Identifying Evolutionary Significant Units in *Spiraea virginiana* Using Microsatellite Markers

by

Logan Crawford Clark

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Approved by:

_____
Matt Estep, Ph. D., Thesis Director

_____
Bridget Tuberty, M.S., Second Reader

_____
Lynn Siefferman, Ph.D., Departmental Honors Director

_____
Ted Zerucha, Ph.D., Interim Director, The Honors College

Abstract

Spiraea virginiana Brit. (Rosaceae) is a rare clonal shrub found in isolated populations within the Cumberland, Tennessee, and Ohio River drainages. This species has been listed as federally endangered since June 1990 due to anthropogenically induced habitat loss and population fragmentation as a result of river damming. Reproduction consists of a mixed mating system that is mostly asexual by ramet formation, with occasional dispersal via vegetative fragmentation downstream. Successful sexual reproduction is limited, and could result from self-fertilization or outcrossing. The species does appear to outcompete other shrub species by vigorous rhizome production and its ability to withstand scouring floods. The lack of sexual reproduction could potentially result in an extremely limited effective population size in each river. This study aims to assess the genetic diversity of *S. virginiana* populations along the New and Cheoah Rivers in North Carolina using eight previously published microsatellite markers. Our results suggest a small effective population size within each of the two rivers. These results are consistent with earlier investigations and could have management implications, possibly treating each river drainage as its own evolutionary significant unit for (ESU).

Introduction

Spiraea virginiana Britt., commonly known as Virginia Spiraea, is a federally listed riparian shrub found in fragmented populations throughout the Southern Appalachians and Cumberland Plateau. It was first described in 1890 from a collection by C.F. Millspaugh along the Monongahela River in Monongalia County, West Virginia (USFWS, 1990). Currently *S. virginiana* is restricted to the Ohio River Basin and is known from 33 watersheds across 6 states including Ohio, West Virginia, Kentucky, Virginia, North Carolina, and Georgia (USFWS, Ogle). This study focuses on two rivers; the South Fork of the New River in the northwestern corner of North Carolina (Watauga and Ashe County) and the Cheoah River in the southwestern corner of North Carolina (Graham County). Although these two rivers are not geographically separated by more than 200 miles they flow in opposite directions, draining two different basins, the Ohio and Tennessee respectively.

Taxonomy and Habitat

Spiraea virginiana is a member of the family, Rosaceae, the rose family. This family includes a wide range of plants used for edible and ornamental purposes. The genus *Spiraea* includes 80 species found across North America, South America, and Asia (Williams, 2003). This species exhibits variable leaf size, shape, and degree of serration, which has resulted in historic confusion between *S. viriginiana* and its sister taxa *Spiraea corymbosa*. The confusion between the two species was later resolved due to differing habitats, *S. corymbosa* preferring more open rocky woods, and clearing edges along the Northern Blue Ridge and Piedmont (USFWS, 1990; Ogle, 1991). Virginia Spiraea has a large and fibrous root system and grows from two to ten feet tall, with arching and upright stems. The leaves are alternate with acute bases, a dark green top, a glaucous (dull grayish-green or blue color) underside,

and vary in their margins from entire to serrate. The degree of serration is usually single, and sometimes curved. The serrations can range from coarse to fine, with a mucronate (abrupt, sharp point) apex. The flowers are cream colored and occur in branched, flat-topped corymb inflorescences (stalks arise at different levels on the main axis and reach about the same height and in which the outer flowers open first) that are 4 to 8 inches wide and flower during June and July, with stamens that are approximately twice the length of the sepals. (USFWS, 1990; Ogle, 1991).

This species is highly adapted to a specific habitat with high sunlight and scour throughout the Southern Appalachians and Cumberland Plateau. Natural populations are only found along scoured banks of high gradient second and third order streams or on point bars, braided features, meander scrolls, and natural levees (Ogle, 1991). *S. virginiana* is a prolific sprouter that forms dense clumps that spread into rock crevices and around boulders. The species occurs within the maximum floodplain, requires high amounts of sunlight, and cannot compete well with larger, shadowing species, like *Phyllostachys spp*. (bamboo) or other common riparian species such as Ninebark *(Physocarpus opulifolius)* and Elderberry (*Sambucus sp*.) that occupy suitable habitat in higher numbers. This species requires disturbance such as flooding or scour strong enough to break off pieces of its rhizome to wash downstream and colonize new habitat. These scour events are also used to maintain its niche by elimination of competing species, while leaving the parent plant which is aided by its large and fibrous root system. (Ogle, 1991).

Reproduction

The reproduction of *S. virginiana* is thought to be largely comprised of asexual propagation via rhizome dislodgement to form downstream ramets (Ogle, 1991). Sexual

reproduction is extremely limited to non-existent in natural populations with no seedlings observed in the field (Ogle, 1991). However, common garden experiments have shown seed set is possible if individuals from different drainages are crossed, suggesting the possibility of a self-incompatibility mechanism to prevent inbreeding (Brzyski and Culley, 2013, Murrell and Anders, 2001, Emery, 2014). Members of Rosaceae are known to have an S-RNase based self- incompatibility system (Ashkani et al. 2016). This pre-fertilization mechanism results in a failure to produce viable zygotes after self pollination due to identical genotypes expressed in the pollen and female sporophyte tissues, which acts as a built in measure to ensure out crossing, which is thought to have evolved to help prevent an inbreeding depression (Ashkani et al. 2016). *S. virginiana* is thought to be a clonal species, which would result in identical genotypes found throughout local populations. This theoretically could limit the local mate availability due to this self-incompatibility mechanism. Low mate availability, which would result in low sexual reproduction, should theoretically result in limited gene flow and high differentiation among populations (Pate, 2010).

Distribution

The present distribution of *Spiraea virginiana* reflects a population structure caused by glacial and interglacial cycles during the Quaternary Period, between 1.6 million years ago and 12 thousand years ago (kya) (Anders and Murrell, 2001). This geologic process drove range expansion and constriction to refugia in the southeast, particularly the Southern Appalachians. This provides a unique opportunity to study the effects of past climate change on plant species that may hopefully aid in understanding some of the current challenges with the changing environment (Ander and Murrell, 2001). As the climate warms it can be

expected to see changes to current ecosystems in terms of both geographic location and demography of populations. By studying plants known to have been affected by previous climatic changes informed management decisions can be better made in order to most effectively conserve biodiversity in the face of modern day climate change.

During this period the Laurentide Ice Sheet pushed ecosystems and plant communities toward more southern locations than their current distributions (Delcourt and Delcourt, 1984). As the ice sheet receded and the climate warmed, these plant communities were able to recolonize the more northern locations of their present range. This would result in the southernmost extant populations harboring more genetic diversity than their northern counterparts due to having been established for the longest period of time, giving them more time to accumulate mutations and therefore more diversity. This increased genetic diversity would make the southernmost populations evolutionary significant units (ESU) for conservation purposes. Studies in another riparian shrub, *Alnus glutinosa*, in Europe found that the southernmost populations harbored more genetic diversity and were a valuable source of evolutionary potential to sustain the species (Lepais et al. 2013).

The Hypsithermal period that followed glaciation about 8.5 kya brought warm and dry conditions to eastern North America which plausibly favored plants adapted to these conditions (Anders and Murrell, 2001). *S. virginiana*'s current riparian habitat and genetic isolation may be due to the events of this time period during which frost churn and increased erosion inhibited a boreal competition, allowing *S. virginiana* to flourish with more suitable habitat available. This species was left restricted to riparian zones where suitable habitat, with scour and high sunlight could be found as the climate changed (Ogle, 1991).

Threats and Conservation

S. virginiana has been listed as federally endangered since June of 1990 and currently has a global ranking of G2, meaning that this species is imperiled and at a high risk of extinction due to very restricted range, very few populations (often 20 or fewer), steep declines in individuals, or other factors. The status of this species is most likely due to a conglomeration of anthropogenic activities coupled with what may have been a historically rare or restricted distribution of the species, due to it's riparian affinity. The only known documented cases of extirpation have been anthropogenically induced by river damming, water recreations and accidental roadside mowing. Three populations have been lost to river impoundments on the Little Tennessee River, Cypress Creek in Alabama, and the Monongahela River in West Virginia (Ogle, 1991). Populations in North Carolina have been destroyed due to road development along the Cheoah River and industry along Hominy Creek in Buncombe County, North Carolina (Ogle, 1991). These documented cases are likely examples of more widespread extirpation events. Habitat alterations have increased the possibility of extinction for this species by suppressing natural stochasticity in the environment, which many riverine species including *S. virginiana* depend on to survive (Ogle, 1991). These artificially created low disturbance conditions favor plant succession and competition, while too much disturbance may exceed the species ability to maintain viable populations (Ogle, 1991). Destruction of suitable habitat, such as this, has led to further fragmentation and isolation of populations. Invasive species, such as like *Phyllostachys spp*. (bamboo) and even *Spiraea japonica* (a close relative*),* outgrow and outcompete Virginia Spiraea. Impoundment of rivers

and decreased snowmelt with warming global temperatures pose further threats to the natural disturbance regime that this species relies on (Pate, 2010).

Previous genetic studies have investigated population structure and evolutionary history using morphometric analysis, RAPDs, ISSR's and microsatellites (Anders and Murrell, 2001; Brzyski and Culley, 2011). These studies found high levels of genetic differentiation among populations, low levels of gene flow, and higher genetic diversity in the southern part of the range suggesting ancestral populations of glacial refugia, and evidence that *S. virginiana* is dominated by a few, large clonal lineages (Anders and Murrell, 2001; Brzyski, 2011). This study aims to investigate the population structure of this imperiled species along the New and Cheoah Rivers in North Carolina in hope of identifying Evolutionary Significant Units (ESU) for in-situ conservation efforts, using 8 previously published microsatellite markers (Brzyski, 2010). This would help aid in management policies and possible augmentation efforts to preserve this Appalachian endemic riparian shrub.

Methods

During the summer of 2016 starting in June several field excursions were made along the New River in Ashe County North Carolina to collect samples from 12 populations of *S. virginiana* using Elemental occurrence (EO) data from the NC natural heritage program. Populations were accessed via boat and 8 leaf samples were taken at each EO and stored in vials of silica gel. Once back the samples were stored in a -80°C freezer where they were kept until needed for DNA extraction. Similar methods were used on the Cheoah River samples collected by collaborators at UNC Asheville.

DNA was extracted using a standard CTAB procedure (Doyle and Doyle, 1987). The resulting DNA was quantified using a Nanodrop 1000 (V3.6, Thermofisher, US) and imaged on a 1% agarose gel. The DNA was then screened using PCR and 8 microsatellite markers (Brzyski, 2010). The 96 samples of highest quality were arrayed into a 96 well plate and amplified with the 8-microsatellite markers and four florescent dyes (VIC, NED, FAM, PET). Individual PCR products were then multiplexed into two plates (4 dyes each) using hi-di and LIZ 500 size standards (Applied Biosystems, US). The plates were then sent to Georgia Genomics for separation of labeled fragments. The resulting chromatograms were then scored using Geneious 10.1.2 (Kearse et al., 2012) with the microsatellite plug-in (Kearse et al., 2012). A set of scoring standards was outlined which included identification of peak pattern, intensity of peak height (>500), and the size range of peaks.

The genotype data was then exported into an excel sheet where samples that failed across 5 or more markers were removed from the data set. The PCR reactions for VS8 were also removed, due to a high rate of failure.

The resulting data was then analyzed in excel using GenAlEx 6.503 (Peakall and Smouse 2012) to calculate allele frequencies, a genetic distance matrix, F-statistics, Hardy-Weinberg Equilibrium HWE, Analysis of Molecular Variance (AMOVA), and Principal Components Analysis PCoA. An analysis of genetic structure was run using the Bayesian statistical program STRUCTURE (Pritchard Lab, 2009). A batch run was set at a burn-in time of 500000 with 5000000 reps. The burn-in time is how long to run the simulation before collecting data to minimize the effect of the starting configuration and the number of reps after burn-in is how long to run the simulation after burn-in in order to get accurate parameter estimates. The resulting analysis was then re-organized through STRUCTURE

HARVESTER online interface (Earl and vonHoldt, 2012). This software calculates the most appropriate number of genetic groups (ΔK value) from the data. Once the number of groups was calculated (K=2) STRUCTURE was run again with the K value set to 2 for the same burnin time and reps. The resulting data was then re-drawn in the online interface, PopHelper, in order to create the bar plot showing the distribution of genetic groups (Francis, 2016).

Results

The data analysis in GenAlEx revealed very low genetic diversity averaging less than 2 (1.87) alleles per locus with a range of 1.43 to 2.00 (Table 1). Three private alleles were identified in the New River and five in the Cheoah River. There were two private alleles at EO-02 and one in Unknown EO 2. EO-02 displayed the highest average alleles per locus as well as the highest average effective alleles (alleles occurring at equal frequencies in the population) with 2.43 and 2.01 respectively. These values are still considered very low in terms in genetic diversity. The lowest average number of alleles was found in EO16 and EO46 both with 1.43 average alleles per locus. The lowest number of average effective alleles per locus was found in EO45 with 1.25. The Analysis of Molecular Variance (AMOVA) indicated the majority, 83%, of the genetic diversity was found within river drainages while the remaining 17% was found among drainages (Figure 1) An average F_{st} value of 0.92 across all markers was obtained. The F_{st} values reflect the variance in allele frequencies among populations, small values mean that the allele frequencies within each population are similar (Holisinger and Weir, 2009). The PCoA analysis explained 43.58% of the variation across the 1st two axes, but showed very little clustering of populations and no distinct grouping between drainages (Figure 2). Eight shared-multilocus genotypes were

identified across all samples, suggesting some level of clonal reproduction (Table 3). These were identified within the same EO, between EOs, and even between the two drainages. The STRUCTURE HARVESTER analysis identified two distinct groups (k=2) within the dataset (Figure 3). This analysis also suggested a second highest ΔK value for k=8, but it was decided to use the higher ΔK for k=2 originally assuming that these two groupings may fall out as river drainages. Each EO showed admixture of varying degrees of these two groupings, which did not correspond to river drainage.

	Sample Size	Average Alleles per Locus	Average Effective Alleles per Locus	Private Alleles
EO01	3	1.857	1.477	0
EO02	8	2.429	2.045	2
EO15	4	1.714	1.569	0
EO16	4	1.429	1.395	0
EO17	6	2	1.682	0
EO44	7	1.714	1.319	0
EO45	6	1.571	1.249	0
EO46	4	1.429	1.297	0
EO48	6	1.857	1.498	0
Unknown Pop 1	6	2	1.488	0
Unknown Pop 2	4	1.857	1.648	1

Table 1. Allelic Diversity by EO

	Sample Size	Number of Alleles per Locus	Effective Alleles per Locus	Private Alleles
New River	58	3	1.726	3
Cheoah	14	2.571	1.952	5

Table 2. Allelic Diversity by Drainage

ID	Clones	EO
A	2	44
B	3	48, Unknown 2
C	3	44,45,46
D	2	17
E	2	Unknown 1, Cheoah
F	4	44,45, Unknown 1
G	4	1,44,45
H	3	1,2,45

Table 3. Observed Shared Multilocus Genotypes

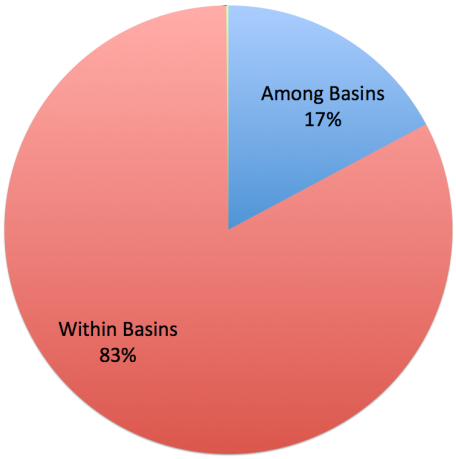

Figure 1. AMOVA of data divided by drainage

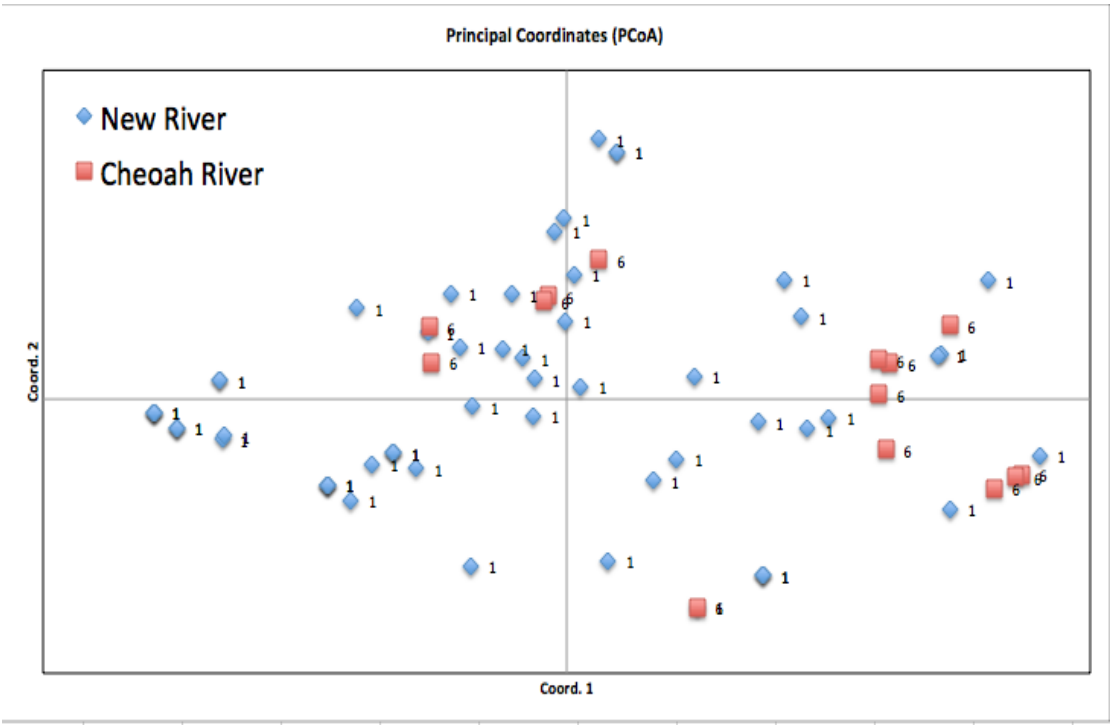

Figure 2. Principle Coordinate Analysis

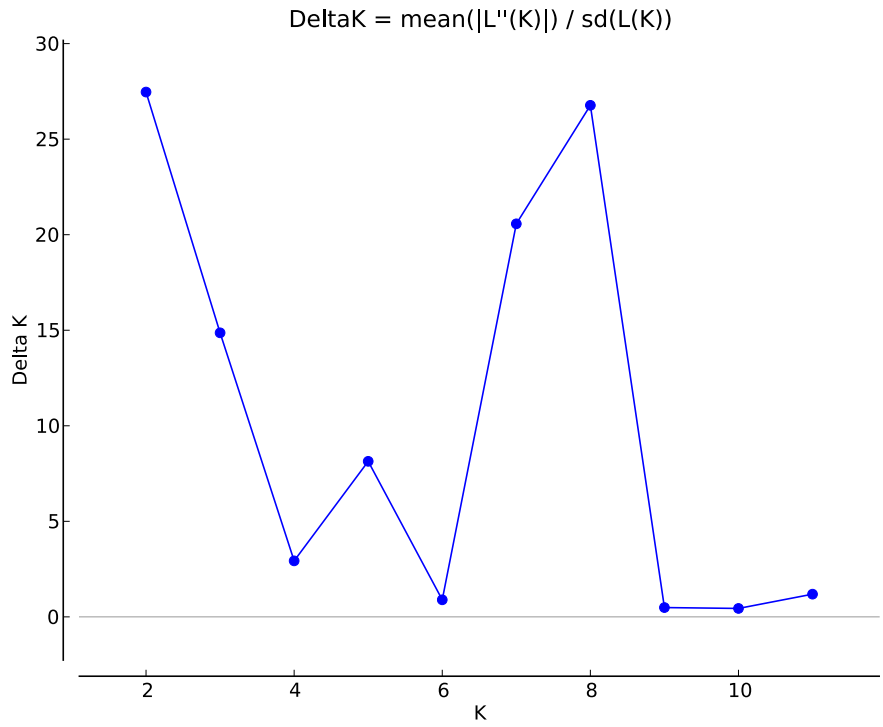

Figure 3. ΔK Values generated in STRUCTURE HARVESTER

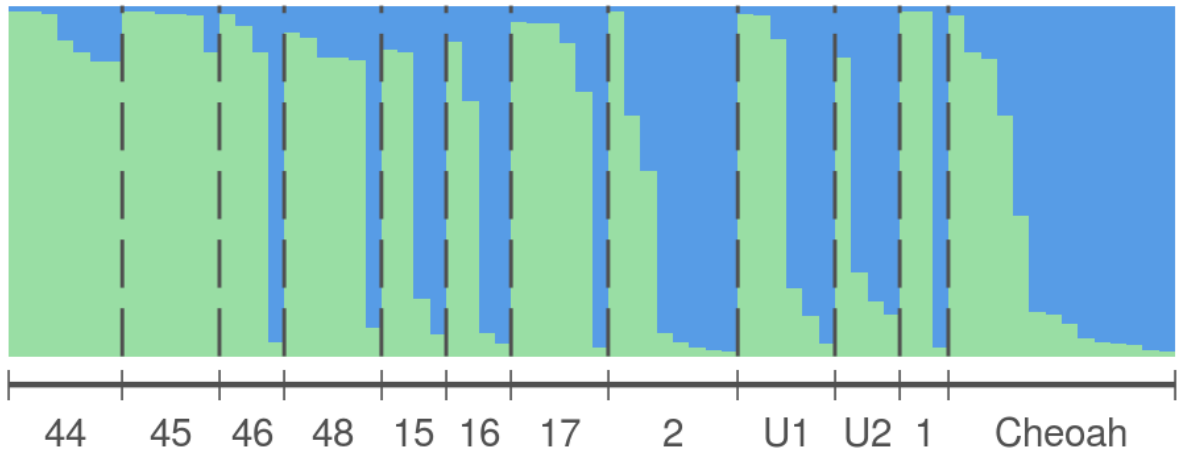

Figure 4. STRUCTURE Analysis with EOs organized with flow of the New River, south to north, with the Cheoah outgroup

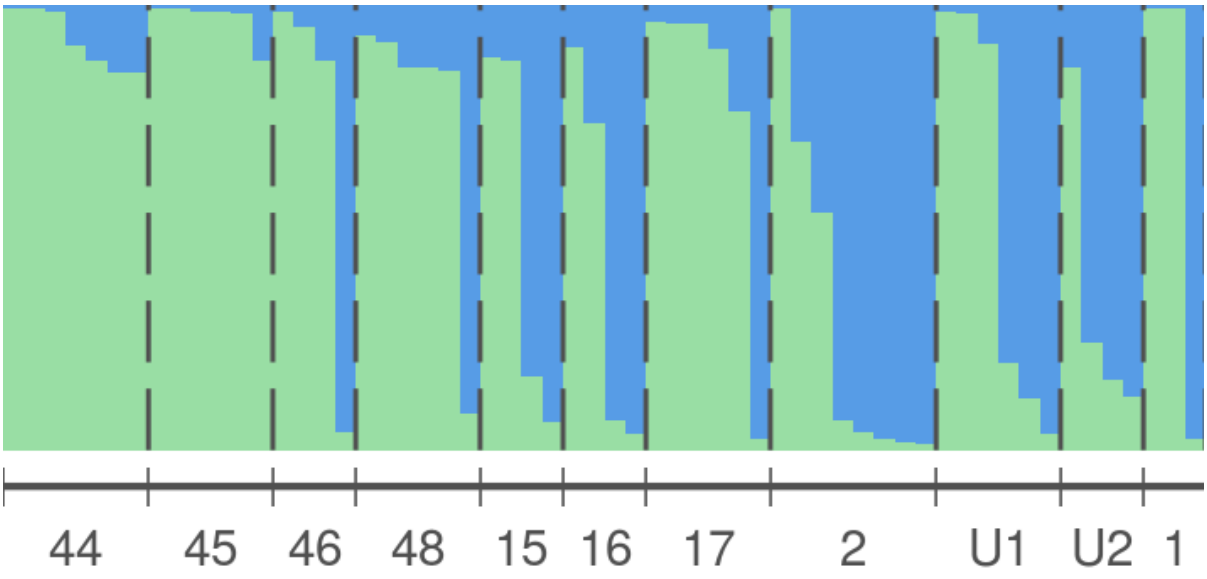

Figure 5. STRUCTURE Analysis with EOs organized with flow of the New River, south to north

Discussion

Spiraea virginiana may not be as clonal and genetically isolated as once thought. These results did not support the original hypothesis of population differentiation by drainage. Although evidence of clonality was documented, not to the extent that was expected based on previous work. Low genetic diversity was observed and the unique alleles identified in each drainage suggest the populations are currently isolated, but there is evidence of admixture between drainages suggesting shared ancestry. The higher number of private alleles found in the more southern Cheoah River would also support the glacial refugia hypothesis, suggesting that the Cheoah populations have theoretically been isolated for a longer period of time, giving them more time to accumulate mutations resulting in unique alleles. These unique alleles have the potential to contribute to this species' evolutionary potential into the future.

The PCoA analyses (Figure 2) showed very little clustering of populations or divergence between drainages. This result does not support the original hypothesis of population differentiation. Based on previous studies it was expected that *S. virginiana* would be a "green fish", genetically isolated within each river drainage (Anders and Murrell, 2001). The data collected in this study displays contrasting evidence and requires further investigation. These drainages appear to be more genetically similar than originally thought. Levels of clonality were identified using shared multilocus genotypes, which are individuals sharing the same genotype across all markers within the data (Table 3). These were individuals who could have been in the same EO, different EOs, and even individuals in separate river drianages. This does support previous claims of clonal reproduction to some extent, but *S. virginiana* does not appear to be reproducing strictly asexually. Based on

shared multilocus genotypes there appears to be admixture between the two drainages, which could be ancestral. If our original hypothesis was correct it would be reasonable to have found one shared genotype in each EO (reflecting asexual clones), but in contrast some EOs contained more than one multilocus genotype and these genotypes were shared across drainages. This could potentially mean that there is more sexual reproduction than originally thought, but the very low allelic diversity does not support that notion. Further investigation is currently underway to better understand the reproductive strategies and barriers in this species.

Asexual Reproduction by Ramet Formation

The STRUCTURE analysis (Figure 4), with each EO organized in accordance with the flow of the New River from South to North, shows a migration from one distinct genetic identity toward a higher level of admixture with the second genetic group moving downstream. This observation does not support the original hypothesis of downstream ramet formation. If this species was indeed reproducing via clonal fragments it would be reasonable to expect to see the same genetic group identity maintained downstream. The cause of this phenomenon is unclear, but re-examination of the dispersal hypothesis in this species is needed. These findings could point to the possibility of more sexual reproduction occurring within the species than previously thought.

Augmentation Towards Species Recovery

Based on this data and analyses, there is no reason to oppose population augmentation. There appears to be little differentiation between these two drainages with some multi-locus genotypes shared. Further analysis is recommended across the entire range before cross drainage augmentation is attempted. It is recommend that clones used in

augmentation efforts be sourced from the same drainage in an effort to maintain any local adaptation and prevent the possibility of an outbreeding depression (Ellstrand and Ellam, 1993). The ultimate goal of any restoration effort made with this species should be to establish and maintain a viable population that requires a minimal amount of management intervention (NC Plant Conservation Guidelines, 2005). More research is currently underway in an effort to understand what reproductive barriers this species may be facing. This information will be critical in the long-term preservation of Virginia Spiraea.

During fieldwork it was noticed that a majority of landowners mow all the way down to the rivers edge eliminating any riparian habitat or buffer zone in which this species lives in order to get a better view of the river. *S. virginiana* is known to help reduce erosion and produces attractive flowers during the summer, possibly making them a desirable addition to landowner's property. Additionally, signs advertising "Save the New River" regarding a proposed relocation of a sewage effluent release were also observed. What better way to tap into the effort to save the New River than preserving a unique piece of its ecology, a beautiful Southern Appalachian endemic? Therefore, the possibility that USFW offer clones of these plants, available from both Appalachian State University and UNC Asheville greenhouses, to land owners to plant along the riparian zone of their properties is offered as a suggestion. This project would involve the public in an effort to preserve our biodiversity and educate them on better land practices, while also bulking the sheer numbers of this species, which is crucial to the long term preservation of *Spiraea virginiana*

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