WESTERN CAROLINA UNIVERSITY

A STUDY OF THE SYSTEMATIC RELATIONSHIPS BETWEEN MEMBERS OF THE *TRILLIUM ERECTUM* COMPLEX

A thesis presented to the faculty of the Graduate School of
Western Carolina University in partial fulfillment of the
requirements for the degree of Master of Science in Biology

By

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ABSTRACT

Systematic relationships among members of the *Trillium erectum* complex (*T. cernuum* L., *T. flexipes* Rafinesque, *T. simile* Gleason, *T. rugelii* Rendle, *T. erectum* L., *T. sulcatum* T. Patrick, and *T. vaseyi* Harbison) are not well described. For the purposes of conservation and cataloguing biodiversity it is important to know the phylogenetic relationships among taxa and quantify gene flow among these hybridizing taxa. A study of pollinator fidelity and geographic isolation was conducted used in combination with cladistic relationships inferred from chloroplast DNA sequencing and genetic distance inferred from allozyme electrophoresis data. The level of genetic divergence typically seen among species within the family Trilliaceae was considerably higher than that observed among members of the Erectum complex. Allozyme data suggest a high degree of gene flow among sympatric taxa, and local habitat selection pressures. However, these data also support the hypothesis of assortative mating between different floral colors as a factor maintaining species distinctiveness. The Erectum complex taxa appear to be sets of hybridizing groups in states of incomplete speciation.

INTRODUCTION

Along the southern range of the Appalachian Mountains are found 11 described species of pedicellate *Trillium*; six of these are known to hybridize with varying levels of introgression (Case and Case, 1997). These six taxa, along with *Trillium cernuum* L., a species restricted to northeastern North America, form the monophyletic Erectum (group) complex of the genus *Trillium* (Farmer and Schilling, 2002). Phylogenetic relationships within the Erectum complex have not yet been discerned; researchers have not been able to find informative genetic variation among taxa. Members of this group can hybridize and the hybrids appear developmentally normal and interfertile, yet these taxa are considered distinct (Case and Case, 1997). From this situation arise questions of how taxonomic groups are distinguished as species and what barriers maintain species distinctiveness. Certain species are found in sympatric populations (*T.erectum* and *T.rugelii*), while the ranges of other species barely overlap (*T.vaseyi* and *T.sulcatum*). The production of fully developed, fertile offspring through hybridization suggests there is no genetic basis for reproductive isolation and these taxa are not fully diverged (Grant, 1981; Coyne and Orr, 2004). This complex appears to behave as a syngameon or semi-species as described by Grant (1981), having no evidence of intrinsic reproductive barriers, hybridization that could lead to introgression, and distorted morphological boundaries (Grant, 1981). Distributions of taxa within the *Trillium* Erectum complex suggest there may be extrinsic geographical (between allopatric taxa) or ecological isolating mechanisms (between sympatric taxa). Examples of possible geographic

barriers could be the Appalachian Mountains or the Tennessee River, and examples of ecological isolating mechanisms can range from selection for different habitats, pollinators, or temporal isolation (Coyne and Orr, 2004). These extrinsic barriers are not complete and are known to be "leaky," allowing for introgression, if there are not intrinsic reinforcement mechanisms (Grant, 1981; Coyne and Orr, 2004). Extrinsic mechanisms are also reversible, meaning that disturbance or removal of a barrier could lead to homogenizing of populations into a single conspecific gene pool, translating into the loss of species diversity.

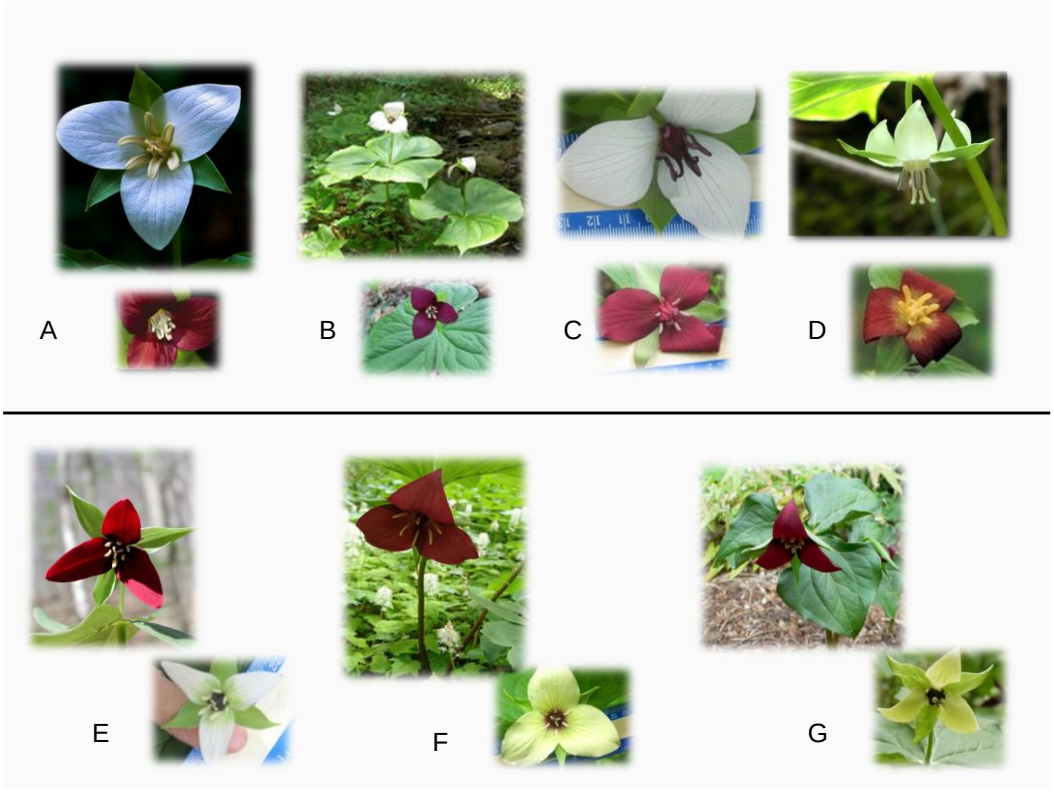
The objectives of this study were threefold: qualify barriers to gene flow within the *Trillium* Erectum complex, quantify levels of introgression among taxa, and to identify whether discrete taxonomic units exist as defined under the Biological Species Concept (Mayr, 1982). Each of these objectives will be addressed in the following chapters of this thesis. Chapter One contains background information on the biology and taxonomy of the Erectum group and summarizes previous research. Chapters Two and Three address the question of extrinsic reproductive barriers and seek to describe those that may occur, including pollinator isolation and geographic and ecological isolation. Chapters Four and Five examine gene flow among taxa using two forms of molecular data, cpDNA sequences and allozyme profiles. Finally, Chapter Six contains a discussion of species boundaries in the Erectum group in light of my findings from the previous chapters.

CHAPTER ONE: BACKGROUND

Background to taxa

The genus *Trillium* is comprised of long-lived herbaceous flowering plants; all but one species (*Trillium camschatcense* Ker Gawler, found in Asia) are found in North America (Case and Case, 1997). *Trillium* plants typically produce one or more scapes (depending on age) from a rhizome (Hanzawa and Kalisz, 1993; Case and Case, 1997). Each scape bears a single, trimerous flower, which may be erect or declined, and three leafy bracts. The true leaves are found below ground on the rhizome. The flowers are perfect, with a flask- to globular-shaped, superior ovary and six stamens that vary in size and shape depending on the taxa (Barksdale, 1939). Petal and sepal colors can vary from bright white to deep purplish reds; they also may be yellow or streaked with a variety of colors (Figure 1; Table 1, Appendix A). The individual taxa have slightly staggered flowering times starting usually from early March (but as early as late January) up until late June (but as late as August) in the Appalachian habitat (Case and Case, 1997). Each taxon can have a wide array of pollinators that varies by species, habitat, or time of day (Gonzales et al., 2006; Griffin and Barrett, 2002). *Trillium* are generally self-compatible but with varying levels of inbreeding depressions that result in highly reduced seed set or fruit production (Wright et al., 2008; Sage et al., 2001).

Figure 1: Photographs of each member of the Erectum complex with their respective color variants. A: *T. flexipes*; B: *T. simile*; C: *T. rugelii*; D: *T. cernuum*; E: *T. erectum*; F: *T. vaseyi*; G: *T. sulcatum*. Pictures of *T. cernuum*, *T. flexipes* taken by Frederick Case and pictures of *T. sulcatum* by Thomas Patrick.

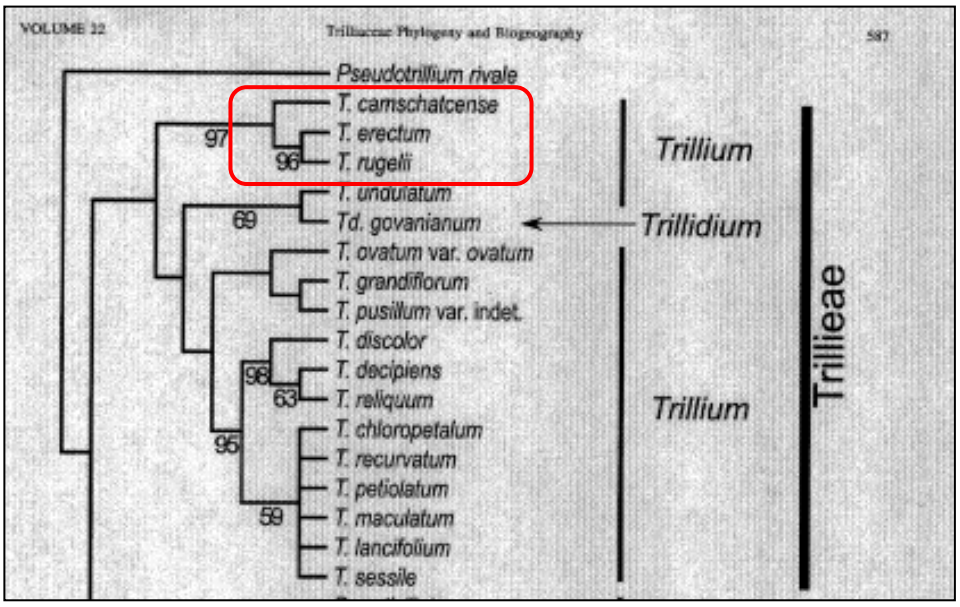


Trillium species produce secondary metabolites that are both harmful and beneficial to humans. Asian species of *Trillium* are being used to develop multidrug resistance inhibitors for cancer treatments from the steroidal alkaloids found in the rhizome (Yokosuka and Yoshihiro, 2008). The North American species are known to have antimicrobial, antifungal, and antibacterial properties, and can be used as a uterine

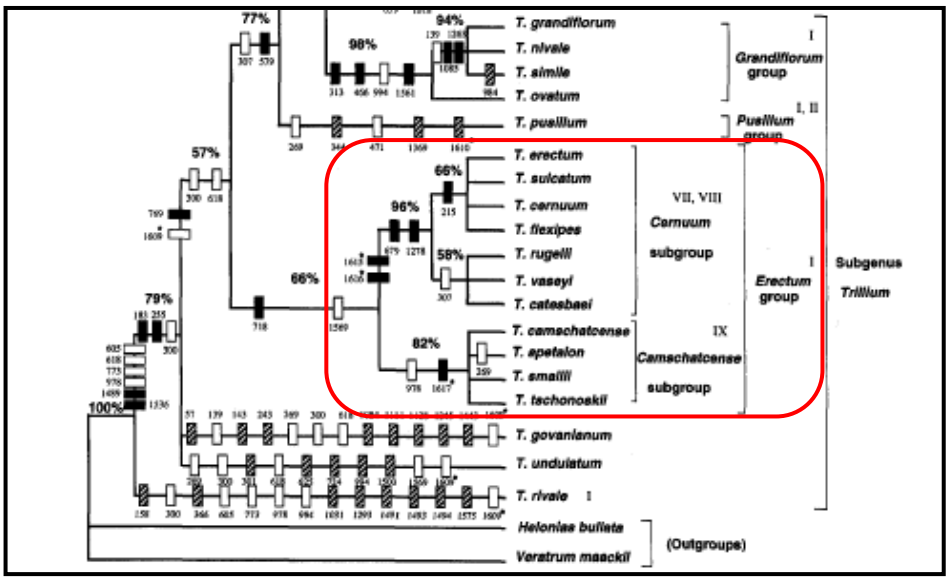
stimulant (Case, 2008). Some parts of the plant can cause serious pain, injury, and even death if consumed (Case, 2008).

Taxonomy

There is much debate surrounding the familial placement of *Trillium* and arrangement of lower taxa within the genus. *Trillium* is currently treated as a member of the family Trilliaceae by A. Weakley (Weakley, 2006), a family which first became recognized in 1846 (Lindley cited in Farmer, 2006). Yet the Angiosperm Phylogeny Group considers Trilliaceae to be synonymous with Melanthiaceae, and does not recognize it as a separate entity (Stevens, 2001). Within the genus *Trillium* the pedicellate flowered taxa (subgenus *Trillium*) and the sessile flowered species (subgenus *Phyllantherum*) (Case and Case, 1997) have been separated. Subgenus *Phyllantherum* is accepted as a monophyletic sub-group of 22 species, and an arrangement of lower taxa within that group has been published and widely accepted (Farmer, 2006). Several methods of cladistic analysis using a variety of data sets have led to resolution of several different clades within the subgenus *Trillium* (Figure2: Farmer, 2006; Osaloo et al., 1999; Ihara and Ihara, 1978). The most recent phylogenetic study by Farmer (2006) is consistent with that of Osaloo et al. (1999, Figure 2) and shows that members of the Erectum complex are monophyletic. Within the Erectum Complex relationships are uncertain.



A



B

Figure 2. Phylogenetic relationships within the pedicellate Trilliums. A: Farmer 2006. B: Osaloo et al. 1999

A classification of lower taxa within subgenus *Trillium* based on morphological characteristics such as ovary shape and color, stigma length and curvature, and stamen

morphology was defined by Barksdale (1939, Figure 3); which similarly to Farmer's and Millam's molecular phylogenetic studies, differentiates *T. undulatum* Willdenow, which has a 3-angled ovary, from the Grandiflorum group (*T. grandiflorum* (Michx.) Salisbury, *T. catesbei* Elliot, and *T. pusillum* Michaux), which has a 6-angled ovary, and the Erectum group (*T. cernuum* L., *T. flexipes* Rafinesque, *T. simile* Gleason, *T. rugelii* Rendle, *T. erectum* L., *T. sulcatum* T. Patrick, and *T. vaseyi* Harbison), which has a 6-angled ovary and rhombic leaves. The evolutionary relationships among taxa belonging to the Erectum group remain unresolved (Millam, 2006; Farmer, 2006), with the current taxonomic divisions between taxa within the complex based morphological differences only.

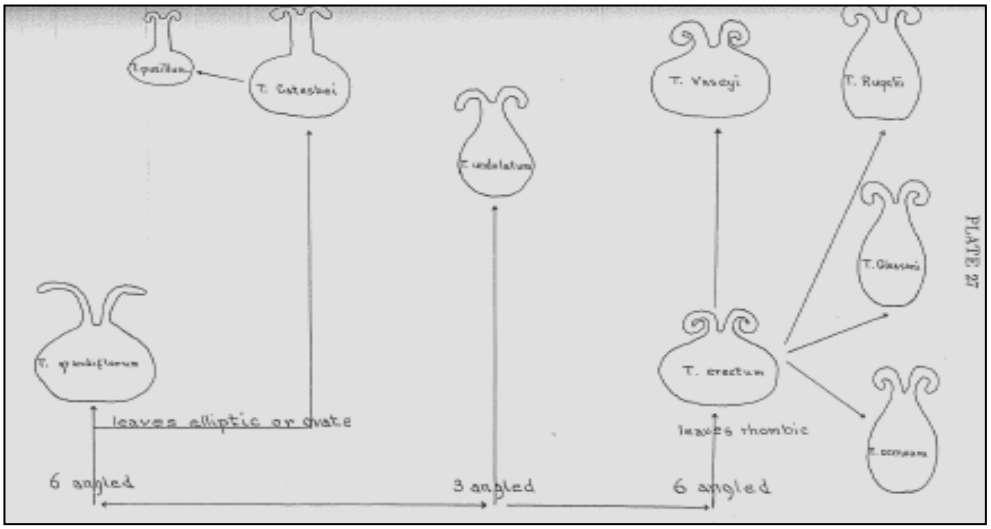


Figure 3. Hypothesis of relationships in pedicellate *Trillium* based on ovary morphology (Barksdale, 1939).

The Erectum Complex has been accepted as a monophyletic group within subgenus *Trillium* (Farmer, 2006, Ihara and Ihara, 1978, Millam, 2006; Osaloo et al., 1999). The complex is composed of the following taxa: *Trillium cernuum, T. flexipes, T. simile,* and *T. rugelii*, which are typically white flowered; and *T. erectum, T. sulcatum,* and *T. vaseyi* which are typically red flowered (Table 1 contains detailed morphological descriptions, Appendix A). The goals of this project were to estimate the phylogenetic relationships among members of the Erectum group, determine if significant levels of introgression are present among taxa, and determine possible ecological factors affecting introgression.

Syngameons & Hybrid Complexes

The prevailing theme behind the Biological Species Concept (BSC; Mayr, 1982) is that species are groups defined by only the populations which are included in a particular gene pool and excludes all others that are intrinsically reproductively isolated from that gene pool (Coyne and Orr, 2004). This species concept defines discrete groups of taxa in the strictest and sometimes the most inclusive sense. This definition has not been satisfactory to some because it may mask the total variation and diversity present. There are many of other species concepts that have been published and widely used in taxonomy that are based on character state, evolutionary cohesion, and interbreeding (Coyne and Orr, 2004). Either way you define a species there are problematic exceptions, such as hybridization and introgression, that blur morphological distinctiveness.

Many groups of taxonomically distinguished sister taxa are known to hybridize naturally and produce fertile offspring (Coyne and Orr, 2004). Groups of naturally hybridizing taxa can be referred to as syngameons (Lotsy cited in Stebbins, 1958), a notable example being the White Oak syngameon found in California (Arnold et al., 2004). These complexes are also referred to as semispecies (Grant, 1981), a term which may be more descriptive of the true nature of their relationship with one another. Grant describes semispecies as occurring during "various intermediate stages of divergence and reproductive isolation, when populations are neither good races nor good species but are connected by a reduced amount of interbreeding and gene flow." (pg. 71 Grant, 1981). Strictly speaking, groups of interfertile populations could be considered conspecific, but Grant (1981) distinguishes these systems by limited gene exchange: "syngameons behave like a well-isolated biological species on their outer boundary, but differ in their more complex internal structure". They are defined as "the most inclusive interbreeding population system in a hybridizing species group" (pg. 74 Grant, 1981). Another type of relationship can exist between hybridizing taxa: the hybrid complex. A hybrid complex is distinct from a syngameon in that hybrid races or species within the group exhibit stable reproduction, and there are high levels of introgression among populations which have distorted the morphological boundaries between parent species (Grant, 1981). Although the famed Louisiana Irises are thought to fit the syngameon model (Arnold et al., 2004), they are probably a more accurate representation of a hybrid complex.

Members of the Erectum complex may be in the midst of a similar state of limbo in their evolution and not much is known about the amount of genetic exchange that occurs among taxa within the complex. The taxonomic limits are described and defined

by morphological characteristics, most of which are variable and overlap among species (Table 1, Appendix A).

"Taxonomy is required to identify and monitor components of plant diversity to ensure conservation and sustainable use" (United Nations' Convention on Biodiversity Article 7, page 20 Leadlay and Jury, 2006). Groups of taxa that are not fully diverged and maintain levels of introgression are hard to differentiate taxonomically for practical use in conservation management. When forming taxonomic groups it is important to realize that assumptions about genetic cohesion and divergence within and among taxa will be made by those creating management plans (Leadlay and Jury, 2006). Taxonomic groups, unless otherwise specified in the literature, may be treated as independent gene pools and managed without regard to historical gene flow between other taxa. Understanding the reproductive relationships between groups of taxa can contribute to information not only on current gene flow and phylogenic relationships but also the future trajectory of the organism and the ecological factors that influence it. Character state delineations do not always offer the same insights (Leadlay and Jury, 2006).

Barriers to Gene Flow

To address questions of species distinctiveness within hybrid complexes and syngameons, it is necessary to know about paths of gene flow within the complex, be able to quantify gene flow, and know something about the barriers to gene flow. Patterns in the fertility relationships of plant species based are on life form and breeding system (Grant, 1981). Grant describes one pattern, the "*Geum*" pattern, as a type of fertility relationship often seen in "perennial herbs without prominent species-to-species

differences in floral mechanism" (Grant, 1981). This pattern is described as closely related species which are interfertile, yet have compatibility barriers within the complex. These taxa are predominantly outcrossed. Also, members of this complex would have little difference in floral mechanism between species, and barriers that do exist among species are due to extrinsic or ecological factors (Grant, 1981). This pattern describes the apparent breeding system in the Erectum complex.

Since the taxa in the Erectum complex of *Trillium* are known to hybridize successfully (producing fertile offspring) in wild populations, it is difficult to determine the mechanisms that reinforce distinct gene pools in sympatric populations. Introgression among taxa could make finding species-specific genetic markers difficult. A particular allele may be fix in a population or taxa but if there is hybridization and back crossing that allele may be shared with individuals outside of the population or taxa and therefore could not be listed as a species or population marker. Measures of genetic distance may correlate more with presence or absence of isolation barriers than with evolutionary relationships. Based on Grant's (1981) model of the typical fertility relationships among perennials like *Trillium*, reproductive isolation barriers could be extrinsic (i.e. ecological). These barriers could hold clues to the driving forces behind divergence in this group. Since hybrids formed in this group are said to be developmentally normal and fertile (Case and Case, 1997), there is no evidence that points to intrinsic barriers to gene flow.

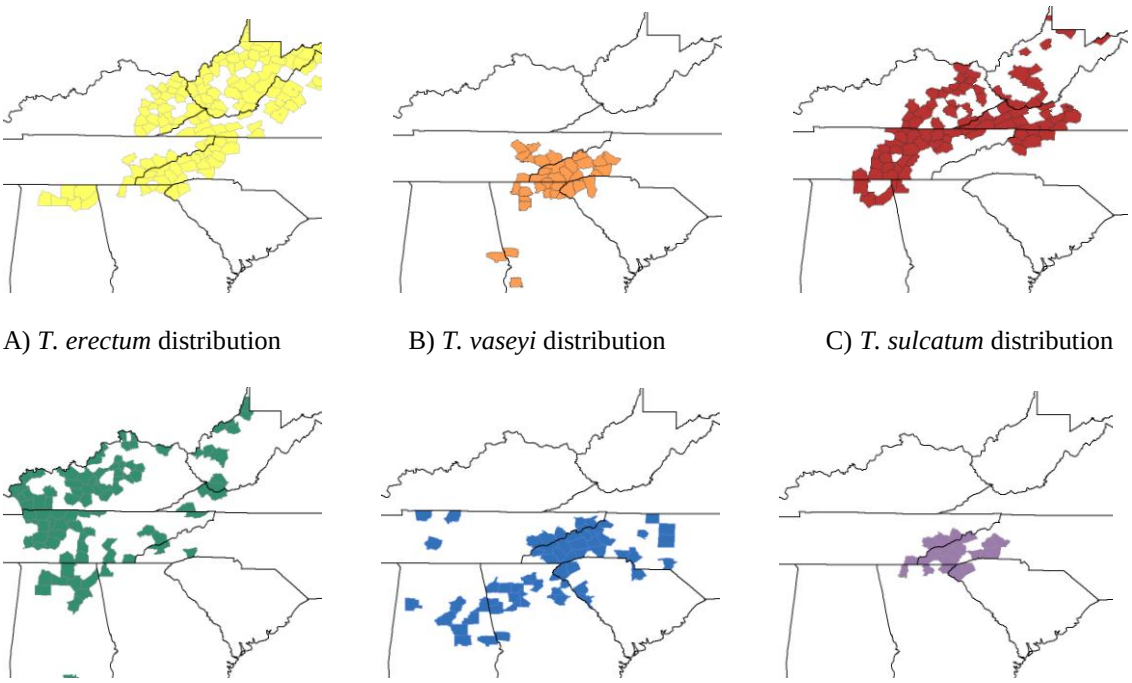
Members of the Erectum complex tend to be found in sympatric populations of taxa from the opposite flower color group. The ranges described for most taxa do not

overlap (or scarcely so) with the ranges of similar flower colored taxa (Figure 4). *Trillium* individuals may have relatively close pollen donors, shown to be within 2.2 m in two species of pedicellate *Trillium* (Irwin, 2001). Seed dispersal by ants is common in this genus and has been shown to influence the genetic structure within populations of *T. grandiflorum* (Kalisz et al., 1999). These patterns suggest taxa not found in sympatric or parapatric populations would have very little gene flow.

Two types of extrinsic barriers to gene flow may exist in *Trillium*: pre-zygotic extrinsic barriers such as habitat isolation and post-zygotic extrinsic barriers such as ecological inviability of hybrids (Coyne and Orr, 2004). The contrasting flower color scheme in the Erectum Complex also suggests the presence of assortative mating via pollinator preference. This type of extrinsic pre-zygotic mating barrier would allow taxa with different colored flowers to remain distinct in sympatry (Coyne and Orr, 2004). Assortative mating by pollinators happens when one particular group of pollinators visits the same floral type in succession (Kearns and Inouye, 1993; Grant, 1981; Coyne and Orr and Orr, 2004). A related concept that might also occur in a model of assortative mating is floral constancy; this is a characteristic of an individual pollinator in which it visits the same floral type repeatedly and in succession (Kearns and Inouye, 1993; Grant, 1981; Coyne and Orr, 2004).

Assortative mating has been documented in red and white campions (*Silene dioica* L. Clairv. and *S. latifolia* Poir.) using dyed pollen (Coyne and Orr, 2004) and between white and pink phlox (*Phlox pilosa* L. *and P. glaberrima* L.) (Grant, 1981). *Phlox pilosa* and *Phlox glaberrima* are both usually pink, but there is a white variant of

P. pilosa. These species are pollinated by the same species of Lepidoptera (Grant, 1981). The white form is usually rare in populations where *P. pilosa* is the only species present, but in sympatric populations with both species the white form is dominant (Grant, 1981). Levin and Kerster (1967, cited in Grant, 1981) studied pollen movement between the species and determined that five times as much pollen was deposited from *P. glaberrima* to stigmas of the pink form of *P. pilosa* as to the white form (Grant, 1981). This gave the white form a selective advantage because it received less pollen from outside of its own species (Grant, 1981). Similarly, in the study by Coyne and Orr (2004), dyed pollen was used to show pollinator isolation between red and white campions (*Silene dioica* and *S. latifolia*), which overlap in some pollinators. The strength of the reproductive barrier between the red and white species was found to be ca. 0.45, where 0 is no pollinator isolation and one is complete assortative isolation (Coyne and Orr, 2004).



A) *T. erectum* distribution B) *T. vaseyi* distribution C) *T. sulcatum* distribution

D) *T. flexipes* distribution E) *T. rugelii* distribution F) *T. simile* distribution

Figure 4. Geographic distributions of *Trillium* Erectum complex taxa.

Rationale for Study

There is no immediate concern for the conservation of most *Trillium* species since they are fairly abundant within their range. Yet because of their small ranges some species are listed as "rare," "threatened," or "vulnerable." *Trillium simile* is listed as "rare" in both NC and SC, a "species of concern" in GA, and global status G3: vulnerable (Weakley, 2010; NatureServe, 2010). *Trillium sulcatum* is a "species of concern" in GA and the global status is G4 (Weakley, 2010; NatureServe, 2010). *Trillium rugelii* is on the NC Watch List and listed as "rare" in SC (Weakley, 2010; NatureServe, 2010).

It is important to consider how likely it is that even the healthiest populations of all species will be maintained in light of present day circumstances, such as *Tsuga*

canadensis die off and climate change. These circumstances have the potential to cause dramatic shifts in the ecological forces that effect Trillium species in eastern forests (Eschtruth et al., 2006; Crookston, 2010). Since *Trillium* species are extremely difficult to cultivate from seed, many are poached from the wild. Deer herbivory is one of the most prominent threats to *Trillium* populations, and management of deer herds could mitigate any further harm (Vellend et al., 2003; Case and Case, 1997). Some species have an exceptionally small range that covers areas susceptible to human development (i.e. habitat loss), and some species have small, fragmented populations that may be sensitive to loss of genetic diversity from the loss of populations and/or suitable habitat.

It is also important to consider that *Trillium* species are managed by state and federal organizations according to current taxonomic delineations. There is the possibility that these delineations are too narrow and do not include all interbreeding populations. In other words they would not include all the individuals (or all of the genetic diversity) necessary to sustain a minimum viable population, and management decisions based on this could be detrimental to species diversity and population health. Evaluate these species now, while they are still in sustained populations; and we can determine what ecological and geographical features vital to maintaining diversity, seems wise. If these species do become endangered, biologists the current body of knowledge, regarding *Trillium* life history characteristics and gene flow among and within taxa, is inadequate for creating proper management plans. It is recommended that information about genetic diversity within and among taxa be used to guide conservation science (Leadlay and Jury (2006).

Key barriers that influence genetic diversity within and among taxa in healthy populations need to be examined. Once evaluated, this knowledge would also not only provide valuable information to the scientific community for the subgenus *Trillium*, but also contribute to the overall body of knowledge regarding biogeography in the Southern Appalachians and to speciation mechanisms in general.

Study Site

The Southern Appalachian Mountains contain the highest diversity of *Trillium* taxa in North America, making this an ideal place to study the relationships and gene flow among sympatric populations. Field sites were chosen based on their isolation from anthropogenic disturbance, population size (25-100 individuals), and abundance of *Trillium* taxa present. Sites included Standing Indian Wildlife Management Area (Macon County, NC N34.99972, W-83.46778), Balsam Mountain Preserve (Jackson County, NC N35.39, W83.2), Wolf Creek Watershed area (Jackson County, NC), Great Smoky Mountains National Park (Swain County NC, N35°33'01.98", W83°29'33.08" and Sevier County, TN N35.66833, W-83.4725), Frozenhead State Park (Anderson County, TN N36.256, W-84.4690), Chattahoochee National Forest Chattooga district Black Rock Mountain State Park (Rabun County, GA; N34.908146, W -83.409536), Sumter National Forest Whetstone district, Whitewater Falls area (Oconee County, SC N34.8619 W-83.1919), Western Carolina University picnic area (Jackson County, NC; N35.315632, W-83.188382), Nantahala Gorge along Hwy 64 (Swain/Madison Counties, NC; N 35.335304, W -83.622677). Since these taxa are known to hybridize in native populations

with parapatric sister taxa, individuals from the interior and the perimeter of populations were collected.

CHAPTER TWO: POLLINATOR ISOLATION

Objective

The objective to this portion of the study is to identify extrinsic interspecific mating barriers pertaining to pollinator fidelity.

Hypothesis

I hypothesize that assortative mating or floral constancy based on flower color is exhibited by pollinators, and provides a potential barrier to gene flow between red flowered and white flowered taxa. This will be tested by tracking the distribution of marked pollen and by pollinator observations. If this is true then I would expect to see an insignificant amount of pollen transferred between red and white flowers.

Materials and Methods

Pollinator observations were attempted by performing observations of patterns of floral constancy among taxa and individuals. Visual observations of pollinator behavior were made to see if pollinators would consistently visit flowers of a similar type. Two 10m x 10m plots in Balsam Mountain Preserve, Jackson County, NC, were used to test for floral constancy by tracking dyed pollen. One plot was done in April 2009 and the second in May 2009. Histochemical dyes were injected into the anther flaps prior to dehiscence; the pollen grains absorb the dyes (which are visible to the naked eye) (Kearns et al., 1993). Twenty-eight individuals of each white taxa and each red taxa in both plot had their anthers stained. The first plot contained *T.grandiflorum* (stained orange) and

T.erectum (stained blue); the second plot contained *T.erectum* (stained blue), *T.vaseyi* (stained green), and *T.rugelii* (stained magenta). The second plot contained two red species and only one white species but frequency of red and white individuals was similar. After two weeks of pollinator activity (to allow for pollen tube growth) the stigmas were collected and analyzed. We were only able to collect data from half of the individuals in one plot. One plot was highly disturbed and most individuals trampled by hikers; the other plot was in a more remote drainage but there was a high amount of insect herbivory on the anthers and stigmas. Quantitative data were scored for the different colors of pollen seen on each stigma and the approximate percentages of each present. A chi-squared contingency table was used to test the significance of cross pollination among taxa and among different colored taxa. .

Results

Eight out of 45 stigmas collected contained pollen from an individual with a different petal color (18%, Table 2, Appendix B). The resulting test statistic was $X^2 = 16.2$ (*df=1, p=0.05, x=3.84*); therefore we reject the null hypothesis that pollination was random between red and white flowered individuals. Eight of 30 stigmas (27%) collected contained pollen from an individual with the same color flower but from a different taxon. The resulting test statistic was $X^2 = 3.27$, *df=1, P=0.07*; therefore we fail to reject the hypothesis that pollination was random between different taxa of the same flower color. During the course of this study approximately 12 hours of observations were done to try to confirm the types of pollinators that were described by Barksdale (1939). Lepidoptera, Hymenoptera (ants, carpenter bees, bumble bees, sweat bees, and carpenter ants), and several species belonging to the order Araneae were all observed in or on

Trillium reproductive organs (although the spiders were never observed moving from one plant to another). Another interesting observation is that while it has been reported that some insects eat the elaiosome from the seeds (Kalisz, 1999), there was not a large amount of insect herbivory on the ovary itself. By mid to late June, all or at least some part of the petals, stigmas, styles and stamen had been eaten from almost every individual, yet most of the ovaries were untouched.

Discussion

The lack of significant pollen dispersal to plants of different floral color supports assortative mating between different color forms of these taxa. The data also supports a higher significance of pollination by flowers within the same color petals opposed to random pollination. This supports the hypothesis that similar colored taxa are genetically isolated by an incomplete and unstable extrinsic barrier, assortative mating via pollinator. Also, when you consider that these taxa are considered to be separate species it is interesting to note that the degree of cross pollination among all taxa, at least as judged by the presence of non-self pollen on the stigma, is significant ($X^2= 7.2$, $df=1$, $P=0.007$). The presence of pollen from other taxa does not confirm cross fertilization. The germination of neither conspecific nor heterospecifc pollen was able to be observed so there is no data on the successful fertilization of plants pollinated by conspecific pollen verse plants pollinated by other taxa. Since hybrids are known to form in the wild, it is known that cross fertilization occurs. But since it is not known if all pollen, conspecific and from other taxa, is equally as likely to germinate it cannot be assumed that assortative mating via pollinators is the only mechanism acting as a barrier to gene flow.

CHAPTER THREE: GEOGRAPHIC AND ECOLOGICAL ISOLATION

Objectives

The objectives of this portion of the study are to take an exploratory look at identifying extrinsic interspecific mating barriers pertaining to geographic isolation and dissimilar habitat characteristics and quantifying the relationship between potential geographic and ecological mating barriers with the presence of certain taxa.

Hypothesis

I hypothesized that taxa of the same flower color in the Erectum complex are found in different habitat types, occupy separate ecological niches, or have substantial geographical barriers between them that minimize introgression between taxa. If this is true then I would expect to observe substantial differences in the habitats of taxa with the same floral type. These differences in habitat preference might include mutually exclusive conditions pertaining to geologic formation, watershed, average precipitation, average temperature, soil type, or forest type.

Materials and Methods

Initial observations were made by entering known locations of all six taxa from herbarium data and field site collection data into a GIS shape file format imported over a US thematic base map, and then overlaying layers of USGS data on temperature, hydrology, topography, and geologic formation/soil type data. Values for each USGS category at each plant location were extracted using Spatial Analyst (ArcGIS ver. 9.3.1,

2009. Redlands, CA: Environmental Systems Research Institute) and appended into a table. The data were analyzed in R (R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria. ISBN 3-900051-07-0) by creating a Multicategory Logit Regression model (Agresti, 2007) and calculating an Analysis of Deviations table (ANOVA function in R), then forming effect plots that show the probability of a member of each taxon being located in each habitat condition.

Results

Values extracted from USGS data layers for geologic formation, watersheds, average precipitation, average yearly temperature, soil type, and forest type are listed in Table 3 (Appendix C). The Tennessee River outlined the border between the ranges of same colored species (Figure 5).

Figure 5: County-level distribution maps depicting an example of geographic isolation between taxa. The map to the left depicts geographic isolation between two red flowered species; the red blocks represents the distribution of *T. sulcatum* and the orange blocks represents the range of *T. vaseyi*. The map on the right depicts geographic isolation between two white flowered species; the green blocks represents the range of T. *flexipes*, and the blue blocks represents the range of *T. rugelii*. The center map shows the Tennessee River in blue.

An ANOVA Analysis of Deviance Table was calculated in R (Table 4). The analysis shows that there is a significant amount of deviation between the habitat characteristics of species in three categories: average precipitation, watershed, and geologic formation.

Table 4: Analysis of Deviance Table (Type II tests).

Significance codes: 0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1

Variable	Chi Sq Value	DF	P value	Siginificance
Avg._Temp.	4.924	6	0.5535946	ns
Avg._Precip.	23.183	6	0.0007374	***
Watershed	33.953	12	0.0006862	***
Forest_Type	8.285	18	0.974	ns
Geologic_Formation	143.53	72	1.14E-06	***

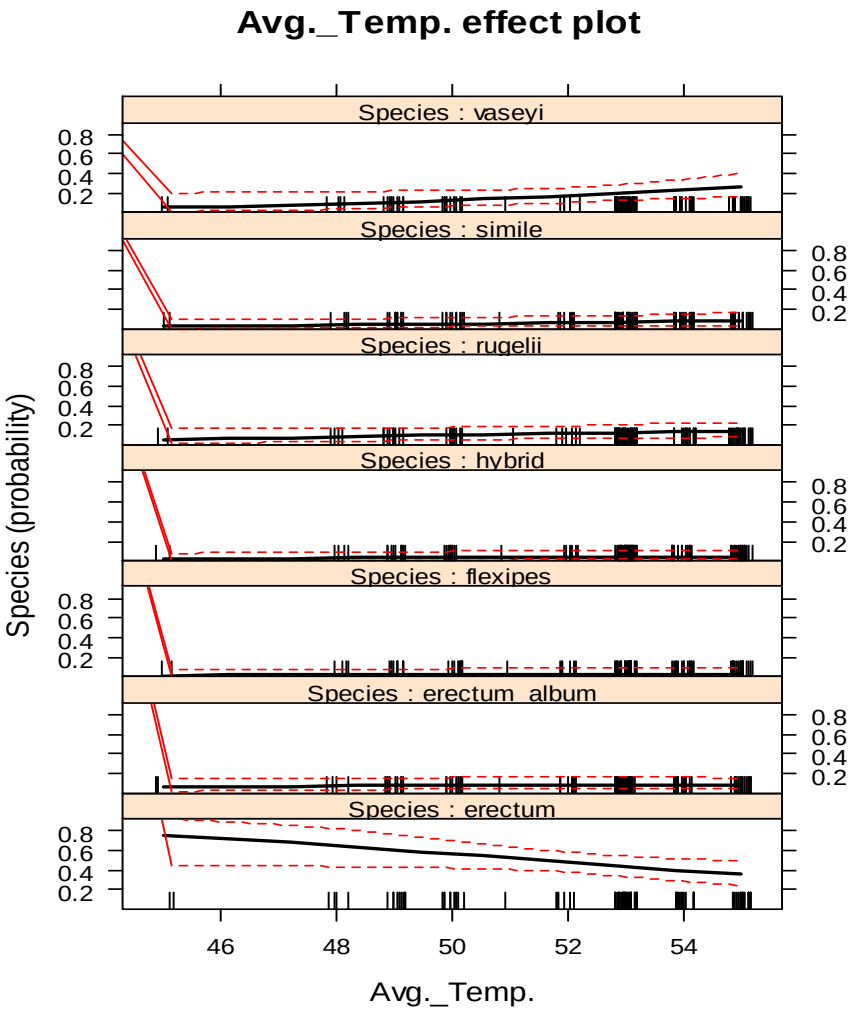


Figure 6. The Y-axis represents the probability of finding each species based upon the average yearly temperature (in degrees Fahrenheit) of a location (X-axis). The lines within the box for each species are actual values recorded.

The data in Figure 6 no not show a significance between particular habitat temperatures and the probability of find *T. flexipes*, *T. rugelii*, *T. erectum* var. *album*, and *T.simile*. The data do show that there is a higher probability of finding *T. erectum* in locations with lower average temperatures and of finding *T.vaseyi* in locations with higher average temperatures.

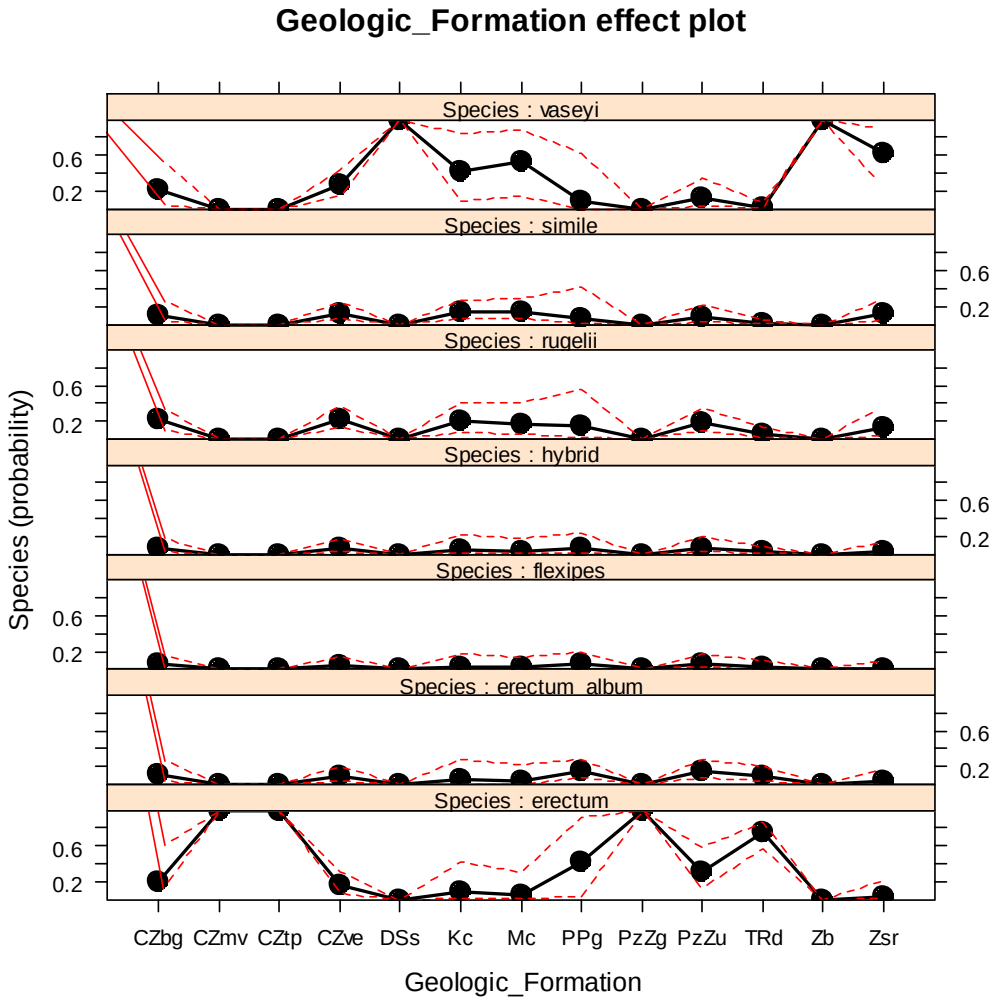


Figure 7. The Y-axis represents the probability of find each species based upon the geology type of a location (X-axis).

The data in Figure 7 show a low degree of probability of finding *T. flexipes*, *T. rugelii*, *T. erectum* var. *album*, and *T.simile* in one particular geologic formation over another. The data also shows a high probability of finding *T.vaseyi* and *T. erectum* over particular and opposing geological formations from one another.

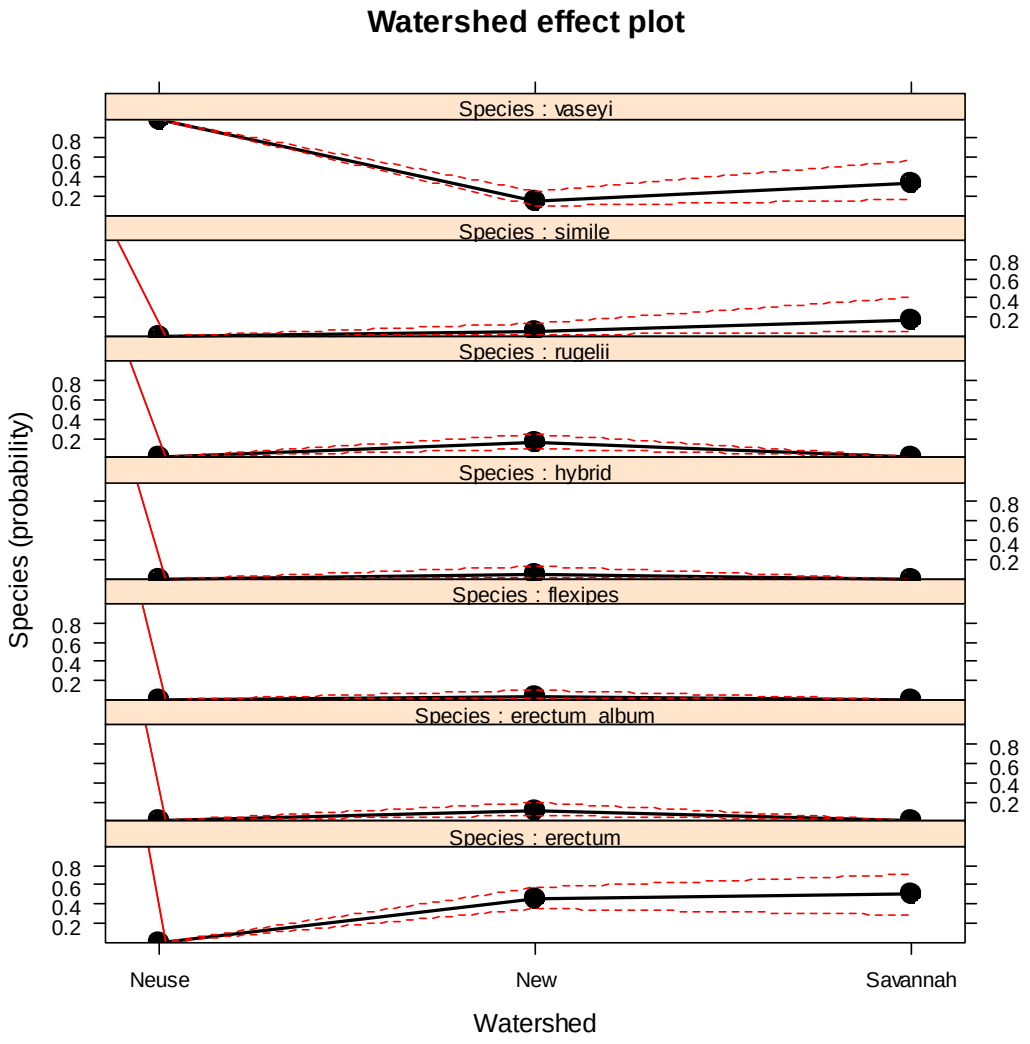


Figure 8. The Y-axis represents the probability of finding each species based upon the watershed, the X-axis, in which it was located ("New" stands for "New River" watershed).

The data in Figure 8 show a higher probability of *T. vaseyi* being in the Neuse watershed, of *T. erectum* of being in the New River or Savannah watersheds, and of *T. simile* and *T. rugelii* of being in the Savannah watershed over the others.

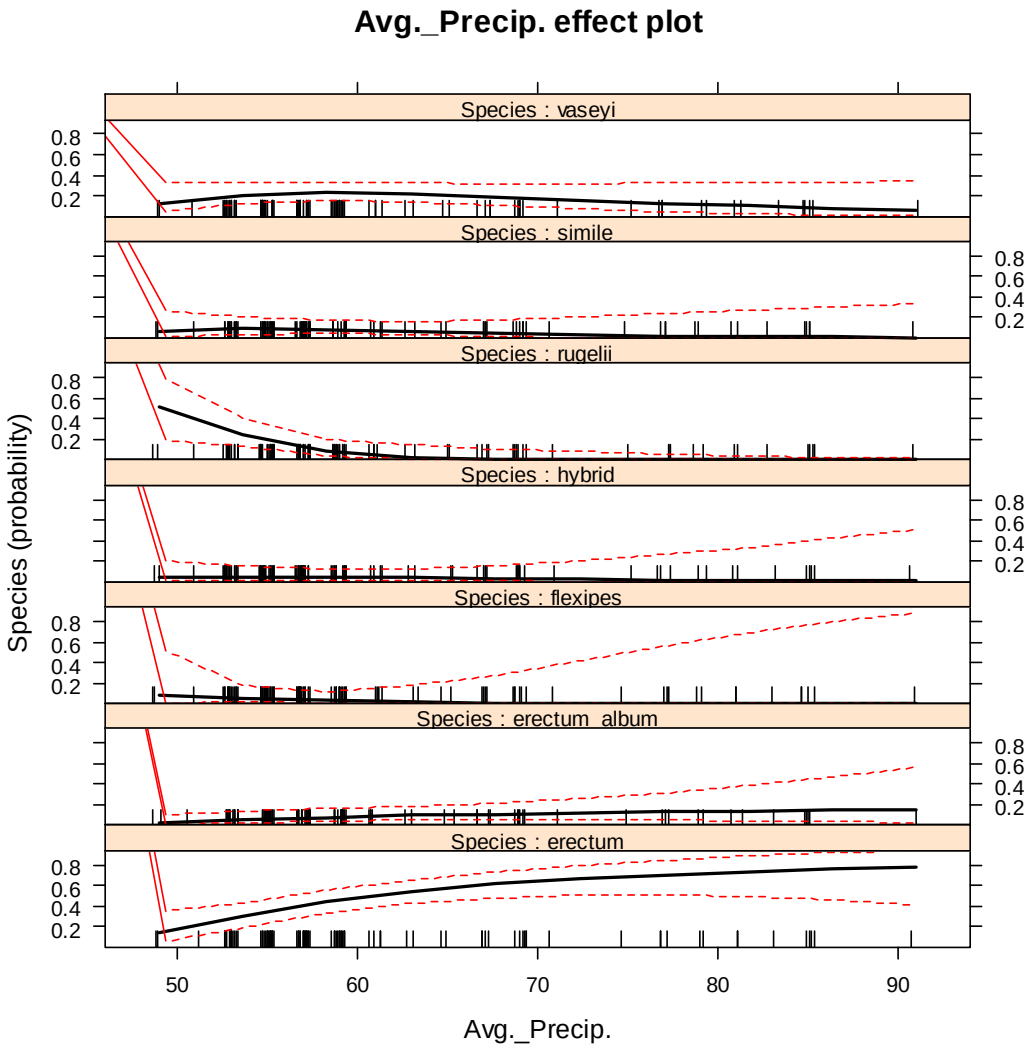


Figure 9. The Y-axis represents the probability of finding each species based upon the average amount of precipitation recorded in inches, the X-axis, where it is located. The lines within the box for each species are actual values recorded.

The data in Figure 9 show a higher probability of finding *T. rugelii* in locations with lower average precipitation. The probability of finding *T. erectum* in a location increases as the average precipitation increases.

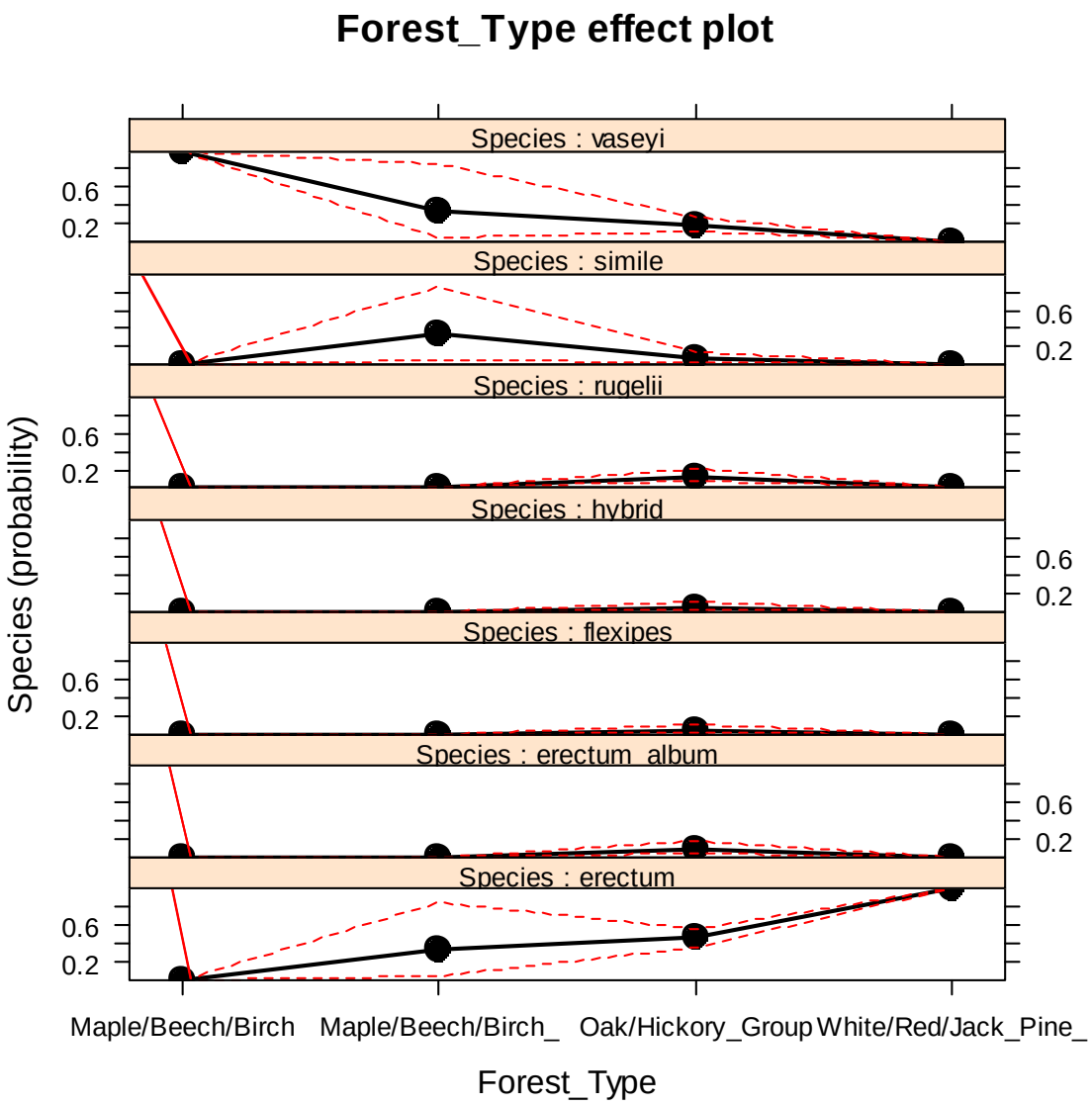


Figure 10. The Y-axis represents the probability of find each species based upon the USFS Forest Type, on the X-axis, of the location in which it is located. There are two subsections of the Sugar Maple/Beech/Yellow Birch forest type which were not distinguished in the metadata for the layer in ArcGIS.

Figure 10 shows that *T. vaseyi* is most likely to be found in a Sugar Maple/Beech/Yellow Birch forest then other forest types. *Trillium rugelii* is slightly more likely to be found in a Sugar Maple/Beech/Yellow Birch forest then other forest types.

Trillium erectum is slightly more likely to be found in an Oak/Hickory forest and most likely to be found in a White Oak/ Red Oak/ Jack Pine forest than other forest types.

Discussion

It is well known that geology influences the composition of plant communities (Kruckeberg, 2002). Geologic formation may be the most significant variable distinguishing the distributions of each *Trillium* species in this study. Precipitation and watershed also significantly deviated among taxa. This indicates that, as well as selection for separate soil types or bedrock, these taxa may have varying ecological needs regarding moisture. The Tennessee River may be acting as a barrier to gene flow between taxa of the same flower color, effectively separating two red flowered species from each other and two white flowered species from each other. *Trillium vaseyi* is most often significantly associated with specific habitat types (Table 3) but there is also a high deviation in habitat types. A possible explanation could be that it has a preferred habitat type but that it is phenotypically plastic enough to allow it to exist in a variety of habitats. While not empirically tested in this study, individuals of *T.vaseyi* were observed to be smaller (scapes <12inches tall and flowers smaller than 1inch in diameter) in size when in sympatric populations and larger (scapes up to 28inches tall and flowers almost 2inches in diameter) in parapatric populations. This has led me to speculate that in the populations where it is larger and it is also the only taxa in that locality, it is in the preferred habitat and can outcompete other taxa. In localities where it is sympatric and not as robust in size, it is not as fit and cannot outcompete sister taxa. The most prominent deviation in habitat type seen is between two red flowered taxa that occupy the same geographic range, *T. erectum* and *T. vaseyi*, but have dissimilar habitat

characteristics (Table 4). During field observations, there were never more than a dozen individuals of *T. erectum* or *T. vaseyi* in sympatric populations with one another (Table 7, Appendix D). From the literature available there are certain species that are known from field observations to be consistently found in certain habitat types (Case and Case, 1997). These have yet to be empirically tested to determine whether or not similar colored taxa select for dissimilar habitat conditions. In this preliminary work, it appears that at least two taxa similar colored taxa are selecting for dissimilar habitat conditions. This supports the hypothesis that similar colored taxa gain at least partial reproductive isolation through ecological isolation. In future studies direct examination of habitat characteristics like acidity, elevation, and moisture would be ideal to use in attempting to find differences among taxa habitat selection.

CHAPTER FOUR: CHOLOROPLAST DNA SEQUENCING

Objective

The objectives in this portion of the study are to construct a phenogram and genetic network based on genetic distances from cpDNA sequence data to depict possible phylogenetic relationships among the members of the Erectum complex.

1)

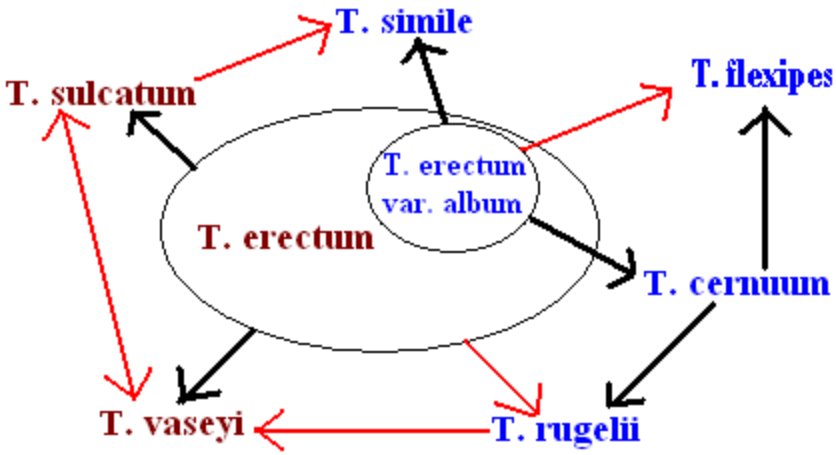


Figure 11. *Hypothesized Evolutionary Relationships*: The black arrows indicate direct ancestor-descendant relationships and red arrows indicate introgression contributing to the speciation event. Red text indicates red-flowered taxa; blue text indicates white-flowered taxa (This proposed model is not intended to represent current gene flow, but to describe historic paths of of gene flow over the development of the complex)

I hypothesize that the common ancestor of the Erectum complex may have been similar to the extant *T. erectum* species. *Trillium erectum* is known to have the widest variation in morphological characteristics in the Erectum group. In taxa such as these,

individuals with divergent phenotypic variations could have become isolated by pollinator preference for specific floral types (Coyne and Orr, 2004). In this model (Figure 11) there would have been two color forms (red and white) of the ancestral taxon, which became isolated by pollinator preference. Within each clade further divisions would have occurred based on habitat preference (black arrows). Morphological differentiation from the ancestor would have been promoted through introgression from other semi-species (red arrows, Figure 11). The red-flowered form of the ancestral *T. erectum* ultimately gave rise to *T. sulcatum, T. erectum*, and *T. vaseyi*; and the white-flowered form gave rise to *T. erectum* var. *album, T. simile, T. flexipes, T. rugelii, and T.cernuum*.

If this model (Figure 11) holds true I would expect to see the most variation, greatest number of haplotypes and greatest number of polymorphic loci to be found in *T. erectum*. I expect to see higher genetic identity between white-flowered taxa then between white-flowered and red-flowered taxa. If these groups within the Erectum complex are distinct taxa, then I would expect to see an average genetic distance within the genera of 0.07, among species within the complex of 0.04 from cpDNA sequence data (based on averages from a combined *mat*K and ITS data set; Farmer, 2006), and 0.97-4.17% variation within the genus (Shaw *et al*., 2005).

Materials and Methods

A total of 203 fresh tissue collections (Table 29, Appendix D) and 12 voucher specimens of five taxa and one variety of *Trillium* were collected from 11 sites: six in NC, three in TN, and two in GA. Voucher specimens are deposited in the herbarium at Western Carolina University (WCUH). For taxa for which fresh leaf tissue could not be obtained, DNA extractions were attempted from herbarium material.

Leaf tissue from individuals of each taxon belonging to the Erectum group was sampled at each field site in which they were present. One leaf was taken from between one and twenty-four individuals from each taxon. Prospective hybrids (individuals with intermediate phenotypes) were also sampled when present. Samples were collected on dry ice, flash frozen in liquid nitrogen, and stored at -70ºC until DNA extractions could be made.

Total DNA was obtained from leaf tissue frozen in liquid nitrogen using a modified CTAB extraction (Doyle and Doyle, 1987) and herbarium specimens using either a DNeasy Plant Mini kit (Qiagen Corporation, Valencia, California). Primers for the *rpl*32-*trn*L intergenic spacer region (Shaw et. al 2007) were used to amplify non-coding cpDNA using standard polymerase chain reaction (PCR). The PCR cycle consisted of the following steps: template DNA denaturation at 80°C for 5 min, 30 cycles of denaturation at 95°C for 1 min, primer annealing at 50°C for 1 min, a ramp of 0.3°C/s to 65°C, and primer extension at 65°C for 4 min; followed by a final extension step of 5 min at 65°C (Shaw et. al 2007). Successful amplification was confirmed by running samples in a 1% agarose gel stained with ethidium bromide and quantity was measured

using a micro-volume spectrophotometer (Nanodrop). The samples were purified prior to sequencing using QIAquick PCR Purification Kits (Qiagen Corporation, Valencia, CA). The samples were then loaded with the sequencing primers on to 96-well plates and shipped to the Genomic Sciences Laboratory (NC State University, Raleigh, NC) for sequencing and capillary electrophoresis on a 3700 Genetic Analyzer (Applied Biosystems). Subsequent electropherograms were edited and aligned in Sequencher software (GeneCodes Corp., Ann Arbor, MI). Sequencher was used to view chromatograms, edit ambiguous base calls, and trim, and align the sequences. PAUP*, vers. 3.1.1 (Swofford, 1993) and TCS, vers. 1.21 (Clement et al., 2000) were used to calculate neighbor-joining phenograms, compute genetic distances, and create a genetic network calculated using the uncorrected-p method. Sequences of *Trillium grandiflorum* and *Trillium camschatense* (downloaded from GenBank) were used as representative outgroups in the analyses.

Results

A total of 108 individuals were successfully sequenced in both directions with the *trnL-rpl32* primers. The total number of bases sequenced was 826, including 49 variable characters in the ingroup and 39 parsimony informative characters. Sequences from forward and reverse primers did not overlap, so each end of the spacer region was analyzed separately. From the forward end (*rpL32* end) 445 bases were sequenced (7 variable and 4 informative), and this included a portion of the *rpL32* coding region. From the reverse end (*trnL* end) 381 bases were sequenced (42 variable and 39 informative) and all were within the intergenic spacer region.

No species-specific haplotypes were observed. A genetic network was created in TCS linking haplotypes that contain members of several taxa (Figure 12). The individuals belonging to each haplotype are listed in Table 5. The average genetic distance between *T. camschatcense* and members of the Erectum complex is 0.07. The genetic distance among taxa within the complex is 0.003. The genetic distance between members of the same taxon is 0.0007. Results from an ANOVA comparing variation within a taxon to variation between ingroup taxa were not significant (*df=1; F=0.0919, p=0.721*). Results from an ANOVA comparing variation within the complex with variation between the members of the complex and the outgroup were highly significant (*df = -1; F= 170.45, p= 2.2e¯16*). The average Nei's pairwise genetic identity between red and white taxa is 99.74, between red taxa is 99.62, and between white taxa is 99.81. *Trillium vaseyi* was found to contain the most genetic variation within a taxon. The TCS parsimony network created (Figure 12) shows that there are only two haplotypes that are specific to one taxon. The majority of the individuals in the network clump in to one haplotype and the two largest haplotypes contain individuals collected from multiple taxa and localities. A Neighbor Joining phenogram was created in PAUP (Figure 13). The diagram does not show complete segregation of individuals by taxon or by locality but for the most part individuals identified as *T. vaseyi*, *T. rugelii* and *T.erectum* do group with their own taxa.

Figure 12: Genetic parsimony network created using TCS. Numbers represent the number of individuals belonging to each taxon that were placed in that haplotype. Haplotypes that were not connected to the network are not shown.

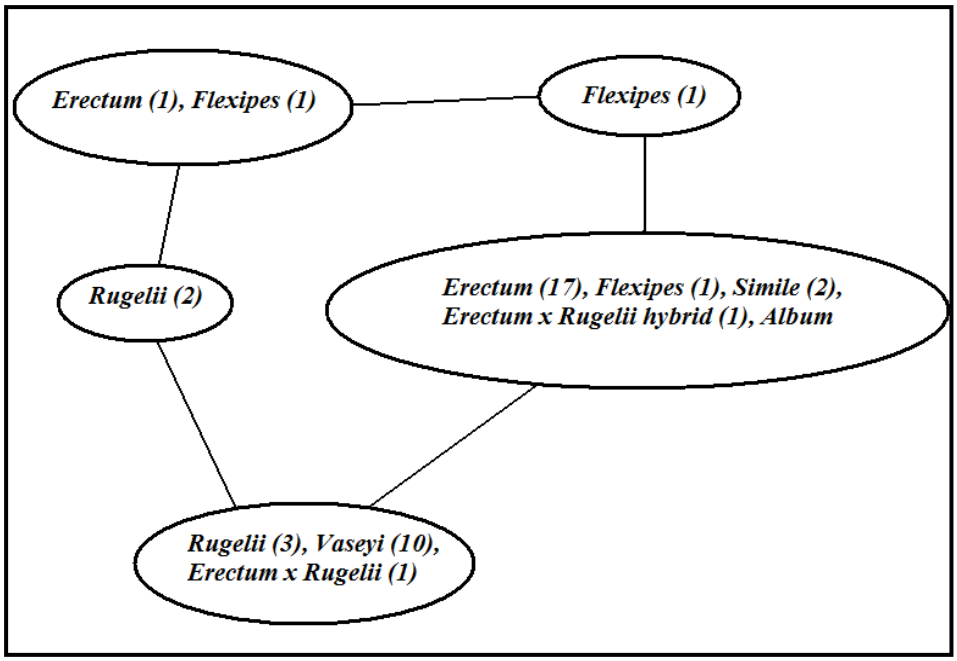
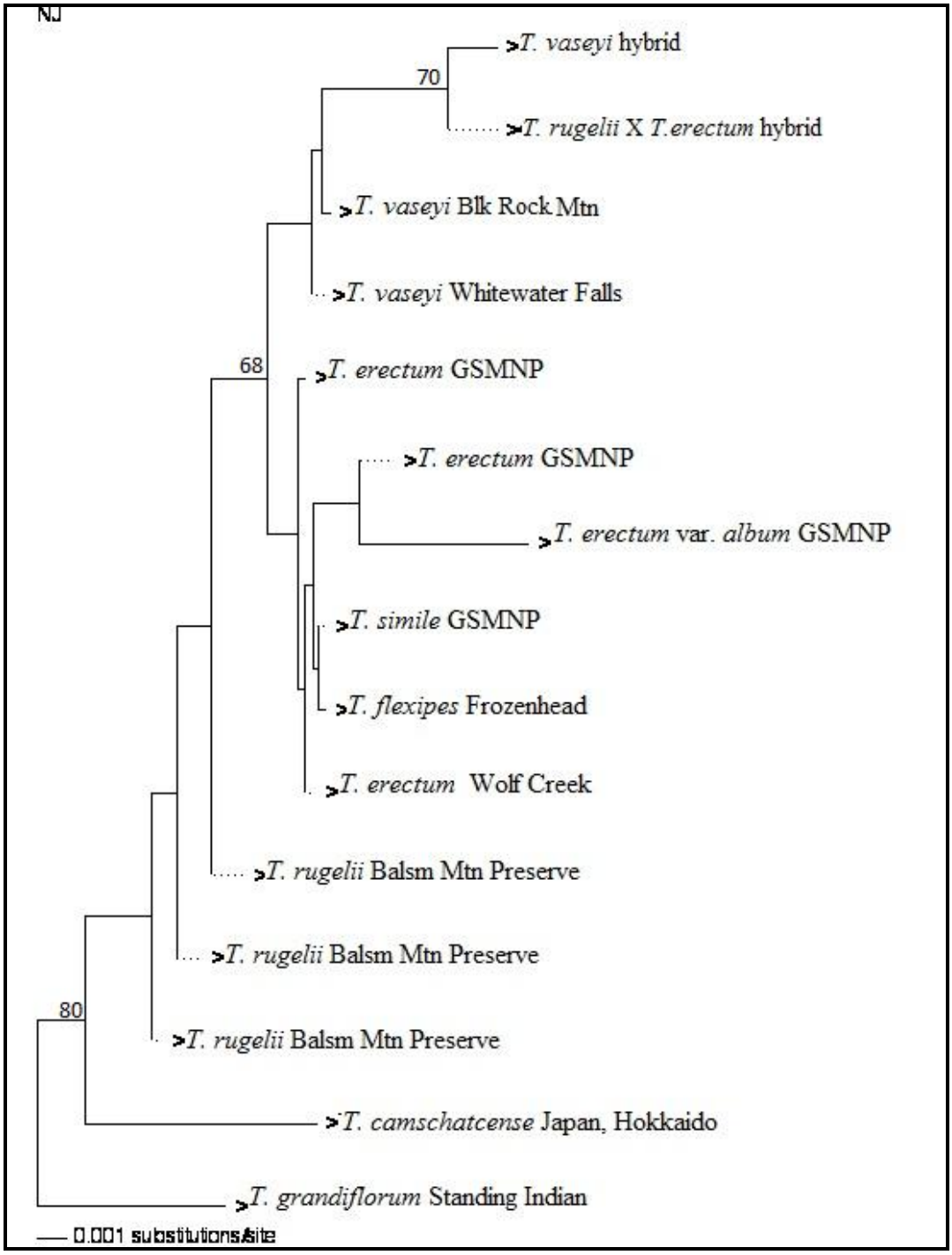


Figure 13: Neighbor Joining Phenogram created in PAUP. The location of each taxon is inserted after the name at the end of each branch. The branch lengths are proportional to genetic distance values.



Discussion

The genetic identity values show that the distance between *T. camschatcense* and its American sister taxa meet expectations based on the assumption that they are separate species in the same genus, but the distance values within the complex are an entire order lower than would be expected even for members of the same species. Previous research by Farmer (2006) using ITS and *mat*K combined sequence data show the average genetic distance among members of the same genus in the family Trilliaceae was 0.07 and within species was 0.04 (Farmer, 2006). The average genetic identity between individuals was 99.86, considering the average identity between members of the complex was 99.93 there appears to be more variation in that one "species" then between all "species". The genetic identity between white flowered taxa was higher than the distance between all members of the complex, this suggest a stronger relationship amongst them as compared to the rest of the complex. Nine haplotypes were created using TCS that included 52 individuals (Table 5). A map was created using ArcGIS (Figure 15). While none of the haplotypes were exclusive to only one taxon or locality when mapped, the groupings do show segregation into a large cluster on the eastern side of the Appalachian Mountains and individuals near the crest and the northwest side in another. The rest of the haplotypes include a few individuals from the foothills at the southern tip of the mountain range. The distribution of cpDNA haplotypes may be more representative of historical relationships due to isolation in glacial refugia (Gonzales et al, 2008) than divergent phylogenetic groupings.

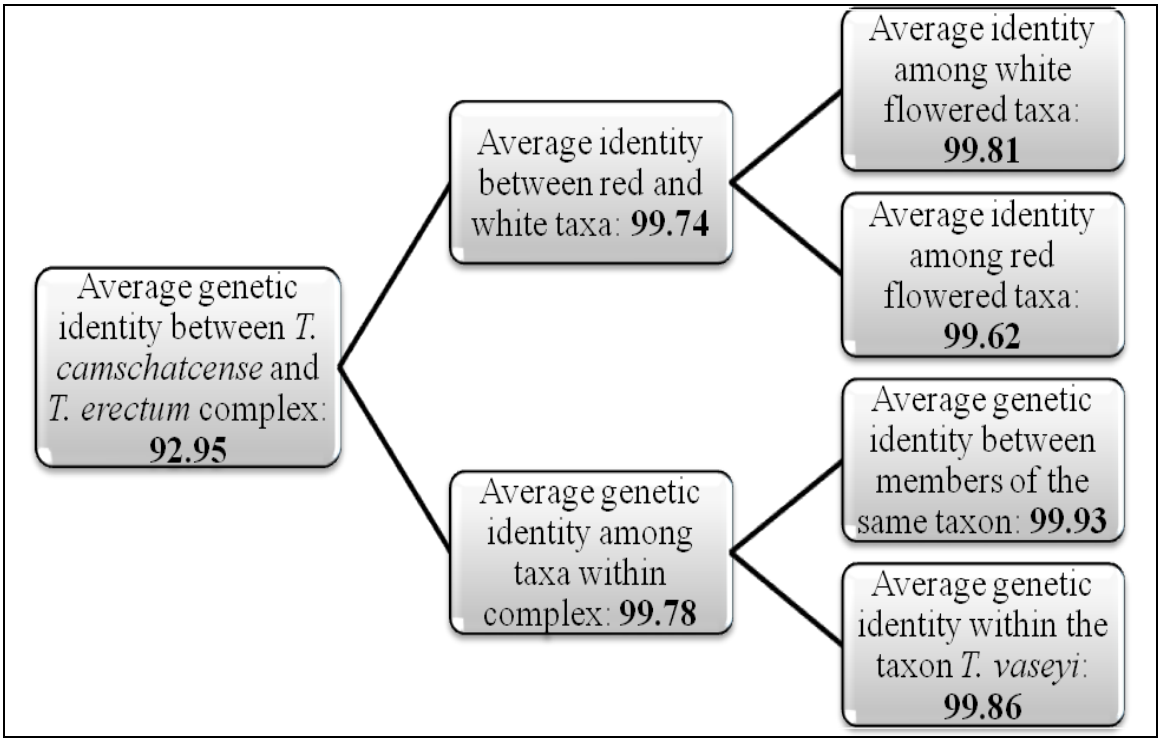


Figure 14: Average Genetic Identity Values.

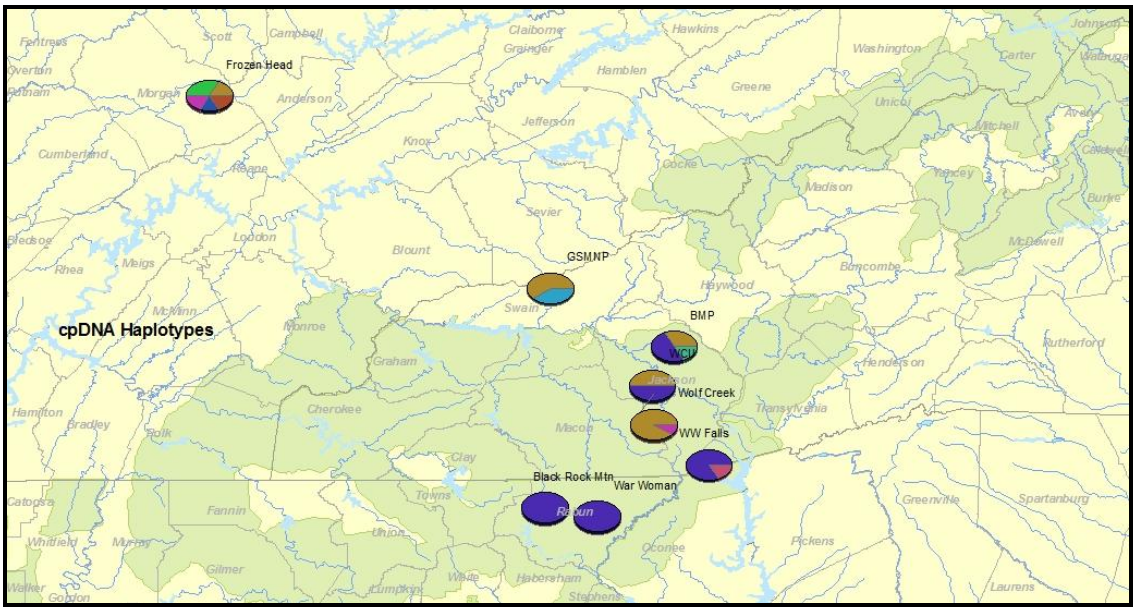


Figure 15: Geographic distribution of haplotypes created in TCS from cpDNA sequence data. Each color represents a separate haplotype.

Table 5: Haplotypes created in TCS based on cpDNA sequences. E: *T.erectum*, A: *T.erectum* var. *album*, R: *T.rugelii*, S: *T.simile*, V: *T.vaseyi*, F: *T.flexipes*

Haplotype	Individuals included
Haplotype1	104A, 110A, 12R, 38E, 107A, 48ExR, 101E, 95E, 112E,118F, 200S, 22E, 29E, 74E, 47E, 73E, 76E, 91E, 37A, 95E, 201S, 29E, 94E, 38E, 28E, 104E
Haplotype2	62V, 50V, 4ExR, 2R, 64V, 164V, 163V, 152V, 10R, 12R, 58V, 147V, 49V, 12R, 149V, 148V, 65V
Haplotype3	301S
Haplotype4	118F, 131F
Haplotype5	70V
Haplotype6	2R
Haplotype7	25E, 118F
Haplotype8	132V
Haplotype9	131F

CHAPTER FIVE: ALLOZYME ELECTROPHORESIS

Objectives

The objectives of this portion of the study is to determine the relationships among syngameons it may be helpful to look for the types of molecular variation typically found within populations of the same species, such as allozyme variation between holotypes (an organism exhibiting the typical character states described for a species) from isolated populations of each species compared with known hybrids (Arnold et al., 2004). This has been used successfully with a variety of other herbaceous plants; determining relationships in *Prosopis* (Bessega et al., 2005; Saidman and Vilardi, 1987), the phylogeny of *Lathyrus* (Brahim et al., 2002), genetic variation and hybridization in *Orchis laxiflora* and *Orchis palustris* (Arduino et al., 1996), measuring genetic distinctiveness and introgression in *Carex* (Tyler, 2003), and for showing patterns of introgression and hybrid speciation in the Louisiana irises (Arnold et al., 1990).

My objectives were to find species-specific markers for as many Erectum species as possible. These may be in the form of fixed alleles or fixed allelic frequencies for particular allozyme loci. To determine the level of introgression, genetic distance, inbreeding, and heterozygosity values for each taxa and locality were calculated.

Hypotheses

If these groups within the Erectum complex are distinct taxa, then I expect to see values of genetic identity to be in the range of 0.956 within species, and genetic identity

values to be in the range of 0.67 (+/- 0.04) between for allozyme data (Soltis and Soltis, 1989; Gottlieb, 1981). I would also expect the genetic distance between different *Trillium* species growing in allopatry to be greater than the genetic distances between different species growing in sympatric populations (Solits and Soltis, 1989); If there is a significant introgression currently occurring, then genetic identity values should be higher among sympatric species than allopatric species.

Materials and Methods

A total of 203 fresh tissue collections (see Table 29, Appendix E for detailed list) and 12 voucher specimens of five taxa of *Trillium* and one variety were collected from 11 sites; seven in NC, three in TN, two in GA. Voucher specimens are deposited in the herbarium at Western Carolina University (WCUH).

Leaf tissue from individuals of each taxon belonging to the Erectum group was sampled at each field site in which they were present. One leaf was taken from between one and twenty-four individuals from each taxon. Prospective hybrids (individuals with intermediate phenotypes) were also sampled when present. Samples were collected on dry ice, flash frozen in liquid nitrogen, and stored at -70ºC until protein extractions could be made.

Allozyme electrophoresis was used to detect the presence of allelic variations in loci that are known to have variations in *T. erectum* (Irwin, 2001; Griffin and Barrett, 2004). Preliminary trials performed in Dr. Jim Hamrick's lab at the University of Georgia in Athens were used to determine which allozyme loci are polymorphic within this species complex. Small portions of leaf tissue ($1cm^2$) were ground in liquid nitrogen

using a mortar and pestle. The proteins were extracted using the buffer from Wendel and Parks (1982). The extractions were absorbed on to Whatman no.3 wicks. After the trials it was determined that horizontal starch gel-electrophoresis would be performed using 12% gels with three buffer systems: system 4 to resolve uridine diphosphoglucose pyrophosphorylase (UGPP); system 34/40 (Cheliak and Pitel, 1984) used to resolve menadione reductase(MNR), diaphorase (DIA), phosphoglucose isomerase (PGI), peroxidase (APER); system -8 to resolve fluorescent esterase (FE). Recipes were modified from Soltis et al. (1983) and Wendel and Weeden, Chapter 1, in Soltis and Soltis (1989); the recipe for DIA and system 34/40 buffer from Cheliak and Pitel (1984). Once the gels were been scored the allele frequencies were analyzed using GenAlEx software (Peakall and Smouse, 2006) by locality, by taxon, by taxon and locality, by color, and by color and locality. Nei's genetic distances, allele frequencies, Hardy Weinberg statistics, haplotypes, an AMOVA, heterozygote frequencies, and F-statistics were calculated in each analyses.

Results

A total of 14 enzymes in three buffer systems were tested on 72 samples. Suitable resolution was achieved in 12 enzymes for a total of 16 possible loci for analysis (Table 6, Appendix D). In the interest of time and money the seven best loci were selected for the final run: diaphorase (DIA), aspartate aminotransferase (ATT), menadione (MNR1 & MNR2), fluorescent esterase (FE), uridine diphosphoglucose pyrophosphorylase (UGPP), glucose-6-phosphate isomerase (PGI). PGI was used despite getting no resolution in the trials since it was believed to have been due to lab error; when PGI was run with the rest of the samples there was still inadequate resolution to be reliably read.

For final analysis, data were collected for four loci from 199 samples (Table 7, Appendix E). There were not enough data obtained from the other three loci to be used in analysis, resolution of bands was not sufficient for accurate data collection. Genetic distances and identities between taxa are recorded in Table 8 and Table 9 (Appendix D). The average identity value is 0.878; the expected for species is 0.67±0.04 (Soltis and Soltis, 1989). The average identity value by locality is 0.75 and the average distance is 0.32 (Table 10 and Table 11). Analysis of molecular variance indicated that the largest amount of variation is found within taxa, 88% (Table 12, Appendix D). *Trillium erectum* and the white variety *T. erectum var. album* shared similar allele frequencies at all loci, yet *T. erectum* showed a slight deficiency in heterozygotes (Table 13, Appendix D). All taxa significantly deviated from HWE in at least one locus (Table 21, Appendix D). When individuals were grouped by locality the populations meet HWE expectations more frequently than expected. When grouped by flower color data show a slight deficiency in heterozygotes (Table 14, Appendix D). For white taxa the mean observed heterozygosity (H_o) = 0.347 SE = 0.09 and the mean expected heterozygosity (H_e) = 0.484 SE = 0.019. For red taxa mean H_o = 0.329 SE = 0.078 and the H_e = 0.483 SE = 0.062. We see higher observed heterozygosity when individuals are lumped by locality rather than taxon. The data show slightly higher genetic identity values than expected when considering them separate taxa. When grouped by locality regardless of taxa, identity values are still fairly high considering some of these populations are hundreds of miles apart or may not contain the same taxa. When analyzing variation within groups of sympatric individuals 16% of the variation is found among localities, 31% among individuals within a locality,

and 54% within individuals (Figure 17 &Table 15, Appendix D). The Fst value among taxa was 0.134 SE=0.016 and the Fst value among localities was 0.318 SE=0.067 (F-statistics are reported in tables 20 and 23, Appendix D).

There were three populations of *T. vaseyi* that were parapatric with other taxa (Whitewater Falls, Warwoman, and Black Rock Mtn) and one population that was sympatric with two other taxa (Rainbow Falls). When you compare the genetic distance between populations of *T. vaseyi* which were parapatric with other taxa they had a higher genetic identity among themselves than with populations of *T. vaseyi* that were sympatric with other taxa (Table 16, Appendix D).

The white flowered form of *T.erectum* shows diverging allele frequencies at two loci compared to the red variety in the population from Clingman's Dome where they are sympatric (Table 27). The allele frequencies of all populations, regardless of allopatry or sympatry, of the white and red do not diverge significantly (Table 19).

Discussion

The data collected for populations of *T.*vaseyi support the hypothesis that the allozyme data are showing introgression between taxa in sympatric populations. The data was not conclusive for the complex as a whole; there was no distinct pattern between sympatry and genetic distance among all taxa. This may have been due to sampling error or lack of power from small sample sizes from certain populations or the patterns of gene flow between individuals vary by taxon compounded by varying amounts of gene exchange occurring in each locality. The Fst values show greater divergence among populations then among taxa; which also supports the hypothesis that there is

introgression and suggests that current introgression could be influencing patterns of identity. This is reiterated in the data from an AMOVA (Figure 18). It shows the largest amount of variation is between individuals, indicating that a large portion of the variation could be more or less random. The variation among taxa is still greater than the variation among localities. When lumped by taxa rather than locality, you see a greater deviation from HWE in the data set. This could suggest that evolutionary forces such as drift, selection, and random mating act more on the local populations as a whole then on each individual taxon.

The differences in allele frequencies of different colored taxa in GSMNP may be due to assortative mating but the similar. In sympatric populations of the white and red varieties of *T.erectum,* the differences in allele frequencies could have been caused by selection (Conner and Hartl, 2004). These data may support previous data on assortative mating. Since only two loci are affected then the cause would have to be some type of selection, drift and gene flow would affect all loci equally (Conner and Hartl, 2004). The higher than expected homozygosity found among all white flowered taxa suggest non-random mating (Conner and Hartl, 2004) which also supports our hypothesis on assortative mating.

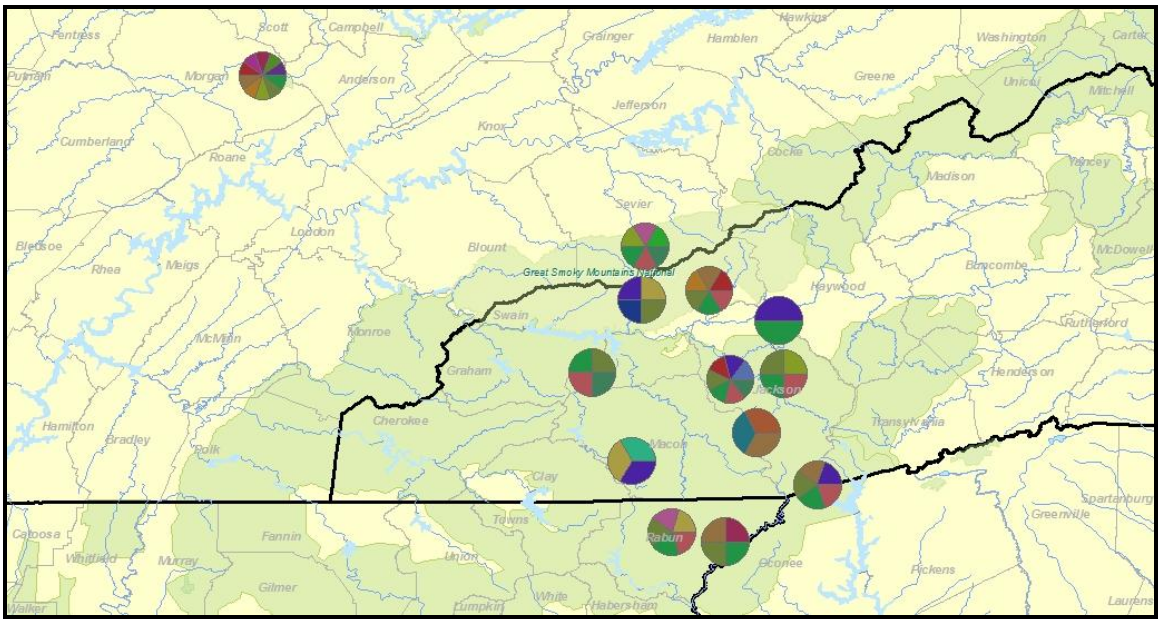


Figure 16: Geographic distribution of haplotypes within localities from allozyme data.

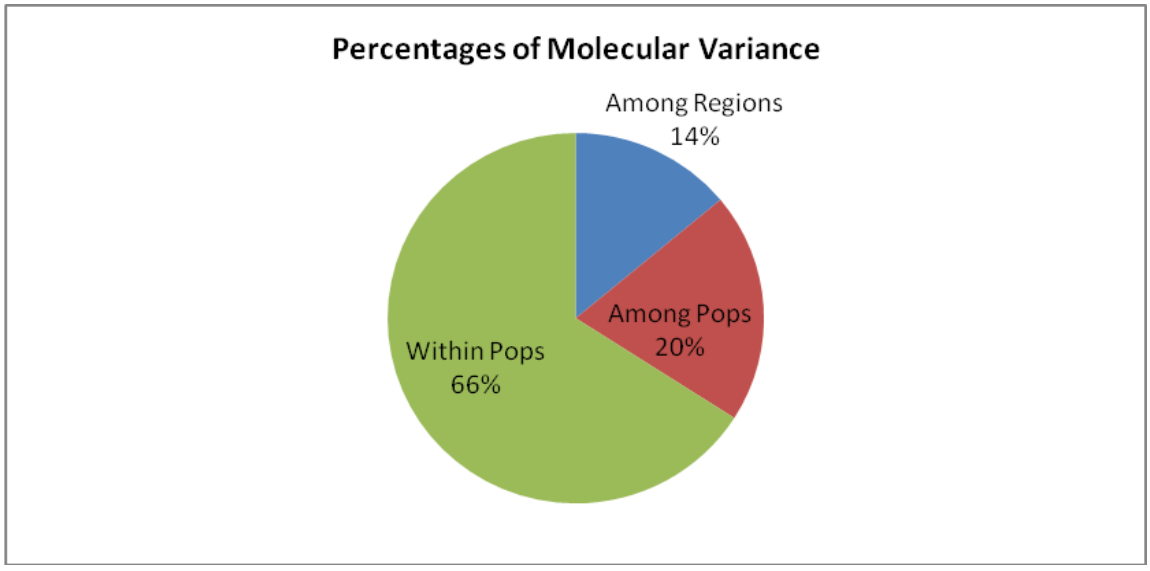


Figure 17: Distribution of Molecular Variance from AMOVA created in GenAlEx. Regions represent variance by locality, populations are comprised of taxa.

CHAPTER SIX: SYNTHESIS AND DISCUSSION

In the study of systematics within the *Trillium* Erectum complex the goal is to be able to answer six key questions about the taxa: 1) what are the taxonomic groups, 3) when did they evolve, 4) where did they evolve and where do they now exist, 5) why are the current populations structured the way they are and 6) how did they become that way? This discussion will attempt to address the questions of who or what are the members of the Erectum complex, when might divergence have begun to occur, and lastly what might have influenced their evolution historically and presently.

Who are the members of the complex and what should be treated as distinct taxonomic units? Currently the taxonomic treatments based on morphology have proved to be impractical for use in the field due to hybridization and development of local ecotypes. I do not disagree that there are at least eight groups of historically divergent taxa within this complex and within each of those groups there is the possibility of further divisions into describable varieties of each taxon. The hierarchical level of classification that each taxon should receive, however, is debatable.

Taxonomy is a decisive component in the quest to quantify biodiversity (Stussey in Leadlay and Jury, 2006). The rank of classification will ultimately impact how the genetic diversity is maintained/ managed by government organizations and how well laws will enable us to protect diversity. Conservation management decisions based on the current taxonomic treatments within the Erectum complex would be based on treating these eight groups as functionally distinct. In other words, each one would be managed

independently of the others. Is this really appropriate considering the complex is more of a web of gene pools with varying degrees of connectivity? Ideally, management activities would "retain existing spatial distribution of genetic variation" (Burgman, pg 20 cited in Akçakaya, 2004). This would not happen if the genetic variation is distributed amongst taxa and then the taxa are all managed as separate discrete entities. Results from this study have shown that populations of a single taxon are not only connected to other populations of the same taxon but connected to other taxa within the same geographic area. Considering the lack of genetic variation found during cpDNA sequencing in this and other studies (Millam, 2006) it is likely that these taxa may not possess the variation within themselves to be successful in their environments. The alleles selected for in each locality based on habitat preference could be spread out over all taxa in a location making them functionally one group. Introgression can promote variability and fitness for one or both taxa involved (Simpson, 2006). For example, populations of *T. vaseyi* overall show a deficiency of heterozygotes, but within sympatric populations containing *T. vaseyi* and other taxa are in Hardy Weinberg Equilibrium (Table 19, Appendix D). This is the downfall of the current taxonomic classification. As for a better solution, there is not one to offer yet; but a taxonomic revision based on gene flow *and* evolutionary lineage seems necessary if these classifications are going to be used in management.

When did these taxa evolve and where? Molecular clock analyses suggest that the complex began to form only a mere 900,000 years ago (Millam, 2006), during the Middle Pleistocene when the last glacial maximum reached the Appalachian mountain range. When glaciation occurred, the ancestral species may have been segregated into populations isolated to the southeastern United States and southeastern Canada. Through

genetic drift and local selection, differentiation could have occurred. If that were true then these taxa would be now in a period of secondary contact after a relatively short period of isolation.

Millam's work suggests that two groups began to diverge 600,000-900,000 years ago, the *T. erectum* clade and the *T. cernuum* clade; divisions within those two lineags began 280,000 and 90,000 years ago respectively. According to the theory of coalescence, the parent taxa should ideally contain the most genetic variation due to founder effects (Nordborg, 2008). Despite our data showing *T. vaseyi* to be the most variable, the scenario provided by Millam still seems the most likely. The sampling area in this study did not span the entire range of certain taxa such as *T.erectum*; therefore the total variation within all taxa was not characterized. Haplotypes created using cpDNA or allozyme data show no distinct pattern regarding refugium, but they are slightly partitioned in the cpDNA haplotypes. Haplotypes depicting glacial refugia for the sessile flowered *T.cuneatum* using cpDNA sequences outline a refugium that occupies the majority of the southern Appalachians (Gonzales et al., 2007). This means that all but one (Frozenhead, TN) of the sample sites in this study were within this previously described refugium.

Why did these taxa evolve in this manner and how are they maintained? In theory there are three possible influences that may have shaped further divergence within the ancestral *T.erectum* and *T.cernuum* clades: isolation, drift and selection. These may have occurred in different orders or all at once. Theoretically individuals could have differentiated based initially on genetic drift following initial found events after glacial isolation. These relict populations would therefore not contain as much of the variation

and plasticity that allows for expansion into new habitats, explaining the smaller distributions of the more recently diverged taxa. Furthermore, during the periods of isolation, variation that was not suited for each particular glacial refugia could have been selected against and migration from parent populations would have ceased, effectively furthering differentiation.

From our data on heterospecifc pollination and other studies on seed and pollen movement (Kalisz et al., 1999; Knight, 2003) it appears that there is to some degree floral constancy via pollinator and a low degree of seed and pollen dispersal. This has several implications. Pollinator isolation is probable barrier keeping the distinct variation between red and white taxa, but this barrier is leaky and impermanent. Short distance seed and pollen movement would lead to populations being highly differentiated within species. This can lead to the ecotypes and variation within each taxon and even complete extrinsic isolation between populations at far ends of the range of a taxon. But a low migration rate between localities does not alone create nor imply variation amongst populations. Fst values show that there is structure within taxa and among populations. This shows support for ecological selection driving divergence among populations regardless of taxa. The data collected in this study on cross pollination suggests that within sympatric populations differentiation between taxa is somewhat maintained if they are of different floral types, but if they have similar floral types then that barrier is weak; either way the barrier is incomplete. Allozyme data support assortative mating based on floral color by showing a decrease in heterozygosity in white flowered groups of individuals. Also, genetic identity values are higher amongst red and amongst white individuals then between red and white individuals.

Allele frequencies and heterozygosity support environmental selection as being two processes that are currently driving the structure of diversity seen in the Erectum Complex. There are slightly higher frequencies of heterozygosity found in allozyme data when taxa are lumped by locality rather than taxon. This means that when grouped by sympatric individuals rather than taxonomic classification, these populations are less likely to violate the assumptions of HWE. There is also evidence that there is selection driving the allele frequencies and that there may be a heterozygote advantage which relates to hybrid vigor (Mitton, in Soltis and Soltis, 1989). The genetic identity values within localities vary, and this suggest that some taxa in certain habitats hybridize more than others. Therefore there may not be the same degree of divergence between all taxa, or there may be greater hybrid success in some habitats over others.

In summation, all of these factors equate to introgression between taxa that already experience within taxon population differentiation due to isolation and varying ecological selection pressures. This scenario is clearly seen between the populations of *T. vaseyi* surveyed in this study. The populations of *T. vaseyi* that are parapatric or allopatric with other taxa are more closely related to each other than to the other taxa, but each population has a distinct allozyme haplotype, which eludes to varying ecological selection. The populations of *T. vaseyi* that are sympatric with other taxa are genetically dissimilar from the other *T. vaseyi* populations. Thus the Erectum Complex appears to be a syngameon: a collection of semi-species with varying degrees of reproductive connectivity. There is no direct evidence for the formation of hybrid species although from field observations it is apparent that hybrid swarms do exist and appear stable and

healthy. It is my opinion that it is not the nomenclature or taxonomic classifications that need to be re-evaluated as much as the categorical rank of species, which implies that these are groups evolutionarily independent from one another. Stuessy describes one of the principles of taxonomy as "using selected features, we determine patterns of relationships that we assume reflect these evolutionary processes." (pg 36, Leadlay and Jury, 2006). The current taxonomic classification groups individuals by morphological similarity, morphology assumingly created by historical evolutionary processes. But introgression, as supported by allozyme data and blurred morphological features in sympatry, might reflect a change in the evolutionary processes at work; therefore a change the patterns of relationships among members of the Erectum complex.

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APPENDICIES

Appendix A

Table 1: Table of morphological characteristics, adapted from chart by Susan Farmer.

	T. erectum	*T. flexipes*	*T. rugelii*	*T. simile*	*T. sulcatum*	*T. vaseyi*
Leaves						
shape	subrhombic	broadly elliptic to obovate	rhombic	broadly elliptic	broadly elliptic to obovate	elliptic
attachment	sessile	subsessile	subsessile	sessile	subsessile	petiolate
Pedicels						
length	short to medium	medium to long	short	medium	long	short
attitude	above to below leaves	above to below leaves	below leaves	above leaves	above to same level as leaves	below leaves
Flower	**Profile**	**Sideview**				
ovary	open	widely agape	open	widely agape	agape	open
	exposed	exserted, bug-eyed	exposed	hidden by petal bases	hidden by petal bases	hidden by filaments
petals	flat, nearly perpendicular to axis	arched to recurved	basally recurved	arched outward	apically recurved	basally recurved
Coloration						
ovary	maroon, white	white	white	white	maroon	maroon
pollen	black	white	maroon	black	purple	purple
	violet to yellow	creamy	maroon	light yellow	purple to yellow	grey-violet to yellow
stamens	maroon	white	maroon	white to tinged red	dark maroon	maroon
Flower fragrance	weakly fetid, as a wet dog	mildly musty	?	weakly green apple	mildly fungal,	strongly funereal
Ratios						
pedicel/ leaf	0.20-0.55	0.45-0.75	0.2-0.3	0.35-0.6	0.45-0.8	0.20-0.45
sepal/ pedicel	0.5-0.8	0.4-0.7	0.8-1.3	0.4-0.7	0.2-0.4	0.5-0.7
filament/ anther	0.4-0.6	0.2-0.5	0.2-0.3	0.3-0.6	0.4-0.7	0.6-1.1
stamen/ pistil	0.8-1.3	0.7-1.4	0.8-0.9	1.2-1.8	0.9-1.6	1.2-2.5

Appendix B

Table 2**:** Pollination data

Color	Taxa	Number of anthers dyed	Number of anthers collected	Pollinated by white flower	Pollinated by red flower
White	*T. rugelii*	28	15	11	4
Red	*T. vaseyi*	28	15	2	13
	T. erectum	28	15	2	13

Appendix C

Table 3: Habitat characteristics extracted at known locations of individuals.

Species	Geologic Formation	Forest Type	Avg. Precip.	Avg. Temp.	Watershed	Soil Type
erectum	CZve	Oak/Hickory Group	55	55	New	5153
erectum	TRd	Oak/Hickory Group	55	55	New	4858
erectum	TRd	Oak/Hickory Group	55	54	New	5769
erectum	TRd	Oak/Hickory Group	55	54	New	6136
erectum	TRd	Oak/Hickory Group	57	53	New	6757
erectum	TRd	Oak/Hickory Group	57	53	New	6552
erectum	TRd	Oak/Hickory Group	57	53	New	6582
erectum	Mc	Oak/Hickory Group	57	53	New	6582
erectum	CZve	Oak/Hickory Group	57	54	New	5897
erectum	TRd	Oak/Hickory Group	63	53	New	7733
erectum	TRd	Oak/Hickory Group	59	53	New	7032
erectum	CZve	Oak/Hickory Group	55	55	New	5153
erectum	TRd	Oak/Hickory Group	55	55	New	4858
erectum	TRd	Oak/Hickory Group	55	54	New	5769
erectum	TRd	Oak/Hickory Group	55	54	New	6136
erectum	TRd	Oak/Hickory Group	57	53	New	6757
erectum	TRd	Oak/Hickory Group	57	53	New	6552
erectum	TRd	Oak/Hickory Group	57	53	New	6582
erectum	Mc	Oak/Hickory Group	57	53	New	6582
erectum	CZve	Oak/Hickory Group	57	54	New	5897
erectum	TRd	Oak/Hickory Group	63	53	New	7733
erectum	TRd	Oak/Hickory Group	59	53	New	7032
erectum	CZmv	Oak/Hickory Group	69	50	New	4342
erectum	PzZu	Oak/Hickory Group	61	53	New	3384
erectum	CZmv	Oak/Hickory Group	69	50	New	4342
erectum	CZmv	Oak/Hickory Group	69	50	New	4342
erectum	CZtp	Oak/Hickory Group	55	55	New	
erectum	CZtp	Oak/Hickory Group	55	55	New	
erectum	CZtp	Oak/Hickory Group	69	50	New	2255
erectum	CZmv	Oak/Hickory Group	69	50	New	4342
erectum	TRd	Oak/Hickory Group	79	52	Savannah	12847
erectum	TRd	Oak/Hickory Group	77	52	Savannah	12826
erectum	TRd	Oak/Hickory Group	85	49	Savannah	13724
erectum	TRd	Maple/Beech/Birch	85	49	Savannah	13373
erectum	TRd	Oak/Hickory Group	67	49	Savannah	11307
erectum	PzZu	White/Red/Jack Pine	77	45	Savannah	806
erectum	PzZg	Oak/Hickory Group	65	48	New	462
erectum	TRd	Oak/Hickory Group	85	48	Savannah	13533
erectum	TRd	Oak/Hickory Group	81	50	Savannah	13723
erectum	TRd	Oak/Hickory Group	79	50	Savannah	3782
erectum	PzZg	Oak/Hickory Group	71	48	New	191
erectum	PzZg	Oak/Hickory Group	67	49	New	152

erectum	PzZg	Oak/Hickory Group	69	49	New	13
erectum	PzZg	Oak/Hickory Group	67	49	New	13
erectum	PzZu	Oak/Hickory Group	75	49	New	4590
erectum album	TRd	Oak/Hickory Group	57	53	New	7145
erectum album	TRd	Oak/Hickory Group	61	52	New	2255
erectum album	TRd	Oak/Hickory Group	57	53	New	7145
erectum album	TRd	Oak/Hickory Group	61	52	New	2255
erectum album	PzZu	Oak/Hickory Group	85	49	New	
erectum album	CZbg	Oak/Hickory Group	57	55	New	
erectum album	CZbg	Oak/Hickory Group	57	55	New	
erectum album	PPg	Oak/Hickory Group	83	45	New	924
flexipes	PzZu	Oak/Hickory Group	55	54	New	
flexipes	PzZu	Oak/Hickory Group	55	54	New	
flexipes	PzZu	Oak/Hickory Group	55	54	New	
hybrid	CZve	Oak/Hickory Group	59	53	New	3107
hybrid	TRd	Oak/Hickory Group	59	53	New	2059
hybrid	CZve	Oak/Hickory Group	59	53	New	1863
hybrid	CZve	Oak/Hickory Group	57	53	New	1799
rugelii	CZve	Oak/Hickory Group	53	55	New	3847
rugelii	CZve	Oak/Hickory Group	53	54	New	3818
rugelii	CZve	Oak/Hickory Group	59	53	New	3107
rugelii	CZve	Oak/Hickory Group	53	55	New	4998
rugelii	CZve	Oak/Hickory Group	57	53	New	1641
rugelii	CZve	Oak/Hickory Group	53	54	New	3291
rugelii	CZve	Oak/Hickory Group	53	55	New	3847
rugelii	CZve	Oak/Hickory Group	53	54	New	3818
rugelii	CZve	Oak/Hickory Group	59	53	New	3107
rugelii	CZve	Oak/Hickory Group	53	55	New	4998
rugelii	CZve	Oak/Hickory Group	57	53	New	1641
rugelii	CZve	Oak/Hickory Group	53	54	New	3291
simile	CZbg	Oak/Hickory Group	59	55	New	
simile	CZbg	Oak/Hickory Group	59	55	New	
simile	Kc	Oak/Hickory Group	61	55	New	
simile	Kc	Oak/Hickory Group	67	53	New	
simile	PzZu	Oak/Hickory Group	51	53	Savannah	2678
simile	Zsr	Oak/Hickory Group	53	50	Savannah	973
simile	Zsr	Maple/Beech/Birch	53	50	Savannah	1922
vaseyi	CZve	Oak/Hickory Group	53	55	New	5191
vaseyi	Mc	Oak/Hickory Group	55	53	New	5757
vaseyi	Mc	Oak/Hickory Group	55	53	New	6262
vaseyi	CZve	Oak/Hickory Group	59	52	New	1787
vaseyi	CZve	Oak/Hickory Group	59	53	New	
vaseyi	CZve	Oak/Hickory Group	53	55	New	5191
vaseyi	Mc	Oak/Hickory Group	55	53	New	5757
vaseyi	Mc	Oak/Hickory Group	55	53	New	6262
vaseyi	CZve	Oak/Hickory Group	59	52	New	1787
vaseyi	CZve	Oak/Hickory Group	59	53	New	
vaseyi	DSs	Oak/Hickory Group	81	55	Neuse	
vaseyi	TRd	Maple/Beech/Birch	77	49	Savannah	13639
vaseyi	PzZu	Oak/Hickory Group	49	53	Savannah	3042

vaseyi	Zsr	Maple/Beech/Birch	53	50	Savannah	992
vaseyi	TRd	Oak/Hickory Group	49	51	Savannah	10956
vaseyi	TRd	Oak/Hickory Group	91	48	Savannah	13379
vaseyi	Zsr	Oak/Hickory Group	53	49	Savannah	1865
vaseyi	Zb	Oak/Hickory Group	65	55	New	
vaseyi	PzZu	Oak/Hickory Group	55	54	New	

Appendix D

Table 6: Allozyme trials. NR: not resolved

Enzyme	Number of loci	of alleles per locus
G-6PD	1	3
PGM	2	2
FE	1	NR
LAP	NR	NR
ATT	2	2-4
PGI	NR	NR
APER	1	2
MNR	1	6
CPER	2	2-4
UGPP	2	1-4
IDH	1	2
SKDH	1	5
DIA	1	4
TPI	2	2

Table 8: Genetic distance values from Allozyme data between taxa. A= *T. erectum var. album*, E= *T. erectum*, V= *T. vaseyi*, R= *T. rugelii*, F= *T. flexipes*, SI= *T. simile*.

A	E	F	R	SI	V	Taxa
0.000						A
0.012	0.000					E
0.198	0.202	0.000				F
0.126	0.154	0.154	0.000			R
0.070	0.118	0.230	0.064	0.000		SI
0.026	0.040	0.240	0.219	0.133	0.000	V

Table 9: Genetic identity values from Allozyme data between taxa. A= *T. erectum var. album*, E= *T. erectum*, V= *T. vaseyi*, R= *T. rugelii*, F= *T. flexipes*, SI= *T. simile*.

A	E	F	R	SI	V	Taxa
1.000						A
0.988	1.000					E
0.821	0.817	1.000				F
0.881	0.857	0.857	1.000			R
0.932	0.889	0.795	0.938	1.000		SI
0.974	0.960	0.786	0.804	0.876	1.000	V

Table 10: Pairwise Locality Matrix of Nei Unbiased Genetic Distance.

BMP	WCU	WFCK	STIND	WWF	GSM	FH	BRM	WWM	MC	OCO	NG	
0.000												BMP
0.397	0.000											WCU
0.500	0.642	0.000										WFCK
0.316	0.077	0.360	0.000									STIND
0.682	0.322	1.040	0.585	0.000								WWF
0.281	0.095	0.468	0.000	0.599	0.000							GSM
0.467	0.110	0.723	0.172	0.248	0.293	0.000						FH
0.529	0.162	0.848	0.324	0.010	0.326	0.141	0.000					BRM
0.534	0.218	0.699	0.334	0.027	0.314	0.308	0.007	0.000				WWM
0.454	0.138	0.725	0.377	0.097	0.338	0.224	0.072	0.057	0.000			MC
0.653	0.145	1.053	0.420	0.056	0.446	0.194	0.026	0.054	0.083	0.000		OCO
0.425	0.121	0.678	0.345	0.140	0.304	0.224	0.104	0.088	0.000	0.117	0.000	NG
0.705	0.180	1.067	0.550	0.043	0.586	0.181	0.048	0.100	0.054	0.041	0.073	RNBWF

Table 11: Pairwise Locality Matrix of Nei Unbiased Genetic Identity.

BMP	WCU	WFCK	STIND	WWF	GSM	FH	BRM	WWM	MC	OCO	NG	
1.000												BMP
0.673	1.000											WCU
0.607	0.526	1.000										WFCK
0.729	0.926	0.698	1.000									STIND
0.505	0.724	0.354	0.557	1.000								WWF
0.755	0.910	0.627	1.019	0.549	1.000							GSM
0.627	0.896	0.485	0.842	0.780	0.746	1.000						FH
0.589	0.851	0.428	0.723	0.990	0.722	0.869	1.000					BRM
0.586	0.804	0.497	0.716	0.973	0.730	0.735	0.993	1.000				WWM
0.635	0.871	0.485	0.686	0.908	0.713	0.799	0.930	0.945	1.000			MC
0.521	0.865	0.349	0.657	0.945	0.640	0.823	0.974	0.948	0.920	1.000		OCO
0.654	0.886	0.508	0.708	0.869	0.738	0.799	0.901	0.916	1.009	0.890	1.000	NG
0.494	0.835	0.344	0.577	0.958	0.557	0.835	0.953	0.905	0.947	0.960	0.929	RNBWF

Table 12: Summary AMOVA table by taxa.

Source	df	SS	MS	Est. Var.	%
Among Taxa	5	63.541	12.708	0.319	10%
Within Taxa	192	567.333	2.955	2.955	90%
Total	197	630.874		3.274	100%

Table 13: Mean heterozygosity and standard error over all loci for each Locality

Pop	Column1	Ho	He
BMP	Mean	0.125	0.219
	SE	0.125	0.129
WCU	Mean	0.331	0.468
	SE	0.133	0.061
Wolf Creek	Mean	0.250	0.250
	SE	0.160	0.144
Standing Indian	Mean	0.600	0.365
	SE	0.216	0.124
WW Falls	Mean	0.238	0.344
	SE	0.085	0.099
GSMNP	Mean	0.333	0.326
	SE	0.152	0.111
Frozenhead	Mean	0.176	0.253
	SE	0.090	0.113
Black Rock Mtn	Mean	0.343	0.495
	SE	0.115	0.071
War Woman	Mean	0.311	0.517
	SE	0.030	0.040
Moses Creek	Mean	0.413	0.341
	SE	0.171	0.117
Oconolunftee	Mean	0.352	0.487
	SE	0.118	0.058
N GORGE	Mean	0.375	0.307
	SE	0.169	0.122
RAINBOW FALLS	Mean	0.342	0.371
	SE	0.123	0.077

Table 14: Mean Heterozygosity and SE over Loci for groups of Red and White Individuals.

Group	Column1	Ho	He
W	Mean	0.347	0.484
	SE	0.090	0.019
R	Mean	0.329	0.483
	SE	0.078	0.062

Table 15: Summary AMOVA table by locality and taxon.

Source	df	SS	MS	Est. Var.	%
Among Regions	12	299.177	24.931	0.755	14%
Among Pops	12	114.884	9.574	1.092	20%
Within Pops	169	603.831	3.573	3.573	66%
Total	193	1017.892		5.420	100%

Table 16: Genetic distance matrix between three populations of *T. vaseyi*.

V-WWF	V-BRM	V-RF	V-WWM	
0.000				V-WWF
0.013	0.000			V-BRM
0.124	0.099	0.000		V-RF
0.021	0.005	0.127	0.000	V-WWM

Table 17: Summary of Chi-Square Tests for Hardy-Weinberg Equilibrium by Locality.
Key: ns=not significant, * P<0.05, ** P<0.01, *** P<0.001.

Pop	Locus	DF	ChiSq	Prob	Signif
BMP	UGPP 1	1	2.000	0.157	ns
BMP	FE1	1	0.222	0.637	ns
BMP	MNR 1	Monomorphic			
BMP	DIA 1	Monomorphic			
WCU	UGPP 1	3	23.037	0.000	***
WCU	FE1	6	28.107	0.000	***
WCU	MNR 1	1	2.798	0.094	ns
WCU	DIA 1	3	0.600	0.896	ns
Wolf Creek	UGPP 1	3	6.000	0.112	ns
Wolf Creek	FE1	3	0.750	0.861	ns
Wolf Creek	MNR 1	Monomorphic			
Wolf Creek	DIA 1	Monomorphic			
Standing Indian	UGPP 1	1	0.918	0.338	ns
Standing Indian	FE1	3	2.222	0.528	ns
Standing Indian	MNR 1	1	3.000	0.083	ns
Standing Indian	DIA 1	Monomorphic			
WW Falls	UGPP 1	1	8.629	0.003	**
WW Falls	FE1	1	2.333	0.127	ns
WW Falls	MNR 1	1	1.976	0.160	ns
WW Falls	DIA 1	3	2.794	0.424	ns

GSMNP	UGPP 1	1	1.852	0.174	ns
GSMNP	FE1	3	3.375	0.337	ns
GSMNP	MNR 1	1	0.750	0.386	ns
GSMNP	DIA 1	Monomorphic			
Frozenhead	UGPP 1	1	0.219	0.640	ns
Frozenhead	FE1	1	6.516	0.011	*
Frozenhead	MNR 1	Monomorphic			
Frozenhead	DIA 1	1	0.083	0.773	ns
Black Rock Mtn	UGPP 1	1	7.783	0.005	**
Black Rock Mtn	FE1	3	2.557	0.465	ns
Black Rock Mtn	MNR 1	1	6.496	0.011	*
Black Rock Mtn	DIA 1	6	5.031	0.540	ns
War Woman	UGPP 1	3	3.953	0.267	ns
War Woman	FE1	3	10.000	0.019	*
War Woman	MNR 1	1	2.090	0.148	ns
War Woman	DIA 1	3	6.366	0.095	ns
Moses Creek	UGPP 1	Monomorphic			
Moses Creek	FE1	1	1.612	0.204	ns
Moses Creek	MNR 1	1	10.164	0.001	**
Moses Creek	DIA 1	3	2.051	0.562	ns
OCO	UGPP 1	1	24.478	0.000	***
OCO	FE1	1	6.912	0.009	**
OCO	MNR 1	1	3.789	0.052	ns
OCO	DIA 1	3	15.264	0.002	**
N GORGE	UGPP 1	Monomorphic			
N GORGE	FE1	3	1.106	0.776	ns
N GORGE	MNR 1	1	7.200	0.007	**
N GORGE	DIA 1	1	0.163	0.686	ns
RAINBOW FALLS	UGPP 1	1	0.099	0.753	ns
RAINBOW FALLS	FE1	3	1.837	0.607	ns
RAINBOW FALLS	MNR 1	1	1.333	0.248	ns
RAINBOW FALLS	DIA 1	1	6.368	0.012	*

Table 18: Summary of Chi-Square Tests for Hardy-Weinberg Equilibrium. Key: ns=not significant, * P<0.05, ** P<0.01, *** P<0.001.

Pop	Locus	DF	ChiSq	Prob	Signif
W	UGPP 1	3	79.861	0.000	***
W	FE1	6	41.684	0.000	***
W	MNR 1	1	5.775	0.016	*
W	DIA 1	6	28.375	0.000	***
R	UGPP 1	3	71.345	0.000	***
R	FE1	6	29.174	0.000	***
R	MNR 1	1	0.044	0.834	ns
R	DIA 1	6	25.676	0.000	***

Table 19: Allele frequencies by taxa. A= *T. erectum var. album*, E= *T. erectum*, V= *T. vaseyi*, R= *T. rugelii*, F= *T. flexipes*, SI= *T. simile*.

Locus	**Allele/n**	**A**	**E**	**F**	**R**	**SI**	**V**
UGPP1	N	53	24	9	20	23	67
	2	0.009	0.063	0.000	0.025	0.000	0.052
	3	0.179	0.188	0.667	0.475	0.370	0.157
	4	0.811	0.750	0.333	0.500	0.630	0.791
FE1	N	49	21	11	17	23	65
	2	0.031	0.048	0.000	0.118	0.000	0.054
	3	0.439	0.548	0.227	0.059	0.283	0.431
	4	0.531	0.405	0.773	0.735	0.696	0.485
	5	0.000	0.000	0.000	0.088	0.022	0.031
MNR1	N	48	19	10	19	22	63
	1	0.635	0.632	1.000	0.421	0.455	0.762
	2	0.365	0.368	0.000	0.579	0.545	0.238
DIA1	N	45	22	10	12	18	61
	1	0.256	0.273	0.000	0.125	0.139	0.451
	2	0.633	0.727	1.000	0.792	0.500	0.451
	3	0.100	0.000	0.000	0.083	0.361	0.074
	4	0.011	0.000	0.000	0.000	0.000	0.025

Table 20: F-Statistics and Estimates of Nm over All Taxa for each Locus

All Pops.	Locus	Fis	Fit	Fst	Nm
	UGPP1	0.633	0.682	0.135	1.595
	FE1	0.319	0.381	0.091	2.509
	MNR1	-0.335	-0.114	0.166	1.257
	DIA1	0.238	0.348	0.144	1.484
	Mean	0.214	0.324	0.134	1.712
	SE	0.202	0.164	0.016	0.275

Table 21: Summary of Chi-Square Tests for Hardy-Weinberg Equilibrium by Taxa. Key: ns=not significant, * P<0.05, ** P<0.01, *** P<0.001.

Pop	Locus	DF	ChiSq	Prob	Signif
E	UGPP1	3	19.510	0.000	***
E	FE1	3	3.239	0.356	ns
E	MNR1	1	4.321	0.038	*
E	DIA1	1	0.617	0.432	ns
F	UGPP1	1	0.000	1.000	ns
F	FE1	1	6.043	0.014	*
F	MNR1	1	0.139	0.709	ns
F	DIA1	1	0.139	0.709	ns
R	UGPP1	1	18.000	0.000	***
R	FE1	6	16.444	0.012	*
R	MNR1	1	5.159	0.023	*
R	DIA1	3	0.625	0.891	ns
SI	UGPP1	1	18.908	0.000	***
SI	FE1	3	5.096	0.165	ns
SI	MNR1	1	4.791	0.029	*
SI	DIA1	3	4.698	0.195	ns
V	UGPP1	3	44.799	0.000	***
V	FE1	6	23.935	0.001	***
V	MNR1	1	4.811	0.028	*
V	DIA1	6	20.306	0.002	**
A	UGPP1	3	19.577	0.000	***
A	FE1	3	1.095	0.778	ns
A	MNR1	1	5.317	0.021	*
A	DIA1	6	10.961	0.090	ns

Table 22: Mean Heterozygosity and SE over Loci for each taxa. A= *T. erectum var. album*, E= *T. erectum*, V= *T. vaseyi*, R= *T. rugelii*, F= *T. flexipes*, SI= *T. simile*. Observed heterozygosity= Ho; expected heterozygosity= He.

Pop		Ho	He
A	Mean	0.401	0.455
	SE	0.114	0.051
E	Mean	0.364	0.449
	SE	0.108	0.033
F	Mean	0.134	0.199
	SE	0.106	0.116
R	Mean	0.374	0.449
	SE	0.143	0.038
SI	Mean	0.369	0.499
	SE	0.145	0.036
V	Mean	0.299	0.468
	SE	0.073	0.066

Table 23: F-Statistics and Estimates of Nm over All Localities for each Locus

All Pops.	Locus	Fis	Fit	Fst	Nm
	UGPP 1	0.470	0.671	0.381	0.407
	FE1	0.086	0.225	0.152	1.394
	MNR 1	-0.310	0.294	0.461	0.292
	DIA 1	0.247	0.456	0.276	0.654
	Mean	0.123	0.412	0.318	0.687
	SE	0.164	0.099	0.067	0.248

Table 24: Allele frequencies by locality.

Locus	Allele	BMP	WCU	Wolf Creek	Standing Indian	WW Falls	GSMNP	Frozenhead
UGPP 1	2	0.000	0.022	0.667	0.300	0.000	0.000	0.000
	3	0.500	0.543	0.167	0.700	0.071	0.750	0.571
	4	0.500	0.435	0.167	0.000	0.929	0.250	0.429
FE1	2	0.000	0.105	0.167	0.100	0.000	0.250	0.000
	3	0.750	0.211	0.667	0.600	0.500	0.667	0.267
	4	0.250	0.605	0.167	0.300	0.500	0.083	0.733
	5	0.000	0.079	0.000	0.000	0.000	0.000	0.000
MNR 1	1	0.000	0.447	0.000	0.500	0.875	0.333	1.000
	2	0.000	0.553	0.000	0.500	0.125	0.667	0.000
DIA 1	1	0.000	0.100	0.000	0.000	0.579	0.000	0.000
	2	1.000	0.833	1.000	1.000	0.368	1.000	0.929
	3	0.000	0.067	0.000	0.000	0.053	0.000	0.000
	4	0.000	0.000	0.000	0.000	0.000	0.000	0.071

Locus	Allele	Black Rock Mountain	War Woman	Moses Creek	Ocono	Nantahala Gorge	Rainbow Falls
UGPP 1	2	0.000	0.182	0.000	0.000	0.000	0.000
	3	0.275	0.091	0.000	0.276	0.000	0.083
	4	0.725	0.727	1.000	0.724	1.000	0.917
FE1	2	0.125	0.100	0.000	0.000	0.000	0.000
	3	0.450	0.600	0.425	0.311	0.400	0.100
	4	0.425	0.300	0.575	0.689	0.575	0.700
	5	0.000	0.000	0.000	0.000	0.025	0.200
MNR 1	1	0.775	0.591	0.543	0.639	0.500	0.750
	2	0.225	0.409	0.457	0.361	0.500	0.250
DIA 1	1	0.395	0.450	0.190	0.395	0.125	0.450
	2	0.447	0.400	0.762	0.342	0.875	0.550
	3	0.105	0.150	0.048	0.263	0.000	0.000
	4	0.053	0.000	0.000	0.000	0.000	0.000

Table 25: Genetic identity matrix for three populations of *T. vaseyi.*

V-WWF	V-BRM	V-RF	V-WWM	
1.000				V-WWF
0.987	1.000			V-BRM
0.883	0.905	1.000		V-RF
0.979	0.995	0.881	1.000	V-WWM

Table 26: Allele Frequencies for three populations of *T. vaseyi*. N= sample size.

Locus	Allele/n	V-WWF	V-BRM	V-RF	V-WWM
UGPP1	N	19	20	9	11
	2	0.000	0.000	0.000	0.182
	3	0.079	0.275	0.056	0.091
	4	0.921	0.725	0.944	0.727
FE1	N	19	20	7	10
	2	0.000	0.125	0.000	0.100
	3	0.553	0.450	0.000	0.600
	4	0.447	0.425	0.786	0.300
	5	0.000	0.000	0.214	0.000
AAT1	N	16	16	8	7
	3	0.219	0.375	0.063	0.214
	4	0.750	0.625	0.875	0.786
	5	0.031	0.000	0.063	0.000
MNR1	N	18	20	9	11
	1	0.944	0.775	0.667	0.591
	2	0.056	0.225	0.333	0.409
MNR2	N	17	16	2	8
	1	0.971	0.875	0.500	0.938
	2	0.029	0.125	0.500	0.063
DIA1	N	18	19	7	10
	1	0.611	0.395	0.357	0.450
	2	0.333	0.447	0.643	0.400
	3	0.056	0.105	0.000	0.150

Table 27: Allele frequencies for red and white forms of Trillium at Clingman's Dome.

Pop	Allele/n	UGPP1	FE1	DIA	MNR1
Red flowered	**1**				0.500
	2	0.000	0.000	0.250	0.500
	3	0.000	0.500	0.500	
	4	1.000	0.500	0.250	
White flowered	**1**				0.500
	2	0.000	0.000	0.000	0.500
	3	0.000	0.167	0.333	
	4	1.000	0.833	0.667	

Table 28: Summary of AMOVA by locality.

Source	df	SS	MS	Est. Var.	%
Among Pops	12	158.447	13.204	0.714	22%
Within Pops	187	486.453	2.601	2.601	78%
Total	199	644.900		3.315	100%

Appendix E

Table 29: Leaf tissue collections

Locality	Species	Quantity
Wolf Creek	*T. erectum*	9
Black Rock Mtn	*T. vaseyi*	26
Balsam Mountain Preserve	*T.erectum var. album*	1
Balsam Mountain Preserve	*T. erectum*	3
Balsam Mountain Preserve	*T. rugelii*	2
Frozen Head	*T. flexipes*	11
Frozen Head	*T. erectum*	4
Frozen Head	*T. vaseyi*	2
GSMNP	*T. erectum*	9
GSMNP	*T. erectum var. album*	8
GSMNP	*T.erectum hybrid*	2
GSMNP	*T. simile*	11
GSMNP	*T. vaseyi*	15
Moses Creek	*T. rugelii*	4
Moses Creek	*T. erectum hybrid*	8
Moses Creek	*T. erectum*	6
Moses Creek	*T.erectum var. album*	7
Nantahala Gorge	*T. simile*	18
Nantahala Gorge	*T. album*	3
Standing Indian	*T. erectum*	3
Standing Indian	*T. erectum var. album*	6
Standing Indian	*T. erectum hybrid*	2
War Woman	*T. vaseyi*	18
WCU	*T. rugelii*	17
WCU	*T. rugelii hybrid*	2
Whitewater Falls	*T. vaseyi*	25
Whitewater Falls	*T. vaseyi (white flowered)*	2
Oconolunftee	*T. erectum var. album*	19
Oconolunftee	*T. simile*	3
Oconolunftee	*T. vaseyi*	2
Oconolunftee	*T. vaseyi hybrid*	1

Table 7: Allozyme data set. N/A: individual not run for that locus, 99: not resolved.

Location	Collection number	UGPP 1	FE1	AAT1	MNR1	MNR2	DIA 1
BMP	1	33	33	N/A	99	N/A	22
BMP	3	44	34	N/A	99	N/A	22
WCU	8	33	99	N/A	99	N/A	99
WCU	11	33	44	N/A	22	N/A	99
WCU	13	33	22	N/A	22	N/A	22
WCU	15	33	22	N/A	12	N/A	99
WCU	16	33	99	N/A	99	N/A	99
WCU	17	33	44	N/A	12	N/A	99
WCU	18	33	44	N/A	22	N/A	99
WCU	19	33	44	N/A	12	N/A	22
WCU	20	33	99	N/A	12	N/A	22
WCU	21	33	44	N/A	12	N/A	99
Wolf Creek	24	22	23	N/A	99	N/A	22
Wolf Creek	30	34	33	N/A	99	N/A	22
Wolf Creek	31	22	34	N/A	99	N/A	22
Standing Indian	39	33	33	N/A	12	N/A	22
Standing Indian	40	23	34	N/A	99	N/A	22
Standing Indian	41	23	34	N/A	12	N/A	22
Standing Indian	42	33	23	N/A	12	N/A	22
Standing Indian	45	23	34	N/A	99	N/A	22
Whitewater Falls	48	44	33	33	11	11	13

Whitewater Falls	49	44	34	44	11	11	13
Whitewater Falls	50	34	33	99	11	11	11
Whitewater Falls	51	44	34	34	11	11	11
Whitewater Falls	52	44	44	44	12	12	11
Whitewater Falls	53	33	33	N/A	11	N/A	22
Whitewater Falls	54	44	44	N/A	99	N/A	22
Whitewater Falls	57	44	34	44	11	11	11
Whitewater Falls	59	44	44	44	12	11	11
Whitewater Falls	60	44	33	44	11	11	11
Whitewater Falls	63	44	33	44	11	11	22
Whitewater Falls	64	44	33	34	11	11	12
Whitewater Falls	66	44	34	45	11	11	12
Whitewater Falls	67	44	34	34	11	11	99
Whitewater Falls	68	44	44	44	11	11	11
Whitewater Falls	69	44	33	44	11	11	12
Whitewater Falls	70	44	44	34	11	11	12
Whitewater Falls	71	44	34	44	11	11	12
Whitewater Falls	72	44	34	34	11	11	12
GSMNP	93	44	33	N/A	99	N/A	22
GSMNP	106	33	23	N/A	12	N/A	22
GSMNP	108	33	23	N/A	99	N/A	22
GSMNP	109	33	33	N/A	12	N/A	22
GSMNP	113	33	33	N/A	99	N/A	22
GSMNP	115	34	24	N/A	22	N/A	22

Frozen Head	115	44	99	N/A	99	N/A	22
Frozen Head	117	33	34	N/A	99	N/A	22
Frozen Head	118	44	33	99	11	11	99
Frozen Head	120	33	44	N/A	11	N/A	22
Frozen Head	121	33	44	N/A	11	N/A	22
Frozen Head	122	33	44	N/A	11	N/A	22
Frozen Head	123	99	44	N/A	11	N/A	22
Frozen Head	124	34	44	N/A	11	N/A	22
Frozen Head	131	34	33	44	11	11	22
Frozen Head	132	44	34	44	11	11	99
Frozen Head	133	99	33	N/A	99	N/A	22
Frozen Head	134	34	44	N/A	11	N/A	22
Frozen Head	135	34	44	N/A	11	N/A	22
Black Rock Mtn	136	44	44	44	11	11	14
Frozen Head	136	33	44	N/A	11	N/A	22
Black Rock Mtn	137	44	34	44	11	11	33
Frozen Head	137	34	44	N/A	11	N/A	24
Black Rock Mtn	138	44	44	34	11	11	11
Frozen Head	138	34	44	N/A	11	N/A	24
Black Rock Mtn	139	33	23	N/A	22	N/A	99
Black Rock Mtn	140	33	24	N/A	22	N/A	22
Black Rock Mtn	142	34	34	34	11	11	24
Black Rock Mtn	146	44	44	33	11	11	13
Black Rock Mtn	147	44	33	34	11	11	12

Black Rock Mtn	148	44	33	34	11	11	22
Black Rock Mtn	149	44	33	44	11	11	22
Black Rock Mtn	150	34	34	34	22	22	23
Black Rock Mtn	151	44	34	44	11	11	12
Black Rock Mtn	152	34	34	44	11	11	11
Black Rock Mtn	153	44	34	44	11	11	12
Black Rock Mtn	154	44	34	33	12	12	12
Black Rock Mtn	156	44	33	34	12	12	12
Black Rock Mtn	157	33	22	N/A	11	N/A	22
Black Rock Mtn	158	33	24	N/A	12	N/A	22
Black Rock Mtn	159	44	34	44	11	11	11
Black Rock Mtn	160	44	34	33	11	11	11
War Woman	162	22	99	N/A	22	N/A	22
War Woman	163	44	34	44	11	11	12
War Woman	165	44	33	34	11	11	99
War Woman	166	24	22	N/A	22	N/A	22
War Woman	168	23	44	N/A	22	N/A	22
War Woman	169	44	33	44	11	11	12
War Woman	171	44	33	44	12	11	11
War Woman	172	44	34	99	11	11	33
War Woman	173	44	34	44	12	12	13
War Woman	174	44	34	44	12	11	11
War Woman	176	34	33	33	11	11	11
Moses Creek	220	44	34	33	12	12	23

Moses Creek	221	44	34	55	12	12	12
Moses Creek	222	44	44	34	12	12	23
Moses Creek	223	44	34	24	12	12	22
Moses Creek	224	44	99	55	12	12	22
Moses Creek	225	44	33	45	11	12	22
Moses Creek	226	44	33	44	12	12	22
Moses Creek	227	44	44	34	12	12	22
Moses Creek	228	44	44	44	12	12	22
Moses Creek	229	44	34	34	12	12	22
Moses Creek	230	44	44	34	12	12	12
Moses Creek	231	44	44	34	11	12	12
Moses Creek	232	44	34	33	12	12	22
Moses Creek	234	44	34	33	12	12	22
Moses Creek	235	44	33	44	11	12	22
Moses Creek	236	44	99	45	12	N/A	99
Moses Creek	237	44	33	45	12	12	12
Moses Creek	238	44	44	44	12	12	12
Moses Creek	239	44	33	44	12	12	12
Moses Creek	240	44	99	99	12	12	12
Moses Creek	241	44	44	34	22	12	22
Moses Creek	242	44	44	44	12	12	99
Moses Creek	243	44	34	34	12	12	12
WCU	246	44	45	45	11	N/A	22
WCU	247	44	44	55	12	N/A	99

WCU	248	44	34	45	12	N/A	12
WCU	249	44	45	55	12	N/A	12
WCU	250	44	44	55	12	N/A	23
WCU	251	44	45	45	12	N/A	12
WCU	253	44	44	44	12	N/A	22
WCU	254	44	44	55	12	N/A	22
WCU	255	44	34	55	22	N/A	23
Oconaluftee	270	44	34	34	12	11	12
Oconaluftee	271	44	34	44	12	12	23
Oconaluftee	272	44	44	34	12	11	11
Oconaluftee	273	44	33	44	11	12	12
Oconaluftee	274	44	44	44	11	12	11
Oconaluftee	275	34	33	33	12	12	22
Oconaluftee	276	44	44	44	12	12	23
Oconaluftee	277	44	44	34	12	11	11
Oconaluftee	278	44	33	34	11	12	12
Oconaluftee	279	44	34	44	11	11	11
Oconaluftee	280	44	34	34	11	12	12
Oconaluftee	281	44	33	34	11	11	12
Oconaluftee	282	44	34	44	11	12	33
Oconaluftee	283	44	34	33	12	12	12
Oconaluftee	284	44	33	34	11	11	11
Oconaluftee	285	44	34	23	12	12	12
Oconaluftee	286	44	44	34	12	99	12

Oconaluftee	287	44	99	55	12	99	22
Oconaluftee	288	44	34	34	12	99	22
Oconaluftee	290	44	33	34	11	11	12
Oconaluftee	291	34	44	34	11	11	23
Oconaluftee	292	44	44	23	11	12	12
Oconaluftee	293	44	44	44	12	12	11
Oconaluftee	294	44	34	44	12	12	11
Oconaluftee	295	44	44	44	12	22	11
Oconaluftee	296	44	33	44	11	11	11
Oconaluftee	297	44	44	99	12	12	22
Oconaluftee	298	44	44	44	12	11	11
Nantahala Gorge	300	44	34	34	12	12	99
Nantahala Gorge	301	44	34	99	12	11	99
Nantahala Gorge	301	44	44	34	12	12	99
Nantahala Gorge	303	44	34	44	12	12	22
Nantahala Gorge	304	44	34	44	12	99	99
Nantahala Gorge	305	44	45	44	12	12	99
Nantahala Gorge	306	44	34	34	12	12	12
Nantahala Gorge	309	44	44	33	12	12	99
Nantahala Gorge	308	44	34	99	22	12	22
Nantahala Gorge	309	44	33	34	12	12	99
Nantahala Gorge	310	44	33	33	22	12	22
Nantahala Gorge	312	44	44	34	12	12	22
Nantahala Gorge	313	44	44	34	11	12	99

Nantahala Gorge	314	44	34	44	12	12	12
Nantahala Gorge	315	44	33	44	12	12	22
Nantahala Gorge	316	44	34	99	12	11	99
Nantahala Gorge	317	44	33	34	12	11	99
Nantahala Gorge	318	44	44	34	12	12	99
Nantahala Gorge	322	44	44	99	11	11	99
Nantahala Gorge	323	44	44	34	12	12	22
Rainbow Falls	330	34	34	34	11	11	11
Rainbow Falls	331	44	34	44	11	11	11
Rainbow Falls	332	44	45	44	11	N/A	22
Rainbow Falls	334	44	44	44	11	12	11
Rainbow Falls	335	44	99	45	12	N/A	22
Rainbow Falls	336	44	44	34	12	N/A	99
Rainbow Falls	337	44	44	44	11	12	11
Rainbow Falls	338	44	44	44	12	N/A	99
Rainbow Falls	339	44	45	44	11	N/A	22
Rainbow Falls	340	44	45	44	12	N/A	22
Rainbow Falls	341	44	45	99	12	N/A	12
Rainbow Falls	342	34	99	44	12	N/A	22
Whitewater Falls	350	44	44	44	22	N/A	99
Whitewater Falls	351	44	44	44	12	N/A	22
WCU	A-maroon thecae	23	99	N/A	12	N/A	22
Oconaluftee	202	34	44	N/A	22	N/A	33

Oconaluftee	203	33	44	N/A	22	N/A	33
Oconaluftee	204	33	44	N/A	12	N/A	23
Oconaluftee	205	33	44	N/A	12	N/A	23
Oconaluftee	206	33	44	N/A	99	N/A	33
Oconaluftee	207	33	44	N/A	12	N/A	23
Oconaluftee	208	33	44	N/A	12	N/A	33
Oconaluftee	209	33	44	N/A	12	N/A	23
Oconaluftee	210	33	44	N/A	12	N/A	23
Oconaluftee	211	33	44	N/A	12	N/A	33
WCU	A-yellow thecae	33	33	N/A	11	N/A	22