THE DEVELOPMENT OF MICROSATELLITE MARKERS FOR THE CULTURALLY AND ECONOMICALLY SIGNIFICANT PLANT, *ALLIUM TRICOCCUM* AIT.

by

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Abstract

Allium tricoccum Ait. is an herbaceous monocot in the Amaryllidaceae family and is most commonly known as a ramp or wild leek. *Allium tricoccum* is traditionally used in Appalachian cultures, but it has gained the attention of the general public over the past two decades. Although not a rare plant, its growing popularity has led to over-harvesting and population declines. The purpose of this project was to begin the development of microsatellite markers for *Allium tricoccum*. An Illumina MiSeq library was produced, and 50 potential microsatellite loci were screened for successful amplification using PCR. This project also screened for marker amplification in the narrow-leaved wild leek, *Allium tricoccum* var. *burdickii*, and the nodding onion, *Allium cernuum*. Nineteen of the 50 (30%) screened markers successfully amplified. This work will provide the tools necessary for population genetic studies of *Allium tricoccum* and will help inform conservation management decisions.

Introduction

People have harvested forest products as a form of survival for generations. Many of these are non-timber forests products (NTFPs), which are all products other than timber and pulpwood that are extracted from forest resources. Examples of NTFPs include teas, tinctures, edibles, floral greens, and maple syrup. NTFPs and the forest resources they come from provide a slew of ecosystem services and are essential to various customs and traditions that define local and regional culture (Lake et al., 2018). In Appalachia alone, hundreds of plant and fungi species are harvested for NTFPs that are important to Appalachian culture (Cavender, 2006). Unfortunately, as more people turn to wild harvesting, many harvested species and the traditions that rely on them are threatened by overexploitation, insufficient protection, and a lack of species-specific research. One example of this phenomenon can be seen in the study of the pungent wild green *Allium tricoccum*.

Allium tricoccum Ait. is a bulbous geophyte in the monocot family Amaryllidaceae and is commonly known as a ramp in its southern range or wild leek in its northern range. *Allium* is a robust monophyletic genus with three distinct clades, 15 subgenera, and over 800 species, 84 of which reside in North America (Wheeler et al., 2013; Li et al., 2010). Moreover, twenty *Allium* species are cultivated as edible crops, including the bulb onion (*A. cepa* L.), garlic (*A. saticum* L.), and the leek (*A. ampeloprasm* L.), and multiple *Allium* species are harvested in the wild for personal and/or market use, most notably *Allium tricoccum* (Burba and Galmarini as cited in Wheeler et al., 2013).

Eighty-one of the 84 North American species belong to the *Amerallium* subgenus, including this study's out-group, *Allium cernuum* Roth (Wheeler et al., 2013). *Amerallium* is one of three subgenera in clade A, which is the most ancient clade and contains taxa that rarely

develop distinguishable rhizomes (Li et al., 2010). *Allium tricoccum* is a member of the *Anguinum* subgenus in clade B, which contains five subgenera, and clade C contains the seven other subgenera (Li et al., 2010). Taxa of both clade B and C usually develop notable rhizomes and bulbs (Li et al., 2010).

The *Anguinum* subgenus exists from South-Western Europe to East Asia and in North-Eastern America (Herden et al., 2015). Though ploidy varies within the subgenus, the base ploidy number of *Anguinum* species is eight (Herden et al., 2015). Herden and colleagues (2015) argue that the subgenus originated in East Asia and underwent its main biogeographical radiation within the last million years, deeming it a relatively young subgenus. Based on sequence comparisons of ITS regions, *Allium tricoccum* is the result of a hybridization event between members of clade A and B around 2.5 million years ago, after which it migrated from East Asia to North America across the Bering Land Basin (Herden et al, 2015).

Today, *Allium tricoccum* is confined to the U.S. East Coast and parts of Southeast Canada. Its range reaches north to Quebec, Canada, south to Northern Georgia, and west to Minnesota (Jones, 1979). It prefers moist and nutrient-rich soils in hardwood mesic forests, though habitat preference varies along its range (Jones, 1979). In its northern extent, it is usually found in landscape depressions, along riverbanks, and in maple-dominated forests, while in its southern extent, it exists on north-facing well-drained slopes at high elevations (Jones, 1979). Commonly co-occurring herbaceous species include blue cohosh (*Caulophyllum thalicroides* L.), false Solomon's seal (*Smilacina racemosa* L.), Trillium (*Trillium sp.*), violet (*Viola sp.*), and stinging nettle (*Urtica dioica* L.) (Chamberlain et al., 2014). Additionally, in its northern extent, *Allium tricoccum* is sympatric with *Allium tricoccum* var. *burdickii* Hanes. The taxonomic

resolution of *A. tricoccum* var. *burdickii* is largely debated, and some taxonomists argue for its separation as a species, including Jones (1979).

Allium tricoccum is a long-living perennial that resides along an underground rhizome in dense clumps that dominate the early spring herbaceous layer (Jones, 1979). It is characterized by two to three basal elliptic to oblong leaves with a red base, a tall flower scape with an umbel of 25 to 50 white to cream-colored flowers, shiny black seeds, and an underground bulb (Jones, 1979). *Allium tricoccum* is unique because it has separate photosynthetic and flowering stages. The leaves emerge late March to early April and persist through May, during which time the plant undergoes photosynthesis and obtains a majority of its nutrients and biomass before the overstory closes (Jones, 1979). As local temperatures warm and sunlight becomes limited, the leaves turn yellow and senesce, facilitating nutrient allocation to the bulb. A study of nutrient allocation in northern populations found that 80% of leaf nutrients are allocated to the bulb of reproductive plants, though the degree of allocation largely depends on environmental conditions, location, and plant size (Nault and Gagnon, 1988). Stored nutrients are later used for flowering in July and fruiting in August to September, and nutrient accumulation in one season influences flowering in the next season (Nault and Gagnon, 1988). Observed pollinators include solitary bees (*Halictus sp.*), sweat bees (*Dialictus sp.*), and occasionally bumble-bees (*Bombus sp.*) (Jones, 1979), but empirical studies of pollination-interactions and mechanisms of seed dispersal do not currently exist.

Although sexual reproduction occurs, it minimally contributes to population growth and maintenance. According to a five-year demography study that measured mortality, seedling recruitment, growth, and sexual and asexual reproduction rates, *Allium tricoccum* is a clonal species that relies primarily on asexual clump division of large and established ramets (Nault and

Gagnon, 1993). Local populations tend to be biennial, and flowering is influenced by available resources and plant size (Nault and Gagnon, 1993). If conditions are favorable, pollination is successful, and seeds can set before the flower scape dies, germination may occur. However, seed germination can take up to eighteen months and requires a warm period followed by cold stratification to release seeds from dormancy and initiate germination (Chamberlain et al., 2014). Following germination, a bulb grows in autumn, and a single leaf emerges the following spring, but seedling mortality is generally high. Seedling growth is influenced by fluctuating soil moisture and temperature, shallow roots, and a small bulb reserve (Vasseur and Gagnon, 1994). If a seed germinates and survives, it can take five to seven years for juvenile plants to reach sexual maturity (Chamberlain et al., 2014). Indeed, *A. tricoccum* has a complex life-history that depends on underground nutrient storage and established ramets for persistence.

In addition to its complex biology, *A. tricoccum* is loaded with health-promoting compounds and minerals, such as allicin, cepaenes, and Vitamin C. The first of these, allicin, is the thiosulfate responsible for the characteristic garlicky taste of many *Allium* species (Borlinghaus et al., 2014). It can lower blood pressure and cholesterol levels, has antibiotic properties, and has been found to induce cancer-cell death in rats (Borlinghaus et al., 2014). The second, cepaenes, are the thiosulfates that contribute to the pungent smell of *A. tricoccum* and act as an antithrombotic agent (Calvey et al., 1998). And lastly, Vitamin C is an antioxidant that can boost the immune system. These compounds and others like them have historically served Appalachian communities who used ramps as a folk remedy, especially following long winters with bleak diets. Because ramps are one of the first greens to emerge, indigenous tribes and European settlers would eat ramps to add variation to their early spring diets. People would often eat ramps as a spring tonic to "cleanse the blood" and remove toxins that accumulated from

eating primarily dry beans and dry meat (Cavender, 2006). Additionally, ramps functioned as a de-wormer, an earache medicine, and a cold remedy (Moerman, 1998). Appalachian communities still eat ramps as a folk remedy, but nutritional benefits are only one aspect of the plant's cultural significance

Indigenous tribes have harvested and used ramps for generations (Moerman, 1998; Lewis, 2012). Cherokee people have a long history of ramp harvesting, which is continued today by the Eastern Band of Cherokee Indians (EBCI) in North Carolina (Lewis, 2012). In addition to medicinal use, indigenous people prepare traditional ramp meals and occasionally preserve them for special dinners and holidays (Lewis, 2012). Ramps are also significant to sense of place and time for indigenous families (Lewis, 2012). When members return annually to harvest from family ramp patches, the process connects them to previous generations of ancestors who did the same (Lewis, 2012). As indigenous people have passed these traditions from generation to generation, they have also passed the knowledge of how to sustainably harvest and maintain family patches. For example, members of the EBCI leave the roots and bulb-tip when harvesting ramps to ensure family patches can persist (Lewis, 2012). Such traditional ecological knowledge (TEK) adds a unique perspective and expertise to the complexities of modern conservation that science cannot achieve alone. The inclusion of traditional ecological knowledge (TEK) may therefore prove useful to modern conservation and the development of sustainable harvesting methods.

In addition to their enduring function in indigenous culture, ramps also served European settlers of the Appalachian region. Although the origin of the term “ramp” is unknown, it is theorized that it is derived from the Anglo-Saxon variant “ramson,” which is the common name for the European *Allium* species, *Allium ursinum* (Core, 1945). Scottish and Irish settlers

emigrated to the New World with knowledge of *A. ursinum* and likely adopted the name as they began using *A. tricoccum* (Core, 1945). Similar to indigenous tribes, European settlers relied on ramps for survival, and the plant continues to play a meaningful role in Appalachian identity (Rivers et al., 2014).

According to an interview-study by Rivers and colleagues (2014), ramps are integral to Appalachian identity and memory. Before ramps became popular, they were a symbol of poverty, because Appalachian families who could not afford to buy greens from the grocery store would instead harvest and eat ramps and other wild vegetables (Rivers et al., 2014). There are even stories about teachers sending children home from school because they reeked of ramps (Rivers et al., 2014). Consequently, a community grew around this Appalachian symbol, which eventually led to the advent of widely attended ramp events, such as ramp suppers, dinners, and festivals (Rivers et al., 2014; Hufford, 1998). During these events, families would gather and prepare ramp meals, boiling or frying them, sometimes in bacon grease, and then serving them with eggs, fish, cornbread, pinto beans, and fried potatoes (Hufford, 1998). These gatherings eventually evolved into ramp festivals, which started in 1934 as a way for community members to celebrate not just ramps, but spring and Appalachian identity (Rivers et al., 2014). Although ramp festivals began as small community gatherings, they quickly gained momentum and evolved into large events featuring folk music, foods, and crafts.

Ramp festivals still occur today, and for many communities and local organizations, they serve as fundraising events (Rivers et al., 2014). For example, Richwood, WV, which is the self-proclaimed "Ramp Capital of the World," holds the Feast of the Ramson. The Feast of the Ramson is one of the largest and most widely attended ramp festivals, attracting thousands of visitors, all of whom bring money into the community (Rivers et al., 2012). However, many

ramp festivals now focus on the culinary aspect of ramps rather than the plant's traditional meaning (Rivers et al., 2014). For example, culinary competitions that celebrate the versatility of ramps have become the focal point of some festivals, like the Hudson Valley Ramp Festival in New York (Ricers et al., 2014). The evolution of the meaning of ramp festivals is likely due to the growing interest in wild harvesting by the general public over the past several decades (Rivers et al., 2014).

Between 2008 and 2012, a third of the current ramp festivals emerged, and there are now nearly a hundred ramp events along the East Coast (Rivers et al., 2014). One can also find ramps on menus in high-end restaurants in cities like Chicago and New York, and it is not uncommon to see ramps at roadside markets and farmers markets (Davis and Greenfield, 2002). As interest in the plant grows, so does the price. In 2002, a pound of ramps cost two dollars (Chamberlain et al., 2014), but today, a commercial harvester can sell a pound of ramps for ten to twenty dollars (USDA and NCRS, 2009). Furthermore, festivals consume between 350 to 750 pounds of ramps (USDA and NCRS, 2009), and commercial distributors can process around 50,000 pounds a season (Baumfleck, 2016). Many of these plants come from wild ramp patches where people harvest entire ramp plants (Davis and Greenfield, 2002).

There are a few possible reasons for this boom in interest. For example, the spread of information about wild harvesting by intrigued "foodies" in the 1990s likely resulted in more publicity about the unique character and taste of ramps. Cooking books, blogs, and newspapers have also provided increased coverage (Rivers et al., 2012). Additionally, as the price has increased, many people consider harvesting and selling ramps for supplemental income, and it is estimated that harvesters can earn upwards of $30,000 selling ramps outside the plant's range (River et al., 2012). As ramps continue to attract a mass of attention, wild ramp populations are

declining, and *A. tricoccum* is now listed as a species of concern in Maine, Rhode Island, and Tennessee (USDA and NRCS, 2019). Additionally, permits are required to commercially harvest ramps in some national forests, including North Carolina national forests (USDA and NRCS, 2009), and there are bans on harvesting in Quebec, Canada, and the Great Smoky Mountain National Park (GSMNP).

The GSMNP began monitoring ramp populations in the late 1980s, and they banned harvesting by the general public in 2002 (Rock et al., 2004; Lewis, 2012). The ban was informed by a study that concluded a ten percent harvest of entire ramp plants every ten years is the maximum sustainable harvesting intensity (Davis and Greenfield, 2002; Rock et al., 2004). This ban resulted in conflict with the EBCI, who in 2007 were barred from family ramp patches within park boundaries (Lewis, 2012). According to members, the study that informed the ban did not consider TEK regarding indigenous harvesting methods (Lewis, 2012). However, a bill was passed in 2016 that allows federally recognized tribes to collect traditionally important plants in national park boundaries, such as ramps (Gathering of Certain Plants or Plant Parts by Federally Recognized Indian Tribes for Traditional Purposes, 2016), but the rule requires elaborate environmental impact studies and agreements on each species of interest. Currently, the EBCI is working with several federal and state agencies to research sustainable harvesting methods and to reach agreements that would allow them to harvest ramps and other traditionally significant species.

Although harvesting is banned in Quebec, Canada, and the GSMNP, many ramp populations are not formally protected. Fortunately, managers and researchers are developing alternative strategies to protect populations. For example, researchers are developing harvesting methods to certify commercial sellers as sustainable harvesters (Baumfleck, 2016). Additionally,

forest farming techniques are being developed for commercial and private ramp cultivation (Davis and Greenfield, 2002). Practices such as these would ideally alleviate some of the pressure placed on wild ramp populations by harvesters. Moreover, inclusion as an NTFP could incentivize more thoughtful management of wild and transplanted ramp patches and support monitoring programs. All of these conservation strategies, as well as more information on seed collection and storage, TEK, and life history, aid in the protection of ramps. This undergraduate thesis project specifically focuses on developing tools to monitor the genetic diversity of *Allium tricoccum*.

There is only one genetic diversity study for *Allium tricoccum* to date, and it is based on isoenzyme markers. The isoenzyme study analyzed six populations of both *A. tricoccum* and *A. tricoccum* var. *burdickii* in different habitats around Ottawa, Canada (Vasseur et al., 1990). Fifteen bulbs for each variety were collected from each population, and fourteen enzyme systems were assayed to test for polymorphism (Vasseur et al., 1990). Six enzyme systems were detected, and two enzyme systems were variable (Vasseur et al., 1990). The study concluded low genetic diversity, and there was no variability between the varieties (Vasseur et al., 1990). However, the study was confined to the plant's northern range, limited by the low resolution of isoenzyme markers, and executed before the plant grew publicly popular. Our study, which is in part funded by the GSMNP, aims to develop microsatellite markers to further examine the genetic diversity of *A. tricoccum*.

Microsatellites, or Short Tandem Repeats (STRs), are co-dominant markers commonly used in population genetic studies. Unlike isoenzyme markers, microsatellites are neutrally selected and therefore highly polymorphic, which enables high-resolution genetic diversity studies within and among plant and animal populations. The purpose of this study is to develop

microsatellite markers for *Allium tricoccum* to analyze genetic diversity, population clonality, and population structure in the GSMNP and throughout its range. The development of microsatellite markers for *A. tricoccum* and subsequent genetic studies are crucial to the proper management of this culturally and economically important plant.

Methods

Plant tissue was collected from five *Allium tricoccum* individuals in the GSMNP, one *A. tricoccum* var. *burdickii* individual in Massachusetts, and one *A. cernuum* individual from the Appalachian State University herbarium (accession no. 1786) during the summer of 2018. The tissue samples were stored and dried in separate 2 mL tubes on 1 mL of silica gel and stored at -80 °C. *Allium tricoccum* and *A. cernuum* tissue samples were ground using liquid nitrogen, and *A. tricoccum* var. *burdickii* tissue samples were ground using sand and a micro-pestle. DNA was extracted from *A. tricoccum*, *A. cernuum*, and *A. tricoccum* var. *burdickii* ground tissue samples via a modified CTAB extraction method (Doyle and Doyle, 1987), and DNA was quantified using an ND-1000 nanodrop spectrophotometer (Thermo Fisher Scientific US) and a 1% agarose gel. Extracted DNA was stored at -20 °C.

High quality *Allium tricoccum* DNA was sent to 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 (Browstein et al., 1996). An M13 primer was also added to the 5’ end of the forward primer.

PCR reactions were prepared in 10 μL using DI H_2O, 5X GoTaq Flexi Buffer, 2.5 mM $MgCl_2$, 800 μM dNTP's, 0.5 μM of each primer, 0.5 units of GoTaq DNA Polymerase, and 30 ng of template DNA. One negative control with water and template DNA from five *Allium tricoccum* individuals, one *A. burdickii* individual, and one *A. cernuum* individual were used for primer screening. A Touchdown PCR program ran under the following conditions: 1 cycle of 94.0°C for 5 minutes, 13 cycles of 94.0°C for 45 seconds, 68.0°C for 2 minutes and decreasing 0.5°C per cycle , 72.0°C for 1 minute, then 25 cycles of 94.0°C for 45 seconds, 53.0°C for 1 minute, and 72.0°C for 1 minute, then 72.0°C for 10 minutes, and PCR ended at 10.0°C. DNA amplification was analyzed with a 1% agarose gel and product size was compared to a 1kbp ladder (Thermo Fisher Scientific US).

Results

Fifty microsatellite primers were screened. Seven reactions were prepared with DNA from five *Allium tricoccum* individuals, one *A. tricoccum* var. *burdickii* individual, and one *A. cernuum* individual. The eighth reaction was prepared with water as a negative control. *Allium cernuum* was the outgroup. Primers that resulted in no amplification or products with nonspecific amplification, inconsistent amplification, too many bands, and product sizes above 500 base pairs were eliminated from the screening process.

Nineteen of the 50 (30%) screened primers successfully amplified: AT01, AT02, AT03, AT04, AT05, AT06, AT07, AT10, AT17, AT18, AT21, AT26, AT28, AT32, AT25, AT37, AT42, AT44, and AT50 (Figure 5). Successful primers had a GC content between 45.5% and 55%, annealing temperatures between 58°C and 61°C, and primer sizes between 19 and 25 base pairs (Table 1). Successful markers consistently amplified in *Allium tricoccum* and *A. tricoccum*

var. *burdickii* individuals. The remaining 31 primers unsuccessfully, and there was no consistent amplification in the outgroup, *A. cernuum*.

Marker	Primer Sequence (5' → 3')	Motif	GC (%)	Annealing T (°C)	PIG tail seq
AT01	F CGGACCTCGTATGCACAAG R GTTTAGGGTACTGTTCATAGGCGG	AAAT	F 47.9 R 55	F 59.064 R 59.645	GTTT
AT02	F GGAGTGTAATTTGCGGCATTG R GTTTGGGTGAAATGGAGAAGGC	AAC	F 47.6 R 47.6	F 59.213 R 59.523	G
AT03	F CTCGACGTGCCTTTGAAGAG R GTTTAGCTGAGCCAACAATGTGAG	AAG	F 55 R 50	F 59.668 R 59.223	GTTT
AT04	F ATCTGGTTCGGGCATTCAAC R GTTTGGTTCGACAGTGGTTGG	AAG	F 50 R 50	F 59.293 R59.368	G
AT05	F GTTCGGACCAATCAACCAGC R GTTTCGACGTACCTTGGAAGTGG	AAG	F 55 R 55	F 60.223 R 60.152	GTT
AT06	F ATTTCCCAAGCGTTCCCATC R GTTTGAAGATGGTGCAGGAGG	AATGT	F 50 R 50	F 59. 287 R 58.558	G
AT07	F GGAGTGAGAAACGTGATGGG R GTTTGGGTGGGTTCATTTATTTGGC	AATT	F 55 R 47.6	F 59.02 R 58.5	GTTT
AT10	F AGTGAGTACGATCAGGCATTG R GTTTCCAAATCGATCCGTTCCC	AC	F 47.6 R 50	F 58.583 R 58.722	GT
AT17	F ACTGGATAGGATGGCTAGTGAC R GTTTCCGATCTGCTCAACAATGC	AC	F 50 R 50	F 59.542 R 59.375	GTT
AT18	F AGTGAGTACGATCAGGCATTG R GTTTCCAAATCGATCCGTTCCC	AC	F 47.6 R 50	F 58.583 R 58.722	GT
AT21	F AGCAACTCCCAAACATCACC R GTTTGGTGTATGCAGGTGAGAC	ACAT	F 50 R 50	F 59.141 R 58.284	GT
AT26	F AAACCCAACACACAGCCAAG R GTTTGCTGCCATTTACCGTCTC	AG	F 50 R 50	F 59.936 R 59.301	GT
AT28	F TGCTTCTTCCAGATCCTTGC R GTTTCTCCTCCGCACTCATTTC	AG	F 50 R 50	F 58.639 R 58.366	GT
AT32	F GCAAACAAATCATGGCCATCC R GTTTGGTGGAACGATGTGGAGTATC	AGG	F 47.6 R 52.4	F 59.47 R 58.929	GTTT
AT35	F AAGTTCGGTGTGAGCAACTC R GTTTGTTCTAACTTGGTGGACGC	AT	F 50 R 50	F 59.163 R 58.875	GTT
AT37	F GGGATTGTTGAGAAAGAAACCG R GTTTGCAAAGCCGAACTAGGTC	AT	F 45.5 R 50	F 58.592 R 59.23	GT
AT42	F AGTGTATGCATATTGTCCGCAG R GTTTGCGTGGCTCCTACTTAAC	AT	F 45.5 R 47.6	F 59.624 R 59.334	G
AT44	F GGGATTGTTGAGAAAGAAACCG R GTTTGCAAAGCCGAACTAGGTC	AT	F 45.5 R 50	F 58.592 R 59.23	GT
AT50	F ACGAGCTAAGTGTCGATCGC R GTTTCGACTTTCACTTGGATCGG	AAT	F 55 R 50	F 60.982 R 58.382	GTT

Table 1. Forward and reverse primer sequences, microsatellite motif, GC content, annealing temperatures, and PIG tail sequence for successful primers AT01, AT02, AT03, AT04, AT05, AT06, AT07, AT10, AT17, AT18, AT21, AT26, AT28, AT32, AT35, AT37, AT42, AT44, AT50.

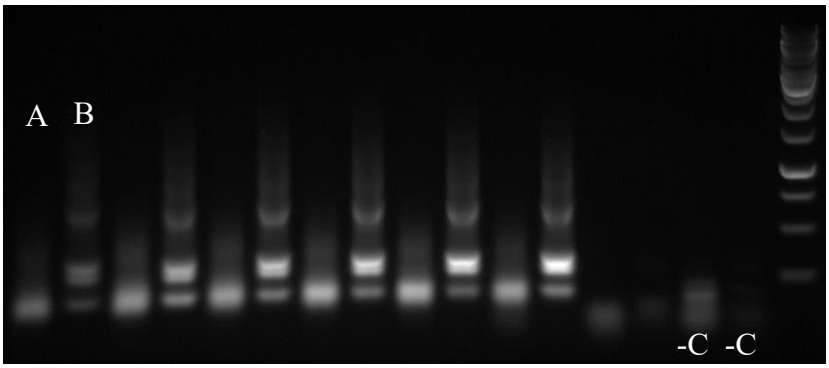

Figure 1. Example of 1% agarose gel with two staggered microsatellite primers with negative controls (-C). (A) AT17 and (B) AT18 successfully amplified.

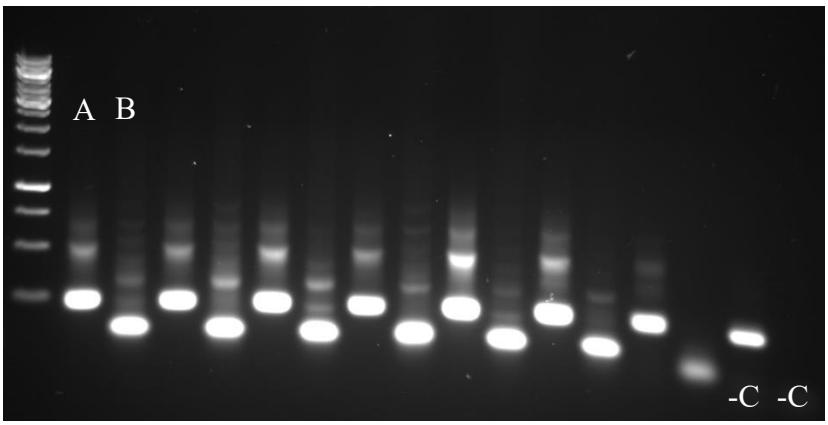

Figure 2. Example of 1% agarose gel with two staggered microsatellite primers with negative controls (-C). (A) AT31 consistently produced bands in the negative control, and (B) AT32 successfully amplified.

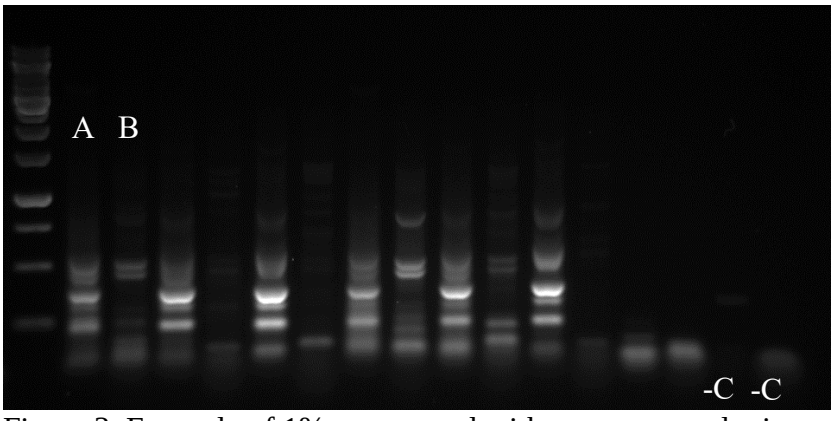

Figure 3. Example of 1% agarose gel with two staggered microsatellite primers with negative controls (-C). (A) AT11 and (B) AT12 unsuccessfully amplified.

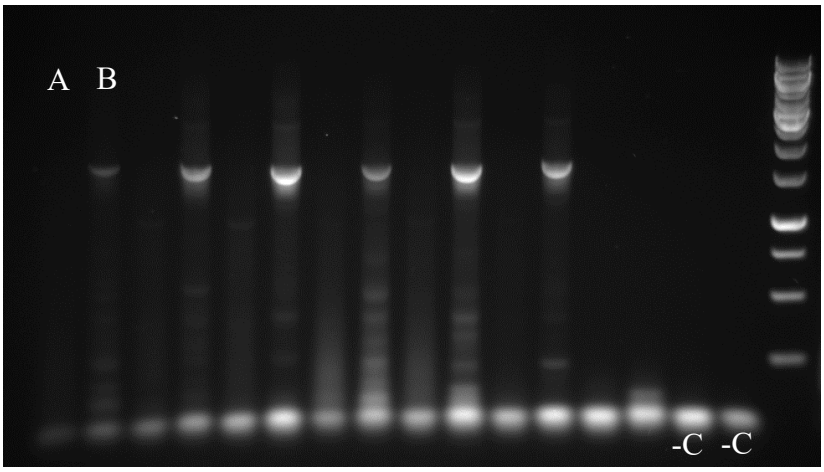

Figure 4. Example of 1% agarose gel with two staggered microsatellite primers with negative controls (-C). (A) AT13 and (B) AT14 unsuccessfully amplified.

Discussion and Conclusion

There was no consistent amplification in the outgroup, *Allium cernuum*, which may be due to DNA degradation in the herbarium specimen and/or its distant phylogenetic relationship with *A. tricoccum*. It would be beneficial to collect fresh samples of *A. cernuum* to conclusively determine the reason for inconsistent amplification. Meanwhile, all successful markers consistently amplified in *A. tricoccum* and *A. tricoccum* var. *burdickii*. Markers that produced bands in the negative control following the replacement of PCR buffers and template DNA may have amplified DNA other than *A. tricoccum* or produced strong primer dimers and were therefore excluded from the screening process.

Following primer screening, successful primers will be fluorescently tagged and genotyped on an ABI3730 to analyze allelic variation and determine polymorphic markers. Once developed, these tools can be used to evaluate genetic diversity and population structure with higher resolution than the isoenzyme markers (Vasseur et al., 1990). Similarly, microsatellite markers can be used to further analyze the taxonomic relationship between *Allium tricoccum* and *A. tricoccum* var. *burdickii* to determine if a species separation is warranted. Additionally, although a demographic study concluded that asexual reproduction by clump division is the

primary mode of population size maintenance (Nault and Gagnon, 1993), these markers can be used to genetically test that hypothesis by evaluating population clonality.

The development of microsatellite markers for *Allium tricoccum* provides an opportunity to better understand the plant's biology, and managers can use these tools to answer important conservation questions. For example, within the GMSNP, managers can use genetic studies to monitor the effect of sustainable harvesting on genetic diversity and population structure. More broadly speaking, genetic studies can determine how the genetic diversity of publicly accessed populations is affected by increased harvesting. Overall, the development of microsatellite markers for *A. tricoccum* can inform management decisions and add to the pool of knowledge needed to achieve a sustainable balance between the conservation of ramps and the rich Appalachian culture that relies on them.

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