smaller populations with increasing selfing rates and when multilocus genotypes are assessed (Jullien et al. 2019, citations therein). When assessing the genetic diversity of a population, the microsatellite markers developed in this study may be able to tease apart some of these confounding factors.

Specifically, these markers will assist in determining the genetic diversity of populations of *A. tricoccum* within Great Smoky Mountains National Park (GSMNP). GSMNP is an over 2,000 km^2 park straddling the border of Tennessee and North Carolina in the USA. Codified as a National Park in 1940, land within the park was traditionally part of Cherokee territory and today borders Cherokee, NC and the Qualla Boundary. Cherokee, NC is the current homeland of the Eastern Band of Cherokee Indians (EBCI).

Here, for over a decade, members of the EBCI have been barred from collecting *A. tricoccum* from patches that have been harvested by generations, going back for thousands of years to be used both as medicine and food. These restrictions were imposed in 2007 after extensive monitoring and, more importantly, the release of a study conducted within the park that stated *A. tricoccum* would be extirpated within the boundaries of GSMNP if collections were not halted (Rock et al. 2004). However, the study in question specifically looked at the

impact of unsustainable harvesting methods in which a forager takes the entire plant, bulb and all, something members of the ECBI do not practice (Lewis 2012). Traditional Ecological Knowledge (TEK) implemented by tribal members stipulates that ramps should be harvested by only taking the leaves and leaving the bulbs behind in the ground or replanting them (Lewis 2012), though some documents indicate the bulbs were at least occasionally consumed (Perry 1974). This process shows how necessary a marriage between the western scientific method and TEK is and how it can often be a powerful integrator of techniques that lead toward sustainable ecological management.

In 2016, members of the ECBI were allowed access to populations of ramps within the park with the stipulation that an assessment of any further impacts to the species would be conducted (Lewis 2012). The microsatellite markers developed in this study have been screened specifically for this purpose with the hope being that members of the ECBI will once again be able to harvest *A. tricoccum* on land that once belonged to them.

When considering all the relevant factors, it is difficult to know what to expect in the analysis of microsatellite loci in *A. tricoccum* from GSMNP. Considering their reproductive methods and the relative isolation of populations in the park, one would think genetic diversity should be low, especially if the sampled populations have a history of being harvested. However, it may be true that *A. tricoccum* have struck a reproductive balance and are able to generate genetic diversity sporadically through sexual reproduction with a majority of reproductive energy put toward vegetative recruitment (Vasseur 2001). Based on preliminary results, it would appear that intensive foraging, isolation, and reproductive methods have led to a decrease in the genetic diversity of *A. tricoccum* within the park as most loci analyzed have been determined to be monomorphic.

Assuming genetic diversity has been lost, it is not a death sentence for *A. tricoccum*. Foraging within the park, especially for the Eastern Band of Cherokee Indians, should continue with an emphasis on sustainable foraging techniques that establish a balance between responsible harvests and population recovery. If the bulbs are left in the ground and 10% or less of a patch is harvested, it allows individuals to store energy for the next year, and while population growth may be slowed, it will not lead to extirpation.

Materials and Methods

Collections

Seven populations of *Allium tricoccum* were located in Great Smoky Mountains National Park (GSMNP) and collected in April and May 2020. Individuals sampled were collected haphazardly, were a minimum of 10 m away from other samples, and distinct populations were separated by at least 1.6 km. This sampling method was adopted due to the highly clonal nature of *A. tricoccum* and their highly localized means of asexual reproduction (Nault and Gagnon 1993, Sitepu 2018). Thirty-five individuals were collected from each population (except for the third population in which only fourteen individuals were collected due to low population numbers), since this yields an informative amount of genetic data in a cost-effective manner (Hale et al. 2012). For each plant, ~1.5 cm^2 of leaf tissue was collected. Care was taken to ensure that only one leaf per bulb was sampled to decrease the chance of sampling from the same individual. Tissue was stored in cryovial tubes with ~1 ml of silica gel and kept at -80°C until DNA extraction.

DNA Extraction

Samples of *A. tricoccum* were ground with sand and a micro-pestle and DNA was extracted via a modified CTAB extraction method (Doyle and Doyle 1987). DNA was quality checked using an ND-1000 Nanodrop Spectrophotometer (Thermo Fisher Scientific, Charlotte, NC) as well as a 1% agarose gel. Extracted DNA was stored at -20°C until PCR amplification.

Microsatellite Marker Development

I used the same development procedure to develop microsatellite markers as did

Gaglianese-Woody (2019) and her methodology is reproduced verbatim here: "High quality

Allium tricoccum DNA was sent to the West Virginia Core Facility for Illumina sequencing.

An Illumina MiSeq library of 983,952 raw sequences was trimmed in FastP using an overlap

analysis, resulting in 888,557 sequences. Filtered sequences were mined in MSAT

Commander version 1.0.8 for microsatellite loci, which identified 1,484 of 9,116 for primer

design. Fifty microsatellite loci were selected for primer design, with primers between 19 to

25 base-pairs long, 45% to 55% in GC content, and a PIG-tail sequence added to reverse

primers for accurate genotyping (Brownstein et al. 1996). An M13 primer was also added to

the 5' end of the forward primer."

To investigate genetic diversity within *A. tricoccum*, a series of 100 microsatellite

markers were screened for the specific taxa between the spring and fall of 2020. Markers

were tested via PCR reaction, which were prepared in 10 μL using DI H_2O, 5X GoTaq Flexi

Buffer™, 2.5 mM $MgCl_2$, 800 μM dNTPs, 0.5 μM of forward and reverse primers, 0.5 units

of GoTaq DNA Polymerase™, and 30 ng of template DNA. Each array consisted of template

DNA from seven *A. triccoum* individuals, one from each population collected, and a single

individual of *A. burdickii* collected from the NE United States to assess any possible cross

amplification between the taxa. A touchdown PCR program ran under the following

conditions: 1 cycle of 94°C for 5 min, 13 cycles of 94°C for 45 s, 68°C for 2 min and

decreasing 0.5°C per cycle , 72°C for 1 min, then 25 cycles of 94°C for 45 s, 53°C for 1 min,

and 72°C for 1 min, then 72°C for 10 min, and PCR ended at 10°C. DNA amplification was

confirmed via a 1% agarose gel, and product size was compared to a 1 kbp ladder. Successful

primers were tagged with one of four different fluorescent tags (FAM, VIC, NED, PET), multiplexed, and combined with HI-DI and a GeneScan Liz 500 size standard (Invitrogen, Carlsbad, California). Multiplexed samples were sent to the Georgia Genomic Facility (Athens, GA) for fragment separation. Chromatograms were analyzed and scored in Geneious (v9.0.5.). Markers were deemed successful based on chromatograms whose peaks were consistent across all eight samples and could be confidently scored due to the uniform nature of peak patterns.

Results

Out of 150 markers screened and developed, only 10 met the previously established criteria to be considered successful. This included chromatograms with consistent peaks across samples that could be uniformly scored. Of these 10, only seven were polymorphic, with the other three displaying monomorphic signatures (Table 2). Six of the seven polymorphic markers cross-amplified in the *Allium burdickii* sample (remaining four samples lacked data in this area). All of the polymorphic markers targeted either di- or trinucleotide sequences.

Locus	Primer Sequence (5' → 3')	Motif	Cross-amplification	Monomorphic/Polymorphic	Allele Size Range (bp)	Fluorescent Label	Tagged	Tag Sequence
AT01	F: CGGACCTCGTATGCACAAG R: GTTTAGGGTACTGTTCATAGGCGG	(AC)$_6$	Yes	Polymorphic	121-123	FAM	Right	GTTT
AT04	F: ATCTGGTTCGGGCATTCAAC R: GTTTGGTTCGACAGTGGTTGG	(AAG)$_9$	Yes	Polymorphic	130-133	FAM	Left	G
AT07	F: GGAGTGAGAAACGTGATGGG R: GTTTGGGTGGGTTCATTTATTTGGC	(AATT)$_6$	Yes	Monomorphic	137-141	FAM	RIGHT	GTTT
AT10	F: AGTGAGTACGATCCAGGCATTG R: GTTTCCAAATCGATCCGTTCCC	(AC)$_8$	--	Monomorphic	140	FAM	Right	GT
AT37	F: GGGATTGTTGAGAAAGAAACCG R: GTTTGCAAAGCCGAACTAGGTC	(AT)$_{13}$	Yes	Polymorphic	222-224	VIC	Left	GT
AT42	F: AGTGTATGCATATTGTCCGCAG R: GTTTGCGTGGCTCCTACTTAAC	(AT)$_{10}$	Yes	Polymorphic	106-108	VIC	Left	G
AT44	F: GGGATTGTTGAGAAAGAAACCG R: GTTTGCAAAGCCGAACTAGGTC	(AT)$_{13}$	--	Monomorphic	220-223	VIC	Right	GT
AT51	F: CACGACGTTGTAAAACGACAGACGCTAGCTTGAGACTGG R: GTTTAAGCGAACTCAGAAATTCCAAC	(AAAT)$_6$	--	Monomorphic	170-174	VIC	Left	GTTT
AT55	F: CACGACGTTGTAAAACGACAGCCAACATCTCCTTTGCGTG R: GTTTCGAGCGAGCAATGAGAAAGG	(AC)$_8$	--	Polymorphic	196-198, 198-200	VIC	Right	GTTT
AT58	F: CACGACGTTGTAAAACGACGTTTACTCTTCCTGCACGCAC R: CCCGAAACCTTTGCACAGTAG	(AC)$_8$	Yes	Polymorphic	131-133	NED	Left	GTTT
AT59	F: CACGACGTTGTAAAACGACTGGTCCCGTTCCAATTTCC R: GTTTGGACGTATTGAGAGAGCGTAC	(AC)$_8$	Yes	Polymorphic	213-223	NED	Right	GTTT
AT88	F: CACGACGTTGTAAAACGACGTTTGGGCTACTCTTCTAATCGGC R: AGAACCGAGGCTCAACAGAG	(AT)$_8$	Yes	Monomorphic	157-161	NED	Left	GTT

Table 2. Successful microsatellite markers.

Discussion

Microsatellite Development in Allium tricoccum

The preliminary results of this study suggest a dearth of genetic diversity in *Allium tricoccum* populations in Great Smoky Mountains National Park. While seven of these markers were determined to target polymorphic loci, eleven of the markers screened were monomorphic in both *A. tricoccum* and *A. burdickii*. This marker development was undertaken with the hope that a subset of 16 working markers would be found. Similar studies have screened comparable numbers of markers and found at least 16 applicable markers (Clark et al. 2019; Gaglianese-Woody 2019), however for as yet unknown reasons, the screening of 150 markers in the current study was unable to hit this threshold.

Despite the need for additional work to elucidate patterns of genetic diversity, the initial findings of this study are biologically plausible for the taxon in question. *Allium tricoccum* is highly clonal, with sexual reproduction only responsible for approximately 2% of population growth (Nault and Gagnon 1993), though it should be noted that Vasseur's (2001) work indicates that populations are able to sporadically generate genetic diversity through autogamous sexual reproduction. Additionally, samples came from GSMNP where populations have been subjected to non-sustainable foraging in the past century (by non-indigenous harvesters), which could have severely reduced numbers of ramps in the park (Rock et al. 2004). With the removal of entire individuals comes the risk of permanently removing their genotypes from the population. If there were at any time an abundance of diversity to be found at the microsatellite loci under investigation, it could have been diminished by overharvesting. The fact that this diversity may have declined and that asexual reproduction is the norm in *A. tricoccum* is not to say that the development and use of these

markers is useless. It would behoove all who work on the taxon to investigate the presence of rare or private alleles, especially for delineating genetically unique populations that should be managed with appropriate precautions.

It is hoped that the development of these markers will also help resolve the taxonomic relationship between *A. tricoccum* and *A. burdickii*. Microsatellite markers have been found to be applicable both within genera and families and have found widespread use within molecular biology (Varshney 2005; Kalia et al. 2011) However, it should not necessarily be assumed that microsatellite markers developed for one species will be applicable to others, as seen in the experimental application of *A. sativum* markers to *A. tricoccum* and their subsequent failure (Sitepu 2018). In an evaluation of the potential for cross-amplification of microsatellite markers, those developed for monocots (in which *Allium* is found) have been found to be less successful in their ability to cross amplify than eudicots, with over 60% of markers amplifying between species within a genus, but less than 20% found to amplify between genera within a family (Barbara et al. 2007).

This should be of particular concern for the *Allium* genus, as it has been found that in some loci, the variation found between species within the genus is comparable to that found between genera within other angiosperm families. However, this is not true for all 850+ species of *Allium*, especially those considered to have undergone recent radiation (Li et al. 2010). In a study on the tropical Indian onion or Pran, a hybrid between *Allium cepa* and an unknown ancestor, researchers looked at the potential for cross-amplification in markers developed for the hybrid. Here, markers developed for the cultivated *A. cepa* hybrid, Pran, worked on wild indigenous species and were able to resolve the genetic diversity between them (Khar et al. 2011). It should be noted that amplification has been found to be less

successful in plants that reproduce primarily through selfing as well as with those that have a short generation time (Barbara et al. 2007). This may be due to the higher likelihood of mutations occurring which then accumulate as a consequence of small population size (Higgins and Lynch 2001). Because both *A. tricoccum* and *A. burdickii* are selfing species, it is possible that complications could arise in the transferability of markers developed for *A. tricoccum* to *A. burdickii*.

The taxonomic line between these two species is currently blurred. While the best evidence for speciation lies in differentially timed anthesis, some scientists remain steadfast that speciation has not occurred and the two actually represent a single taxon (Bell, 2007). Additionally, it has been reported that at the northern extent of its range, the flowering time of the putative *A. burdickii* has been found to overlap with that of *A. tricoccum* (Hanes 1946; Jones 1979). Due to the debate over differences in morphological features (or lack thereof) and characteristics supposedly indicative of one or the other being observed in sympatric individuals, we may assume that radiation is currently underway. This debate should only strengthen the argument for using microsatellite markers to investigate the relationship between *A. tricoccum* and *A. burdickii*. If markers are unable to cross-amplify, this may be evidence in support of those wishing to split the taxa into sister species. If markers do cross-amplify, the pairing of data from *A. triccocum* with that of *A. burdickii* may be able to delineate a species boundary if enough variation is found.

Current and Future Threats to Allium tricoccum

Past studies have highlighted the ability for bulbs of *A. tricoccum* to successfully survive transplantation (Vasseur and Gagnon 1994). This is important because there is the phenomenon where clumps of ramps suddenly die off, aptly referred to as "clump death"

(Nault and Gagnon 1993) which is thought to be brought about by pests (Dion et al. 2016; Lapointe and Dion 2017). Additionally, it has been suggested that while only harvesting the leaves of *A. tricoccum* is the most sustainable way to forage this edible spring ephemeral, populations can handle the loss of entire bulbs as long as a fixed density is left to winter-over (Dion et al. 2016). This brings to mind the intermediate disturbance hypothesis, in which some species flourish with mild to medium amounts of disturbance. It is possible that *A. tricoccum* is such a species, however the amount of disturbance currently experienced by populations is simply too extreme, especially as it has been noted that some foragers of ramps have a tendency to exploit entire patches, without leaving any individuals to repopulate (Rock et al. 2004).

Unfortunately, overharvesting by humans is not the only threat currently posed to *Allium tricoccum*. While the aforementioned clump death is responsible for the demise of specific patches (Nault and Gagnon 1993; Dion et al. 2016; Lapointe and Dion 2018), it is not widely encountered in the literature. However, one pattern does emerge when analyzing growth and reproductive patterns in *A. tricoccum* and that is the influence that seasonal weather variation can have on populations. In Nault and Gagnon's work (1993), an extended spring in 1984 allowed populations to photosynthesize for a longer time period, which subsequently led to scape mortality of only 14%, the lowest of the five years of their study (Nault and Gagnon 1993). In this same study, a particularly hot and dry season in 1988 resulted in a scape mortality rate of 65%. This led the authors to conclude that reproductive success, both sexual and asexual, is resource-limited.

Additionally, the response of the environment to changing conditions directly affects *A. tricoccum* on a physiological level. A 2016 study demonstrated that bulb width and seed

production per bulb were most strongly affected by total light and time of canopy

closure. Delayed canopy closure allowed a longer time for plants to perform photosynthesis

because of the higher light levels, and this in turn delayed leaf senescence in *A. tricoccum*

(Dion et al. 2016). Canopy composition can also influence these outcomes and is correlated

with soil temperature and moisture. An analysis of North Carolina climate trends from 1949-

1998 has shown that precipitation has increased during fall and winter but decreased during

summer, temperatures were warmer than average, the warm season was longer, and the last

ten years analyzed were the wettest in the study period (Boyles and Raman 2003). A more

recent study of North Carolina climate trends predicts a future temperature increase across all

seasons, an increase in the number of very warm nights and hot days, and a decrease in the

number of cold days (Kunkel et al. 2020). This study also predicts that not only will severe

droughts become more intense, but that higher temperatures will lead to increased

evaporation. So, while it is predicted that North Carolina may see more rainfall, increased

chances of droughts as well as increased temperatures could imperil populations of *A.*

tricoccum for which soil moisture is the primary factor affecting their growth and survival

(Vasseur and Gagnon 1994).

One less direct threat to *Allium tricoccum* is the establishment of populations of

invasive plant species. While deer may not directly consume *A. tricoccum* as much as other

species (Anderson 1994), the rapid rise in the North American white-tailed deer population

has altered the abundance, biodiversity, community composition, and ecosystem function of

native understory plants (Knight et al. 2009). When measuring the effects of herbivory by

deer, it was demonstrated that in plots which excluded deer, native plants were dominant,

however in plots which were accessed by deer, invasive herbs became dominant (Knight et

al. 2009). This was attributed to a number of factors. First, that the invasive herbs present were weedy short-lived species that exhibit rapid reproduction and high population growth (Gibson et al. 2002); second, that deer preferentially browsed on native species, and third, that native herbs were negatively affected by soil disturbance due to the non-browsing effects by deer (Knight et al. 2009). So, while *A. tricoccum* may not be a preferred food for white-tailed deer, the effects of their increased presence, as well as that of invasive herbaceous species, potentially threaten the viability of *A. tricoccum* populations.

Another species that has been documented to affect plant assemblages specifically within GSMNP is nonnative wild pigs, *Sus scrofa*. In the park, these pigs have been found to negatively impact both the total plant cover and plant richness in areas they disturb (Rossell et al. 2016). While this same study details disturbances by black bears, *Ursus americana*, and elk, *Cervus elaphus*, the presence of these species was not linked to the negative effects brought about by wild pigs. This study found comparable levels of disturbance to other published work (Rossell et al. 2016).

Restoration of Foraging Rights: A Path Forward

While this study focused on designing molecular tools to understand the population genetics of *Allium tricoccum* in GSMNP, the ultimate goal of this work overall is to restore foraging rights of the EBCI to their traditional harvesting lands within the park. Thus, it is beneficial to discuss the history of foraging laws and their future. Laws around foraging have existed in the United States almost since its inception. While it is easy to think that these laws are purely beneficial, providing security to prized populations of plants, animals, and fungi, the history behind these laws and the cultural restrictions they have put in place are anything but. The earliest forms of such laws came into existence as colonial New Englanders sought

to force the indigenous Powhatan tribe from their traditional foraging grounds and then later as the federal government cracked down on the practice by freed slaves, something that had the potential to generate income and foster personal autonomy (Linnekin 2017).

While foraging is still prohibited in 13 national parks and those that do allow it are not open to foraging of all species, recent efforts have been taken by the National Parks Service (NPS) to open up some lands in an attempt to right past wrongs. In 2018, GSMNP entered into an agreement with the ECBI, allowing them to apply for a total of 36 permits per year for the collection of *Rudbeckia laciniata*, commonly known as the cutleaf coneflower or sochan. Each permit grants the collection of one bushel (2,150 cubic inches) for a prescribed member of their tribe. However, complaints made by members of the ECBI regarding the process included the fact that it required the tribe to pay $68,000 for the environmental assessment that is legally required to open these lands up to foraging.

Despite the fact that foraging laws can be drawn up for unfair and historically racist reasons, they are not completely without merit. While we cannot know the entire demographic history of *A. tricoccum*, as they have been consumed in North America in excess of ten thousand years, there are worrying examples as of late that we can take note of. In the mid-eighties, work was published indicating that several populations of ramps in Quebec had been completely extirpated due to overharvesting by locals (Dagenais 1985). Additionally, in a 2011 *New York Times* article it was reported that local harvesters in the state routinely collect eighteen to twenty-thousand pounds of ramps each year to take to market (Sen, 2011). It should be noted that while many indigenous tribes, including the EBCI, have tried and true methods of sustainably harvesting ramps (Lewis 2012), these

sustainable harvesting methods are not usually employed by foragers of non-indigenous background.

Taken into consideration with the demography studies published by Nault and Gagnon in 1993 and Nantel and Gagnon in 1996, these numbers are worrisome. Demography studies indicate that populations numbering in the low hundreds likely face extinction and that to remain sustainable, harvesting practices would have to take less than 8% of a given population per year, and that even a harvest of 5-15% is enough to bring population growth rates below the equilibrium value (Nault and Gagnon 1993, Nantel and Gagnon 1996). As is now generally accepted in the field of conservation genetics, the sustained growth of a population is not simply due to the number of individuals it contains, but the genetic diversity within (Booy et al. 2000; Jump et al. 2009). Environmental assessments required by the NPS for the collection of previously prohibited foodstuffs look at possible impacts to the taxa in question if foraging is allowed.

As previously stated, the NPS has begun to reverse, or at the very least relax, those laws that prevented the public, indigenous peoples, or both, from foraging on National Park land. Interestingly, in the case of *R. laciniata*, an independent environmental assessment from 2018 reported that traditional sochan collection techniques practiced by the ECBI were actually found to increase seed production, benefitting these populations in the Smokies long term. Other ecological relationships have been found to exist between traditionally foraged taxa and indigenous people such as that shared between the Nisqually people of the Northwest and *Xerophyllum tenex*, also known as beargrass (Hooper 2015).

Unfortunately, as a resurgence in the popularity of foraging grows, public lands like those in the National Parks may be one of the few options left for those looking to forage in the wild. While there have been experimental studies conducted on the reintroduction of such species to increase their numbers and accessibility to them (Shebitz and Kimmerer 2005), national parks may hold genetically unique and robust populations and may be able to aid in such reintroduction efforts. If this proves to be the case, understanding the genetic makeup of populations on NPS land is paramount.

Throughout these examples, there is an unquantifiable metric, the importance of the persistence of cultural traditions and the retention of cultural identity. For many, collecting these non-timber forest products is not just the acquisition of food or crafting materials, but is the continuation of a way of life that dates back thousands of years and the reclamation of these activities keeps that cultural identity alive (Truer 2021).

As the United States grapples with its history of settler colonialism and indigenous genocide, there often seem to be little, if any instructions on how to proceed, but this evolving agreement between the NPS and indigenous tribes sheds light on how these first steps should be taken. *A. tricoccum* is relatively unique in that Scotch-Irish settlers recognized it as being similar to the European native *A. ursinum* and incorporated it into their diets (Core 1945), merging their history of foraging with that of the native peoples. Because of this, *A. tricoccum* has been widely consumed across its range, with festivals dedicated to the spring ephemerals occurring annually in southern and central Appalachia, including Cherokee, NC (Lewis 2012). While non-indigenous foragers may not always have the sustainable techniques to appropriately harvest populations, the deals made between the NPS and tribal peoples are themselves roadmaps. Here, lost knowledge, perhaps first deduced by

people practicing unsustainable harvesting techniques and recognizing the need for something more balanced (Hooper 2015), can be integrated with conservation methods to bring about a reemergence of traditional ecological knowledge (TEK), married with a more ecologically equitable approach to living in a developed world, as well as undoing unjust laws put in place by local and federal officials.

It is necessary to state that any results obtained through the use of these markers should be taken to those who are a part of or work with the EBCI. While the development of molecular markers itself presents the opportunity to conduct a thorough investigation of the species, there is still an inherent cultural and temporal disconnect from these practices. It is the author's belief that the people who have understood the particular life history of *A. tricoccum* for thousands of years (Lewis 2012) should lead in establishing rules and regulations regarding the foraging of this plant. It does not seem by chance that populations were found to be in decline only in the late 20th century. While it could be argued there was no hard data up to this point, common sense would dictate that had indigenous peoples not understood or developed sustainable harvesting practices in regards to *A. tricoccum*, the species would never have persisted up until settler colonialism came to North America. As noted by a member of the Cherokee tribe, ensuring the continued survival of such foodstuffs was a matter of survival for their people (Lewis 2012). That local extirpations closely followed colonization is not lost on the author.

Imperative to this progress is indigenous peoples and park officials working together to tease apart the intricate details of a plant's life history through genetics, ecology (including TEK), and evolutionary biology. This partnership may be the gateway to a greater cultural unification and more widespread respect for native flora that are often looked over, paved

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over, and relegated to the memories of those who carry the knowledge of not just how to exist on the landscape, but to be a part of it.

Generated Waste

This project generated a significant amount of single-use plastic waste. The University of Exeter estimates that labs globally generate 5.5 million tons of plastic waste (Urbina et al. 2015). The lab work required for the DNA extraction and marker development in the current study (including PCR, agarose gel electrophoresis, and nanodrop) generated >6.184 kg of single-use plastic waste. The actual amount generated likely exceeds this estimate as materials such as disposable gloves, and containers for aliquoted reagents were not calculated. Included in this estimate are the most commonly used materials: microcentrifuge tubes, pipette tips, and PCR plates.

The use of such single-use plastics in laboratory settings often reduces costs and while it may not always be feasible for the same reasons to upgrade to re-usable materials, there are methods being developed that might be able to deal with this growing problem (Sawyer 2019). Proteins which break down plastics, certifications which teach researchers to optimize their usage of lab tools and materials, and switching out just a small portion of lab materials that can be sterilized and reused are strategies that labs across the globe are using to lessen their environmental impact (Sawyer 2019; Alves 2020).

While understanding the genetic diversity of populations of threatened species such as *Allium tricoccum* is incredibly important, projects such as this should weigh the pros against the cons of using single-use plastics. At a time when microplastics have infiltrated nearly the entire globe from the Amazon River basin (Andrade et al. 2019) to the Mariana

trench (Chiba et al. 2018), it is the responsibility of scientists to determine the importance of

a research project and whether there are ways to accomplish their goals without using single-

use plastics or to significantly reduce the amount used.

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Vita

David S.K. Camp was born in Nashville, TN. He received his Associate of Science from Nashville State Community College in 2017 and his Bachelor of Science from Appalachian State University in 2019 where he stayed for his Master of Science degree.