

A systematic and biogeographic study of *Trillium* (Melanthiaceae)

A Dissertation Presented for the
Doctor of Philosophy
Degree
The University of Tennessee, Knoxville

Jayne Anne Lampley
December 2021

Copyright © 2021 by Jayne Anne Lampley. All rights reserved.

Acknowledgements

First, I would like to express my appreciation to my advisor, Ed Schilling, for all of his support throughout my program at the University of Tennessee, Knoxville. I especially thank him for his generous assistance with field work and with cultivated *Trillium* specimens, but also for his guidance in teaching and in writing. I would also like to express my gratitude to Randy Small, Meg Staton, and Joe Williams for serving on my committee. I am thankful for their technical guidance and for providing feedback of my dissertation chapters. I am also very grateful for fellow Schilling Lab member, Aaron Floden, for his assistance and expertise, both in the lab and in the field. I also wish to express my tremendous gratitude to Ken McFarland for his guidance in teaching botany laboratory classes at UTK.

In addition, I wish to thank Brian O'Meara, Ben Keck, Mona Papeş, Marcin Nowicki, Ana Maria Sanchez Cuervo, Eric Arnold, Orlando Schwery, Jacob Moutouama, Tyler Poppenwimer, Whitaker Hoskins, Justin Hendy, and Joel Corush for assistance with software or other technical guidance in my research. Special thanks to Susan Farmer, Tom Patrick, Wayne Barger, Brian Keener, Francine and Bruce Hutchinson, Dennis Horn, Ben Crain, Ana Maria Sanchez Cuervo, Fabiola Soto, Patrick Lynch, Russ Graham, Theo Witsell, Misako Nishino, Ken Cameron, Welby Smith, Kaleigh Younge, Susie Egan, Charles Hunter, Chelsea Miller, and Kate Gribbin for help with field work, or for sending data or samples. I also thank the staffs of the following herbaria in the facilitation of this research: TENN, UGA, JSU, UWAL, UNA, AMAL, ALNHS, USCH, MO, and UCHT. Thanks to Joe May and Veronica Brown of the UT Genomics Core for Sanger sequencing and Morgan Schneider and Jennifer Schilling for lab assistance. A special thanks to Margaret Oliver for her help with microscopy and also to Jeff Martin and Kaitlin Palla for their help in caring for greenhouse plants. I apologize if I have omitted anyone inadvertently. I wholeheartedly thank each person for their support.

Funding for this project was provided for by the Hesler Award, Ecology and Evolutionary Biology Department at UTK, and the American Society of Plant Taxonomists. Collection trips were made possible by the Breedlove-Dennis Fund (TENN). The Huntsville Botanical Garden, Holmes *Trillium* Garden is also greatly appreciated for providing live plant material of *Trillium maculatum*.

Finally, I would like to express my deep appreciation to my family for all of their love and support. I could not have finished this work without their help and encouragement. I am truly thankful for each of my friends and family members for cheering me on all of these years while I have been in school.

Soli Deo gloria.

This work is dedicated to my parents.

Abstract

Trillium (Melanthiaceae, Parideae) has a disjunct distribution occurring in eastern and western North America, and eastern Asia. Past studies have examined the phylogeny and historical biogeography of Melanthiaceae and Parideae, however these studies either did not fully examine these aspects within *Trillium* or did not employ sufficiently broad taxonomic or character sampling to clarify relationships among taxa. The first phylogenetic analysis presented in this study provides a resolved phylogeny for *Trillium s.l.* and *Paris s.l.* by using a dataset of 70 plastid coding genes and by sampling broadly from *Trillium s.s.*, *Pseudotrillium*, *Trillidium*, *Paris s.s.*, *Daiswa*, and *Kinugasa*. The results suggested that each of the six genera of Parideae are monophyletic and that *Daiswa* is sister to *Paris* rather than *Trillium* in contrast to some previous studies. The study also illustrated that omitting key taxa fundamentally affects the phylogeny topology, thus changing our understanding of taxonomic relationships. A new subgeneric classification of *Trillium* was presented based on the results of the analysis. In addition, the divergence time and historical biogeographical analyses estimated that diversification in *Trillium* lasted from the Miocene through the Pleistocene and that its origin was centered in eastern North America. The second phylogenetic analysis examined relationships within *T.* subgen. *Sessilium* with an emphasis on the *T. cuneatum* complex. The results of the analysis showed that *T. cuneatum* is paraphyletic and forms a clade including *T. maculatum*, *T. luteum*, and five other separate lineages. Two species, *T. freemanii* sp. nov. and *T. radiatum* sp. nov., were described from two of these lineages. Morphological observations of *T. cuneatum s.s.*, *T. luteum*, *T. maculatum*, and the novel species were compared. In addition, previous morphological classifications of *T.* subgen. *Sessilium* were re-evaluated. The third phylogenetic analysis focused on the disjunct populations of *T. lancifolium* and closely related species, *T. recurvatum*. The results showed that *T. lancifolium* is paraphyletic, forming a clade which included a monophyletic *T. recurvatum* and seven highly supported lineages of *T. lancifolium*. Hypotheses regarding gene flow among populations within river watersheds and physiographic boundaries were discussed and the results of a seed germination experiment were presented.

Table of Contents

Introduction.....	1
References.....	3
Chapter 1 <i>Trillium</i> and Tribe Parideae (Melanthiaceae): Phylogeny and historical biogeography of a Holarctic plant disjunction using plastid protein-coding sequence data.....	5
References.....	27
Appendix A.....	34
Chapter 2 Taxonomic relationships among the Toadshades: A phylogenetic study of the <i>Trillium cuneatum</i> complex	51
References.....	73
Appendix B.....	77
Chapter 3 A phylogeographic analysis of <i>Trillium lancifolium</i> (Melanthiaceae).....	96
References.....	106
Appendix C.....	109
Conclusion	118
Vita.....	120

List of Tables

Table 1.1. A summary of molecular studies of Parideae (Trilliaceae) or <i>Trillium</i>	34
Table 1.2. Table of study taxa.....	35
Table 1.3. Table of geographic categories used in the BioGeoBEARS historical range reconstruction analysis.....	38
Table 1.4. Listing of 70 plastid coding genes used in the plastome matrix dataset.....	40
Table 1.5. A comparison of divergence time estimation studies for events in the evolutionary history of Parideae (Melanthiaceae).	41
Table 2.1. Table of sessile species	77
Table 2.2. A table of samples used in the analysis.	78
Table 3.1. A list of samples used in the study.	109
Table 3.2. Individual gene length differences of genes used in the study.	113
Table 3.3. Observations of fruit flotation and seedling germination in <i>Trillium</i>	113

List of Figures

Figure 1.1. Majority rule consensus tree.....	42
Figure 1.2. Divergence time estimation.....	43
Figure 1.3. Historical biogeographic range reconstruction for Melanthiaceae based on BioGeoBEARS.	44
Figure 1.4. Historical biogeographic range reconstruction for Melanthiaceae showing degree of probability for each inference	45
Figure 1.5. <i>Trillium</i> Erectum group species density	47
Figure 1.6. <i>Trillium</i> subgen. <i>Sessilium</i> species density	48
Figure 1.7. <i>Trillium</i> Grandiflorum group species density.....	49
Figure 1.8. <i>Trillium</i> subgen. <i>Delostylium</i> species density	50
Figure 2.1. Images of the overall appearance of the species of the Cuneatum Complex.	85
Figure 2.2. Bayesian inference phylogenetic tree.	86
Figure 2.3. <i>Trillium cuneatum</i> complex map.....	87
Figure 2.4. Androecium of the species of the Cuneatum Complex	88
Figure 2.5. Stamens of the species of the Cuneatum Complex	89
Figure 2.6. Images of the ovaries of the species of the Cuneatum Complex.....	90
Figure 2.7. Stigmatic surface comparison of the species of the Cuneatum Complex	91
Figure 2.8. Images of <i>Trillium freemanii</i>	92
Figure 2.9. Map of <i>Trillium freemanii</i> distribution.....	93
Figure 2.10. Images of <i>Trillium radiatum</i>	94
Figure 2.11. Map of <i>Trillium radiatum</i> distribution	95
Figure 3.1. A map of the distribution of <i>T. lancifolium</i>	114
Figure 3.2. Images of <i>Trillium lancifolium</i> showing the morphological heterogeneity among various populations.	115
Figure 3.3. Bayesian inference phylogenetic tree using MrBayes showing relationships of <i>T.</i> <i>lancifolium</i> and the Thin Rhizome Group.	116
Figure 3.4. A map of the locations of <i>T. lancifolium</i> populations sampled in the study.	117

Introduction

Trillium (Melanthiaceae) is an economically important monocot genus of spring ephemerals of North America and eastern Asia. *Trillium* provides an exceptional opportunity to study complex evolutionary, biogeographical, and ecological mechanisms due to the unique traits and geographic distribution of each of its species. The following study of *Trillium* is arranged into three chapters. The first chapter of the work focuses broadly on *Trillium* and tribe Parideae to resolve the broader phylogeny of this group. This study includes an estimation of divergence times and historical range reconstruction for Parideae and *Trillium* along with a comparison of past studies. A subgeneric reclassification of *Trillium* is also presented. The second chapter focuses on relationships within *T.* subgenus *Sessilium* with a primary emphasis on the taxonomy and relationships of the putative *T. cuneatum* species complex. Two novel species are described from cryptic entities of the *T. cuneatum* complex. The third chapter is an examination of the putative *T. lancifolium* species complex and sister species *T. recurvatum*. The spatial and phylogenetic relationships among populations of *T. lancifolium* are evaluated for possible correlations to physiographic boundaries and river watershed systems of the southeastern US. An observational fruit flotation and germination study are presented which tested the hypothesis that floodplain dwelling species, *T. lancifolium*, may endure occasional water inundation due to flooding events which might allow for seed dispersal along river drainages.

The first chapter seeks to resolve the phylogeny, timing of divergences, and historical biogeography of *Trillium* and allies. *Trillium* includes approximately 49 species and belongs to tribe Parideae, along with up to five other genera: *Paris*, *Daiswa*, *Kinugasa*, *Trillidium*, and *Pseudotrillium*. These are all mainly forest understory herbs of the northern hemisphere. Many of these taxa have duly garnered attention because of their various folk medicinal usages, horticultural appeal, and large genomes sizes (Pellicer et al., 2014; Huang et al., 2016; Tamura, 2016). Collectively, the tribe displays the classic eastern North American – eastern Asian disjunction pattern. Previous molecular-based phylogenetic studies have come to conflicting conclusions regarding relationships within Parideae leading to taxonomic confusion and controversy rather than resolution. These results may be due to limited data and taxon sampling. For example, *Pseudotrillium* was omitted in some studies, but included in others, which in comparison showed differing relationships in *Paris* s.l. and *Trillium* s.l. (Huang et al., 2016; Kim et al., 2016; Ji et al., 2019; Kim et al., 2019; Yang et al., 2019; Ling and Zhang, 2020). This aspect as well as other limitations of previous studies likely led to notable disagreements in divergence estimations and historical biogeographic analyses. This study uses 70 plastid protein-coding sequence data as the basis of Bayesian and Maximum Likelihood phylogenetic inferences, a divergence timing estimation performed in BEAST, and a historical range reconstruction performed in BioGeoBEARS. In addition, the chapter addresses the phylogenetic relationships between *Paris*, *Daiswa*, *Kinugasa*, and *Trillium*, the monophyly of *Trillidium*, the subgenera within *Trillium*, and the relationship of *Trillium ovatum* to *T. hibbersonii*.

The second chapter examines a putative species complex via a phylogenetic study of *Trillium cuneatum* and *T.* subgen. *Sessilium*. *Trillium cuneatum* displays a widespread distribution, spanning seven states of the southeastern US inhabiting rich, upland woods with limestone soils at lower elevations (Freeman, 1975; Case, 2003; Meredith et al., 2020; Weakley, 2020). *Trillium cuneatum* has been noted for being highly variable, though the explanation for this is unknown (Freeman, 1975; Case, 2003). Recent phylogenetic analyses showed *T.*

cuneatum Raf. (Melanthiaceae) as paraphyletic, suggesting that it may represent one or more cryptic species (Schilling et al., 2013, Schilling et al. 2019). In addition, previous population studies found significant genetic structure among *T. cuneatum* populations based on limited plastid data (Gonzales et al., 2008; Wallace and Doffitt, 2013). The goals of this study are to obtain a species level phylogeny of *T.* subgen. *Sessilium*, to analyze the putative *T. cuneatum* complex including *T. luteum* and *T. maculatum*, and to assess morphological and geographical correlates to clades delineated in the phylogeny. Finally, morphology-based concepts of *T.* subgen. *Sessilium* are explored in detail in relation to the results of molecular-based phylogenetic inference.

The third chapter focuses on the disjunct populations of *T. lancifolium* and closely related species *T. recurvatum*. The regional endemic, *Trillium lancifolium*, has a discontinuous distribution of isolated populations, although widespread, which makes it distinctive among *Trillium* species. *Trillium lancifolium* is typically found in rich woodlands typically in floodplains beside creeks or rivers (Small, 1897; Case, 2003; Weakley, 2020). Populations of the species occur within more than a dozen river watersheds and several physiographic provinces of the southeastern US (Kartesz, 2015; Meredith et al., 2020). In addition, the species displays variable morphological traits and two peak blooming periods (Case and Case, 1997). Results of recent phylogenetic studies have provided evidence to support a paraphyletic *T. lancifolium* that also includes *T. recurvatum*, a species widely accepted as distinct (Schilling et al., 2013; Schilling et al., 2019). *Trillium lancifolium* and *T. recurvatum* belong to an informal morphological group known as the Thin Rhizome group which also includes *T. oostingii* and *T. tennesseense* (Freeman, 1975; Schilling et al., 2013). The objectives of this study are to obtain a resolved phylogeny to assess relationships within the Thin Rhizome group, to examine the species-level taxonomy of *T. lancifolium*, to evaluate the hypothesis that there are two taxa within *T. lancifolium* s.l. which are separated by physiographic barriers, to evaluate the hypothesis that rivers and watersheds have contributed to diversification in *T. lancifolium* by affecting patterns of gene flow, and to observe the capability of *T. lancifolium* to survive occasional flooding via a fruit flotation and seed germination study.

References

- Case, F. 2003. "Trillium." In: Flora of North America Editorial Committee (Eds.). *Flora of North America North of Mexico*. Volume 26. Oxford: Oxford University Press.
- Case, F. and R. Case. 1997. *Trilliums*. Portland, OR: Timber Press.
- Freeman, J. 1975. Revision of *Trillium* subgenus *Phyllantherum* (Liliaceae). *Brittonia*. 27: 1-62.
- Gonzales E., Hamrick, J.L., and S.-M. Chang. 2008. Identification of glacial refugia in southeastern North America by phylogeographical analyses of a forest understory plant, *Trillium cuneatum*. *Journal of Biogeography*. 35: 844-852.
- Huang, Y., Xiaojuan, L., Yang, Z., Yang, C., Yang, J., and Y. Ji. 2016. Analysis of complete chloroplast genome sequences improves phylogenetic resolution in *Paris* (Melanthiaceae). *Frontiers in Plant Science*. 7: 1797.
- Ji, Y., Yang, L., Chase, M.W., Liu, C., Yang, Z., Yang, J., Yang, J.-B. and T.-S. Yi. 2019. Plastome phylogenomics, biogeography, and clade diversification of *Paris* (Melanthiaceae). *BMC Plant Biology*. 19: 543.
- Kartesz, J.T. 2015. The Biota of North America Program (BONAP). North American Plant Atlas. Available at <http://bonap.net/napa>. Chapel Hill, N.C.
- Kim, S.-C., Kim, J.S., Chase, M.W., Fay, M.F., and J.-H. Kim. 2016. Molecular phylogenetic relationships of Melanthiaceae (Liliales) based on plastid DNA sequences. *Botanical Journal of the Linnean Society*. 181: 567-584.
- Kim, C., Kim, S.-C., and J.-H. Kim. 2019. Historical biogeography of Melanthiaceae: A case of out-of-North America through the Bering Land Bridge. *Frontiers in Plant Science*. 10: 396.
- Ling, L.-Z., and S.-D. Zhang. 2020. The complete chloroplast genome and phylogenetic analysis of *Paris delavayi* (Melanthiaceae). *Mitochondrial DNA Part B*. 5: 2126-2128.
- Meredith, C.R. and Trillium Working Group 2019. 2020. *Trillium cuneatum*. The IUCN Red List of Threatened Species 2020: e.T146084767A146089210. <https://dx.doi.org/10.2305/IUCN.UK.2020-2.RLTS.T146084767A146089210.en>. (Accessed 17 February 2021).
- Meredith, C.R. and Trillium Working Group 2019. 2020. *Trillium lancifolium*. The IUCN Red List of Threatened Species 2020: e.T146086425A146089255. Available at <https://dx.doi.org/10.2305/IUCN.UK.2020-2.RLTS.T146086425A146089255.en>. (Accessed 25 February 2021).
- Pellicer, J., Kelly, L.J., Leitch, I.J., Zomlefer, W.B. and M.F. Fay. 2014. A universe of dwarfs and giants: genome size and chromosome evolution in the monocot family Melanthiaceae. *New Phytologist*. 201: 1484-1497.
- Schilling, E.E., Floden, A., and S.B. Farmer. 2013. A New Sessile-Flowered *Trillium* Species from Tennessee. *Castanea* 78: 140-147.
- Schilling, E.E., Floden, A., Lampley, J., Patrick, T.S. and S.B. Farmer. 2019. A New Species of *Trillium* (Melanthiaceae) from Central Georgia and its Phylogenetic Position in subgenus *Sessilium*. *Systematic Botany*. 44: 107-114.
- Small, J.K. 1897. Studies in the Botany of the Southeastern United States IX. The Sessile-Flowered Trillia of the Southern States. *Bulletin of the Torrey Botanical Club*. 24: 169-178.

- Tamura, M. 2016. *Kinugasa japonica* Melanthiaceae. *Curtis's Botanical Magazine*. Royal Botanic Gardens Kew. 33: 261-267.
- Weakley, A.S. 2020. *Flora of the Southeastern United States*, edition of 20 October 2020. Chapel Hill: University of North Carolina at Chapel Hill Herbarium (NCU), North Carolina Botanical Garden, University of North Carolina at Chapel Hill.
- Wallace, L.E. and C.H. Doffitt. 2013. Genetic Structure of the Mesic Forest-Adapted Herbs *Trillium cuneatum* and *Trillium stamineum* (Melanthiaceae) in the South-Central United States. *Castanea*. 78: 154–162.
- Yang, L., Yang, Z., Liu, C., He, Z., Zhang, Z., Yang, J., Liu, H., Yang, J., and Y. Ji. 2019. Chloroplast phylogenomic analysis provides insights into the evolution of the largest eukaryotic genome holder, *Paris japonica* (Melanthiaceae). *BMC Plant Biology*. 19: 293.

Chapter 1

Trillium and tribe Parideae (Melanthiaceae): Phylogeny and historical biogeography of a Holarctic plant disjunction using plastid protein-coding sequence data

INTRODUCTION

Molecular phylogenetic estimation has led to new insight into the evolutionary relationships of plant families, genera, and species. However, if our interpretation of evolutionary relationships within a given group is imprecise, subsequent applications based on these inferences such as those of taxonomy, trait evolution, biogeographic analyses, and divergence time estimation will be impacted. Basic factors such as method of taxon sampling, amount of genetic data used, and sample misidentification could lead to misleading or erroneous results. For example, incorrect phylogenetic inference can sometimes be attributed to inadequate or incomplete taxon sampling, especially in cases of long-branch attraction (LBA) and intensive gene sampling (Hillis, 1998; Hedtke et al. 2006; Heath et al., 2008). Increasing taxon sampling has been shown to decrease terminal branch lengths (thus affecting divergence time estimation) and to increase the accuracy of reconstructed phylogenies under maximum parsimony and maximum likelihood methods (Graybeal, 1998; Rannala et al., 1998; Pollock et al., 2002; Zwickl and Hillis, 2002), though sensitivity to taxon sampling is low in clades with fewer than 20 taxa (Poe, 1998). Some researchers have sought to solve the problem of inaccurate phylogenies by greatly increasing the amount of genetic data in an analysis to combat incongruence (Rokas and Carroll, 2005). Other studies, however, have shown that even large genomic data sets could result in unresolved phylogenies (Soltis and Soltis, 2004; Baurain et al., 2007; Philippe et al., 2011). An incorrect phylogenetic inference would be largely misleading in ancestral state or biogeographic reconstruction if various members of particular clades are misplaced. A prime example of the issues that plague our current understanding of a group can be found in the monocot tribe Parideae (Melanthiaceae).

Previous molecular-based phylogenetic studies have provided a solid foundation of the tribal relationships within Melanthiaceae (Pellicer et al., 2014; Kim et al., 2016b; Kim et al., 2019), but the relationships within Parideae continue to be problematic. The conflicting conclusions of these studies regarding Parideae relationships have led to further taxonomic confusion and controversy rather than resolution. A likely cause is that these molecular studies were based on limited amounts of sequence data, and taxon sampling was also problematic (see Table 1). The monotypic *Pseudotrillium* was omitted in some studies, and sampling of major clades within *Trillium* was often also incomplete, leading to striking disagreements among phylogenetic inferences in regard to sister relationships in *Paris s.l.* and *Trillium s.l.*, for instance whether *Daiswa* was sister to *Trillium* or to *Paris*. Correspondingly, studies which analyzed character trait evolution, divergence times, and historical biogeography based on these limited phylogenetic inferences came to differing conclusions. For example, the divergence time estimation within Melanthiaceae, one of the oldest monocot families (Givnish et al., 2016), varied among recent studies. The crown age of Melanthiaceae differed for example, ranging from 84.8 my (Santonian age of Late Cretaceous) (Givnish et al., 2016) to approximately 92.1 my (Turonian age of Late Cretaceous) (Kim et al., 2019), and the estimations of lower-ranking taxa also showed some differences: the split between *Trillium s.l.* and *Paris s.l.* ranged from 17.3 my (Miocene) (Givnish et al., 2016) to 22.5 my (Miocene) (Kim et al., 2019) to 38.21 my (Eocene) (Yang et al., 2019). (See also Melanthiaceae crown age results from Liliales studies: Vinnersten and Bremer, 2001, mean crown age 42 or 54 my; Janssen and Bremer, 2004, mean crown age 97 my; Mennes et al., 2015, mean crown age 54 or 59 my). Given the limitations of previous studies, a re-examination of the phylogeny of this group with adequate taxon and character sampling is warranted.

Melanthiaceae is a diverse family on many levels

Although Melanthiaceae is relatively small, it is a family of great contrasts and variability in terms of geography, genome size, and morphology. It comprises five tribes (Melanthieae, Xerophylleae, Chionographideae, Heloniadeae, and Parideae) and includes approximately 17 genera and 172 species (Zomlefer et al., 2001; Stevens, 2021). The family has a wide distribution primarily across Europe, Asia, and North America, with the exception of *Schoenocaulon* A. Gray, which extends just south of the equator to Peru (Pellicer et al., 2014; Zomlefer et al., 2006; WFO, 2021). The family has the distinction of having the greatest variability in genome size of any land plant family (Pellicer et al., 2014). The smallest genome in Melanthiaceae can be found in Melanthieae (*Schoenocaulon texanum* Scheele, 1C value=0.66 pg) while the largest genomes are only found in Parideae (the largest of these, *Paris japonica* (Franch. & Sav.) Franch. [*Kinugasa japonica* (Franch. & Sav.) Tatew. & Sutô], 1C value=152.23 pg) (Pellicer et al., 2014). These genome expansions are reportedly caused by a proliferation of transposable elements, and in some taxa, polyploidization magnifies the expansion effect further (Pellicer et al., 2014). In addition, Melanthiaceae members are morphologically diverse. Parideae wholly comprised the family Trilliaceae based on morphology, however this family was sunk into Melanthiaceae and designated as a tribe based on results from molecular studies (APG, 1998). This was controversial because Including Trilliaceae as a tribe in Melanthiaceae made it very difficult to describe Melanthiaceae using key morphological features.

A few studies of historical biogeography have focused on Melanthiaceae due to its complex distribution patterns, notably including the well-known eastern North American – eastern Asian disjunction pattern. Molecular and fossil data suggest that most eastern North American – eastern Asian disjunctions came about during the climatically stable Miocene epoch (Wen, 1999). Subsequent to that time, disruptive glaciation cycles of the Pleistocene epoch led to novel environments which likely promoted lineage diversification, though the hypothesis of climate-driven speciation is controversial (Adams, 1997; Hewitt, 2000; Rull, 2008; Lomolino et al., 2017). Past studies have suggested that Melanthiaceae first arose in North America during the Cretaceous. Givnish et al. (2016) concluded that the Melanthiaceae lineage arose in eastern North America and from there, the ancestral Parideae lineage shifted to western North America followed by movement again to eastern North America and eastern Asia in the ancestor of *Paris*, *Trillium*, and *Trillidium*. Kim et al. (2019) suggested that North America, in general, was the probable ancestral area for Melanthiaceae, Melanthieae, Parideae, and Xerophylleae, though the models used could not clarify the origin of the other two Melanthiaceae tribes. Despite these studies, however, little is known about the geographic shifts within the major lineages of *Trillium* and whether diversification was continuous or restricted to a specific time period, such as the Pleistocene.

Generic delimitation in Parideae has been unsettled, in part because of conflicting results of phylogenetic studies. There is a lack of consensus on whether or not *Daiswa* and *Paris* are distinct or should be treated simply as a single genus (Takhtajan, 1983; Kato et al., 1995; Osaloo et al., 1999; Farmer and Schilling, 2002; Farmer, 2006; Ji et al., 2006; Huang et al., 2016; Kim et al., 2016; Tamura, 2016; Yang et al., 2019). There is also disagreement regarding the sister group to *Trillium* (Farmer and Schilling, 2002; Givnish et al., 2016; Huang et al., 2016). Huang et al. (2016) suggested that contrary to previous studies, *Trillium* is sister to *Daiswa* rather than *Paris*. It was suggested in one study that *Trillium* was not monophyletic: *Trillium kamtschaticum* Pall. ex Pursh [*T. camschatcense* Ker Gawl.] was put in a clade with *Daiswa* Raf.

and *Paris* representatives, all Asian species, while North American *Trillium sessile* was inferred to be sister to *Kinugasa japonica* of Japan in a second clade (Kato et al., 1995).

Another taxonomically controversial Parideae genus, *Trillidium*, is a phylogenetic lineage of special interest due to its extreme floristic disjunction pattern. *Trillidium govanianum* (Wall. ex Royle) Kunth can be found at high elevations (2800 – 3960m) in the Himalayan mountain countries (Fukuda, 2001; eFloras, 2019). *Trillidium undulatum* (Willd.) Floden & E.E. Schill. [*Trillium undulatum* Willd.] occurs in southeastern Canada, New England states of the US and extends further south at higher elevations in the Appalachian mountain range. Recent studies have placed the taxa together based on molecular, chromosomal, and palynological evidence (Fukuda, 2001; Farmer and Schilling, 2002; Farmer, 2006; Weakley et al., 2018; see also Osaloo and Kawano, 1999) while others maintain both species in *Trillium* (Pellicer et al., 2014; Kim et al., 2016; Kim et al., 2019; Yang et al., 2019). For example, Yang et al. (2019) does not support recognition of *Trillidium govanianum* based on their phylogeny which suggests that the taxon is sister to North American sessile *Trillium* species, and rather that Asian *Trillium tschonoskii* is sister to *Trillidium* + sessile *Trillium* species. Resolving the relationships between *T. govanianum*, *T. undulatum* and the rest of Parideae will permit the elucidation of this biogeographic mystery.

Although monotypic *Pseudotrillium* is a rare endemic, it could hold the key to phylogenetic studies. *Pseudotrillium* inhabits just six counties of southwestern Oregon and northern California in the Siskiyou Mountains (USDA, NRCS, 2021; Case and Case, 1997). *Pseudotrillium rivale*, previously placed in *Trillium*, was elevated to a genus based on molecular phylogenetic results and due to its morphological apomorphies (Farmer and Schilling, 2002). When used in molecular phylogenetic studies, *P. rivale* has been shown to be basally diverged in Parideae (Kato et al., 1995; Osaloo et al., 1999; Osaloo & Kawano, 1999; Farmer & Schilling, 2002; Pellicer et al., 2014; Kim et al., 2016; Givnish et al., 2016; Kim et al., 2019). Omission of this species in taxon sampling, however, may be the cause of some of the disparity among phylogenetic studies noted above (Kato et al., 1995; Huang et al., 2016; Yang et al., 2019).

***Trillium* Taxonomy and Biogeography**

Trillium includes approximately 49 species, which display significant intra- and inter-continental geographic disjunctions among areas of eastern and western North America and eastern Asia. Conventionally, *Trillium* has been divided into two main groups: species with pedicellate flowers, sometimes referred to as *T.* subgen. *Trillium*, and species with sessile flowers, *T.* subgen. *Sessilium* Raf. (Case, 2003; Reveal and Gandhi, 2014). This seemingly logical and simple subdivision obscures significant variability within the genus, particularly in the pedicellate species.

An attempt to subdivide the pedicellate trilliums was made by Gleason (1906). He recognized three informal groups based on stigma and ovary morphology. His *T. grandiflorum* type group (with truncate or rounded ovary not deeply angled, and uniformly slender stigmas), which we shall refer to as the Grandiflorum group, included *T. grandiflorum* (Michx.) Salisb., *T. ovatum* Pursh, *T. scouleri* Rydberg ex Gleason which is currently considered to be part of *T. ovatum*, but is under review), and *T. nivale* Riddell. As a group, these are disjunct between eastern and western North America (Gleason, 1906; Farmer, 2006, 2007). He also included species in this group that are now thought to be in separate genera: *Trillidium* (*Trillidium undulatum*) and *Pseudotrillium* (*P. rivale*). The *Trillium erectum* type group (with sharply six-lobed ovary, and sessile, recurved, tapering stigma), which shall be referred to as Erectum group

here, has members occurring in both Asia and eastern North America but is not known from western North America. The Erectum group is the only *Trillium* clade represented in Asia: species can be found in eastern Russia, China, Japan, Korea, Taiwan, and the Himalayan region of east India (Case and Case, 1997). Lastly, Gleason recognized a *T. Catesbaei* type group, which corresponds to *T.* subgen. *Delostylium* Raf. (with uniformly slender stigmas and a definite style), and has members which occur only in the eastern US (Rendle, 1901; Gleason, 1906; Farmer and Schilling, 2002; Farmer, 2007). *Trillium* subgen. *Sessilium* species are disjunct between eastern and western North America (Freeman, 1975; Case and Case, 1997).

Trillium shares the eastern North American – eastern Asian disjunction pattern with at least 64 other genera, but, in contrast to most clades with this observed pattern of distribution, the higher species diversity for *Trillium* is in the eastern US, rather than in eastern Asia (Case and Case, 1997; Wen, 1999; Meng et al., 2021). There are a few other examples of a reverse diversification – disjunction pattern with more species in North America, for example *Carya*, which originated in North America before somehow crossing to eastern Asia around 21.58 mya (Zhang et al., 2013). Some have suggested that rates of molecular evolution as well as selection pressures are equal in sister disjunct species of eastern Asia and eastern North America and are therefore not the reason for differential species richness between the two regions (Melton et al., 2020). *Trillium* may have originated in eastern North America, but Farmer (2006) suggested that *Trillium* may have originated in the Pacific Northwest based on a morphological phylogeny showing that *Pseudotrillium* is sister to the *Trillium* lineage. Lending support to this possibility, Xiang and Soltis (2001) proposed that many temperate angiosperm lineages originated in western North America as well as eastern Asia.

Trillium ovatum Pursh is another problematic Parideae taxon with an unresolved taxonomy. As broadly circumscribed, *T. ovatum* s.l. is highly variable and can be found in a relatively wide distribution in western North America, where it is the only pedicellate *Trillium* representative (Case & Case, 1997). Morphologically part of the Grandiflorum group, molecular phylogenetic studies have also shown that *T. ovatum* is closely related to eastern North American *T. grandiflorum* (Osalo et al., 1999; Osalo and Kawano, 1999; Farmer and Schilling, 2002; Ji et al., 2006; Pellicer et al., 2014). *Trillium ovatum* s.l. has been split into several infrageneric taxa based on morphology. Notably, a dwarf-sized pedicellate *Trillium* and rare endemic from western Vancouver Island, British Columbia, *T. hibernsonii* (T.M.C. Taylor & Szczaw.) D. O'Neill & S.B. Farmer [*T. ovatum* var. *hibernsonii* (T.M.C. Taylor & Szczaw.) G.W. Douglas & Pojar] has been considered a variety of *T. ovatum*, although it differs from that species in its consistently shorter stature, initial pink flower color, and its habitat of coastal, mossy cliffs and river boulders (Wiley, 1969; Taylor and Szczawinski, 1974; O'Neill, 1995; Case and Case, 1997; Douglas and Pojar, 2001; Case, 2003). The relationship of *T. hibernsonii* with *T. ovatum* was examined in this study.

Objectives

The main objectives of this study are: 1) to make a broadly based estimate of the phylogeny of Parideae, by employing an adequate sampling of taxa and genetic data, 2) to test the hypothesis that *Daiswa* and *Kinugasa* are distinct genera from *Paris* and that *Trillium* is sister to a clade comprising *Paris* and *Daiswa*, 3) to clarify the phylogeny of subgeneric clades within *Trillium*, 4) to confirm the relationship of *Trillidium govanianum* and *T. undulatum*, and to test that *Trillidium* is distinct from *Trillium*, 5) to resolve the relationship of *T. ovatum* and *T. hibernsonii*, 6) to use the resolved phylogeny to estimate evolutionary divergence times within

Trillium in relation to related taxa, and 7) to analyze the biogeography of these taxa by historical geographic range reconstruction inference to understand the movements between infrageneric groups within *Trillium* and to test the hypothesis that *Trillium* originated in eastern North America.

MATERIALS AND METHODS

Taxonomic sampling

Representatives of the five Melanthiaceae tribes were sampled as follows: a proportion of species from each of the six genera of Parideae (*Paris* (2/5), *Daiswa* (12/20), *Kinugasa* (1/1), *Pseudotrillium* (1/1), *Trillidium* (2/2), and *Trillium* (13/49) including representative members of each putative subgroup, two or more genera of each of tribes Melanthieae, Heloniadeae, and Chionographideae, and two species from the only Xerophylleae genus, *Xerophyllum* (see Table 2). *Fritillaria* (Liliaceae) and *Lilium* (Liliaceae) representatives were used collectively as an outgroup. One eudicot outgroup, *Verbesina aristata* (Elliott) A. Heller, was also used for divergence dating purposes. Sixteen samples, mostly North American taxa, were obtained from living collections that were then frozen at -80°C or preserved in silica gel (Table 2). In our experience, extraction of DNA from herbarium specimens of *Trillium* has been unsuccessful. Twenty-four samples, all Asian taxa, were included using data from Genbank and data for three others was obtained from the Sequence Read Archive (SRA), of the National Center for Biotechnology Information (NCBI).

Molecular Methods

Whole genomic DNA was extracted from samples, each approximately 0.1 g of leaf material flash frozen in liquid nitrogen, using DNeasy Plant Minikits (Qiagen, Inc., Valencia, CA, USA) following the manufacturer's protocol. A set of DNA extractions, used previously by Farmer (2006), was available for use in this study, having been kept in cold storage since that time at -20°C. Sample concentration was evaluated using a NanoDrop 2000 spectrophotometer (Thermo Scientific, Waltham, MA, USA).

Sequencing and read assembly

Library preparation and next generation sequencing (NGS) services for 22 samples were performed by Global Biologics, LLC (Columbia, MO, USA). Reads were generated via the Illumina HiSeq sequencing platform. Samples included one Melanthiaceae outgroup taxon, one dicot outgroup taxon, Parideae sister taxa, *Trillium* subgen. *Delostylium* taxa, *T.* subgen. *Sessilium* taxa, and other *Trillium* taxa (see Table 2).

To obtain sequences of plastid coding genes from the NGS reads, a combination of de novo assembly and map to reference protocols were used, features available in the Geneious v. 8.1.9 software platform (<https://www.geneious.com>). First, NGS sequence sets were grouped together to generate paired end reads in Geneious. Then, De novo assemblies were performed and consensus sequences from the resulting high quality contigs with the highest assembled read counts were extracted and cross-checked with the Blastn search (NCBI) program. Only contigs which closely matched to other *Trillium* or Parideae taxa were kept for further analysis. Second, paired end NGS read data were assembled in separate runs to reference chloroplast genomes which had been divided into 15 10k bp segments using the Map to Reference aligner in Geneious. Reference chloroplast genomes used for this process included *Trillium cuneatum* KR135077, *T. decumbens* KR534612, *T. tschonoskii* KR780076, and *Daiswa forrestii*

KX784043, available via Genbank. Next, contig consensus sequences from the de novo assemblies and consensus sequences from the fifteen 10k bp plastome segment reference-guided assemblies were collectively assembled to one of the closely related reference plastomes, in full, using the Map to Reference aligner. If these sequences did not fully cover the reference plastome in certain areas of the alignment, these gap areas were targeted for further reference-guided assembly using the NGS reads. Assemblies were generated repeatedly in order to “walk” across the gaps until the full plastome could be produced. Once a draft plastome sequence was obtained with high confidence, Geneious was used to predict its gene annotations by comparing regions with >65% similarity to those of a fully annotated reference plastome. Seventy coding genes were then extracted from each of these annotated draft plastome sequences for each taxon and then each were positioned in the forward direction and concatenated (Table 4). Each gene was also individually aligned and alignments and start and stop codons were manually checked. The 70 plastid coding genes used in this study were selected for ease of alignment by observing which ones were present in a majority of taxa in the study. For example, omitted marker *rps16* has been noted to be missing in both Melanthieae and Chionographideae (Yang et al., 2019). The coding gene *ycf1* is highly variable and might prove informative, but was not included in the plastome matrix in order to make the alignment process more straightforward (Dong et al., 2015). In addition, coding sequences from only one of the Inverted Repeat regions were used in the study. Genes which occurred over two or more intervals were concatenated in the forward direction and aligned side by side. In some instances, neighboring genes *psbD* and *psbC*, and *ndhK* and *ndhC* were found to overlap each other. These were extracted individually in each case without truncating, to correspond with the full Geneious-predicted annotations.

Plastid coding sequence data for three additional samples, *Helonias bullata*, *Xerophyllum asphodeloides*, and *Veratrum nigrum*, were obtained from transcriptomic data deposited in the SRA (www.ncbi.nlm.nih.gov/sra), available via NCBI (see Table 2 for SRA reference numbers). The data were converted to DNA reads and assembled in Geneious in a similar fashion as with the newly obtained NGS reads. Likewise, annotations were performed, genes were extracted, and sequences concatenated in Geneious.

Phylogenetic analyses

Sample sequences were aligned using MAFFT v7.017 (Katoh & Standley, 2013) with the default settings, adjusted by eye, and concatenated all within Geneious. Phylogenies were inferred via maximum likelihood and Bayesian statistical analyses. The plastome data matrix was composed of 70 plastid coding sequences from 43 samples either newly obtained from this study, downloaded from Genbank, or downloaded from SRA and assembled (Table 2). The jModelTest v. 2.1.10 (Guindon and Gascuel 2003; Darriba et al. 2012) application using the Akaike Information Criterion (AIC) estimated the best-fit model of nucleotide substitution for the sequence data to be the high-parameter GTR + I + G model (general time reversible with gamma distribution and a proportion of invariant sites). The Bayesian analysis for the dataset was performed in BEAST v.2.6.0 (Bouckaert 2014). BEAST package BEAUTi was used to create the executable file for BEAST (Drummond et al., 2012). Markov Chain Monte Carlo sampling was done every 2500 generations for 50 million generations. Tracer v.1.7.1 (Rambaut et al. 2018) assessed the percentage of first trees in the sampling to discard as “burn-in,” in this case, 15%, or 3,000 samples. TreeAnnotator program v2.3.2 within BEAST was used to summarize the posterior sample of trees generated in the Bayesian analysis into the final maximum clade credibility tree. FigTree v.1.4.4 (<http://tree.bio.ed.ac.uk/software/figtree/>) was

used for graphical tree display. The Maximum Likelihood (ML) analysis was implemented by RAxML v.8.2.11 or v.7.2.8 (Stamatakis, 2014) in Geneious. The Maximum Likelihood analyses for the plastome matrix executed 1200 bootstrap replicates. Finally, a majority rule consensus tree was obtained for the data set.

Divergence-time estimation

Bayesian estimation of divergence times was performed on the plastome matrix dataset (43 taxa and 70 coding chloroplast regions) with the BEAST v.2.6.0 software package (Bouckaert, 2014). A Relaxed Clock Log Normal clock model was used in the analysis, which allows for independence among branches in substitution rates, along with a Birth-Death tree model prior. For the time calibration priors, we used estimates from Magallón et al. (2015), as currently there are no known *Trillium* fossils (Iles et al, 2015). In the Magallón et al. study (2015), an Angiosperm time tree that was calibrated using 137 fossils estimated that the median stem age of Melanthiaceae is 84.19 my with a 95% HPD min/max of 66.99/103.34 my, and that the median age of the divergence of eudicots, basal grade dicots, and monocots is 135.8 my (HPD 132.8/138.8). In BEAUTi, a taxa subset was created which included all taxa except *Lilium*, *Fritillaria*, and the eudicot outgroup taxon. For this “Melanthiaceae Set,” a log normal prior distribution was chosen to constrain the time calibration (M=84.19 and S=0.111). This allowed for uncertainty in the maximum estimated age of the clade. A “Monocot” subset, with all taxa exclusive of the Dicot outgroup taxon, was created as above except with a time calibration of M=135.8 and S=0.012. The use of root age priors has been recommended to improve the accuracy of divergence estimations, so this second calibration was used in the current study (Sytsma et al., 2014). A GTR substitution model and a Gamma site heterogeneity model were used with 4 gamma categories, and other default settings. A Gamma distribution was used for the UCLD Mean (mean of the branch rates under an uncorrelated lognormal relaxed molecular clock model).

Biogeographic Reconstruction

To determine the origins and sequential movements of this North American – East Asian disjunct group, the Bayesian-inferred phylogeny with divergence-time estimation done for the data set as implemented in BEAST, was used in an historical biogeographical reconstruction analysis via the BioGeoBEARS v.1.1.1 package in R (Matzke, 2018). The Dispersal-Extinction-Cladogenesis (DEC) model which uses two parameters, d (dispersal rate or rate of range addition) and e (extinction rate or rate of range loss), was implemented in the analysis (Ree and Smith, 2008). Model-testing was not applied as some have argued that these tests make unrealistic comparisons (Ree and Sanmartin, 2018).

The 40 taxa featured in this analysis were coded for their current presence in one or more of eight geographic zones (including Circumboreal region - Old World, Circumboreal region - New World, Eastern Asiatic region, North American Atlantic region, Rocky Mountain Floristic region, Mediterranean region, Irano-Turanian region and the Indochinese region, see Table 3) drawn up based on Takhtajan’s floristic regions (1986). Takhtajan’s biogeographical work, which divided the world’s land masses into 35 detailed floristic regions each defined by their generally consistent composition of plant species, seemed to be a reasonable guide to coding the taxa and more precise than purely continental classification. A number of sources were consulted to confirm a precise distribution of each taxon including: Flora of North America and Flora of China (eFloras, 2019), Kew Science Plants of the World Online (POWO, 2019), United

States Department of Agriculture PLANTS Database (USDA, NRCS, 2021), Biota of North America Program (Kartesz, BONAP, 2015), Northern Ontario Plant Database (Meades et al., 2019), Global Biodiversity Information Facility (*Veratrum* L. in GBIF Secretariat, 2019; *Chionographis* Maxim. in GBIF Secretariat, 2019), Fuse and Tamura (2016), and Schilling et al. (2017).

RESULTS

Plastome alignment

The aligned 70 coding gene plastome matrix had a sequence length of 61,807 bp. Most of the genes exhibited at most slight variability in lengths, with a few exceptions (e.g. *accD*, *atpB*, *ndhB*, *ndhF*, *psaB*, *psbA*, *psbC*, *rpoA*, *rpoB*, *rpoC1*, *rpoC2*, *rps4*, *rps11*, *rps18* and *ycf2*, all with >85 bp length difference). This was confirmed in gene region *accD* for *T. cuneatum* and *T. decumbens* by direct sequencing. In *accD*, a 980 bp truncation at the beginning of the sequence was observed in *T. cuneatum*, *T. decumbens*, and *T. catesbaei*. The gene varied in ungapped sequence length among samples from 747 bp (in the three truncated samples) to 1605 bp (*Daiswa chinensis*). Each sample had standard start and stop codons for this gene region. *Ycf2* also had standard start and stop codons and an aligned sequence length of 7,892 bp. The region showed a great deal of variability: ranging from a 6,216 bp sequence (*T. cuneatum*) to 7,143 (*T. decumbens*).

Phylogeny using Maximum Likelihood and Bayesian Inference

The alignment of 70 plastome coding regions for 40 Melanthiaceae representatives was used in the first phylogenetic analysis (Table 4). The results from the analyses using RAxML and BEAST are shown on the same phylogenetic tree in Fig. 1. There was agreement between the results of these two methods. In both phylogenetic reconstructions, Melanthiaceae was monophyletic. Melanthieae, represented by *Veratrum*, was sister to all of the other Melanthiaceae tribes. Chionographideae was placed sister to Heloniadeae, while Xerophylleae was sister to Parideae. Within Heloniadeae, North American *Helonias bullata* was recovered as sister to clade *Ypsilandra thibetica* + *Heloniopsis tubiflora* of eastern Asia. Within Parideae, *Pseudotrillium* was sister to a clade of the five remaining Parideae genera. The two *Paris* s.s. species formed a clade with high support. *Paris* s.s. was sister to the *Kinugasa* + *Daiswa* clade. *Kinugasa* was sister to a monophyletic *Daiswa*. The lowest branch consensus support in the analysis (bootstrap of 56.5) was found within *Daiswa* for a clade which included two branches, each having high support (bootstrap of 92.6 and 100, respectively). The first of these included species of east China and westward to Tibet, e.g. *D. yunnanensis*, and a second branch contained species of mainly southern China and Indochina, e.g. *D. dunniana*. Within a clade that was sister to the *Paris* s.l. clade, *Trillidium* was sister to a monophyletic *Trillium* clade. Four distinct clades within *Trillium* were recovered. One clade corresponded to the Erectum Group. The lineage including western North American *Trillium hibernsonii* was placed within this Erectum Group clade with high support as the first diverging branch, sister to a lineage of Asian + North American Erectum Group species. The Erectum Group clade was sister to a clade with three evident monophyletic *Trillium* groups, corresponding to the Grandiflorum group, *T.* subgen. *Delostylium* and *T.* subgen. *Sessilium*. Within the Grandiflorum Group, *Trillium nivale* was sister to a branch with *T. ovatum* + *T. grandiflorum*. The Grandiflorum Group was sister to a clade with mixed support (1.0 pp/67.2 bootstrap) which included *T.* subgen. *Sessilium* and *T.*

subgen *Delostylium*. The newly described *T. georgianum* was placed with *T. catesbaei* in *T.* subgen. *Delostylium*.

Time Divergence in BEAST

The median stem age prior for Melanthiaceae in the divergence time estimation implemented in BEAST using the plastome dataset was 84.19 mya (see Fig. 2). The crown age, then, for Melanthiaceae (i.e. when Melanthieae diverged from the rest of Melanthiaceae) was inferred to be 59.07 mya (95% highest posterior density (HPD) 32.07 – 81.45 mya) in this analysis. Melanthieae members *Veratrum nigrum* and *V. oxysepalum* diverged from each other 19.98 mya (HPD 5.64 – 37.51 mya). The crown age for the remainder of Melanthiaceae (i.e. when the lineage of Chionographidae + Heloniadeae diverged from Xerophylleae + Parideae) was estimated to be 48.15 mya (HPD 26.04 – 68.5 mya). Chionographideae and Heloniadeae diverged 35.06 mya (HPD 17.7 – 57.71 mya). Within Chionographideae, *Chionographis* and *Chamaelirium* diverged 10.98 mya (HPD 1.24 – 23.1 mya). In *Heloniadeae*, *Helonias* diverged from the *Heloniopsis* + *Ypsilandra* clade 12.83 mya (HPD 5.18 – 23.26 mya). Xerophylleae diverged from Parideae 36.89 mya (HPD 18.9 – 54.92 mya). The two *Xerophyllum* species diverged 12.61 mya (HPD 2.73 – 27.14 mya).

Within Parideae, the lineage leading to *Pseudotrillium rivale* diverged from the remainder of Parideae at 18.84 mya (HPD 8.33 – 30.46 mya). *Trillidium* diverged from *Trillium* 14.59 mya (HPD 6.75 – 23.79 mya). Asian *Trillidium govanianum* and eastern North American *T. undulatum* diverged from each other approximately 10.29 mya (HPD 2.7 – 18.12 mya). *Trillium Erectum* Group diverged from the remainder of *Trillium* around 12.86 mya (HPD 5.91 – 20.96 mya). Within Erectum Group, *T. hibbersonii* diverged early from the rest of the group at 9.11 mya (HPD 3.81 – 15.23 mya); Asian species in Erectum Group diverged from their North American counterparts approximately 5.0 mya (HPD 1.64 – 9.3 mya). The two Asian Erectum Group species in this study, *T. camschatcense* and *T. tschonoskii*, were estimated to have diverged from each other more recently (0.06 mya; HPD 0 – 0.17 mya) than American Erectum Group representatives *T. cernuum* (2.08 mya; HPD 0.63 – 3.84 mya) and *T. rugelii* + *T. sulcatum* (these latter two diverging 1.42 mya; HPD 0.43 – 2.71 mya). Grandiflorum Group diverged from sister clade *T.* subgen. *Delostylium* + *T.* subgen. *Sessilium* about 10.91 mya (HPD 4.89 – 17.93 mya). Within Grandiflorum Group, *T. nivale*, of north-central and northeastern US, diverged from eastern North American species *T. grandiflorum* + western North American species *T. ovatum* around 8.93 mya (HPD 3.8 – 14.93 mya). *Trillium grandiflorum* + *T. ovatum* diverged 6.75 mya (HPD 2.46 – 11.77 mya). *T.* subgen. *Delostylium* and *T.* subgen. *Sessilium* diverged around 9.64 mya (HPD 4.13 – 15.89 mya). The two species of subgen. *Delostylium*, both of the southeastern US, diverged around 6.32 mya (HPD 2.1 – 11.3 mya), while the two species of subgen. *Sessilium*, also of the southeastern US, diverged more recently around 4.62 mya (HPD 1.46 – 8.58 mya). *Paris s.l.* broke from *Trillidium* + *Trillium* clade around 16.36 mya (HPD 7.44 – 26.05 mya). *Paris s.s.* diverged from *Kinugasa* + *Daiswa* clade 14.61 mya (HPD 6.69 – 23.82 mya). The divergence between *Paris verticillata* and *P. quadrifolia* was estimated to have occurred 5.68 mya (HPD 1.59 – 10.76 mya). The *Kinugasa* and *Daiswa* lineages separated 9.93 mya (HPD 3.87 – 16.97 mya), while the crown age of *Daiswa* was estimated to be 5.67 mya (HPD 2.02 – 9.91 mya; also when basal-most species *D. tibetica* diverged). *Daiswa rugosa* then broke with the remaining clade of *Daiswa* taxa at 3.27 mya (HPD 1.18 – 5.71 mya), followed by a divergence of the *D. mairei* lineage at 1.6 mya (HPD 0.67 – 2.7 mya). The remaining group of nine *Daiswa* species had an estimated crown age of 1.51 mya (HPD 0.62 –

2.52 mya), with the most recent divergence within the group being between *D. chinensis* and *D. dunniana*, both of China and Indochina, at 0.21 mya (HPD 0.05 – 0.45 mya).

Historical Biogeographic Range Reconstruction

Historical biogeographic range estimates using model DEC are shown in Fig. 3. With a maximum range size of eight, there are 256 possible range combinations (2^8 states). Color-coded labels at each ancestral node indicate the estimated ranges with the highest probability. Pie charts give the relative probability of possible ranges at each node (Fig. 4). According to the analysis, the inferred origin of Melanthiaceae was not resolved, but was in either the East Asiatic or the North American Atlantic regions. The analysis also showed a high degree of uncertainty for Melanthiaceae representatives, *Veratrum spp.*, in the result that the two sample species diverged in the East Asiatic region from a widespread ancestor with a distribution in four regions (Circumboreal Old World, East Asiatic, Mediterranean, and Irano-Turanian). The most recent common ancestor (MRCA) of Chionographidae + Heloniadae most likely originated in the North American Atlantic region, while the MRCA of Xerophylleae also likely inhabited the same. The historic range of the MRCA of Parideae was uncertain: the results of the analysis suggested it originated in a combined three-region range (East Asiatic, North American Atlantic, and Rocky Mountain Floristic). *Paris s.l.* most likely originated in the East Asiatic region. The probable distribution of the MRCA of the clade containing *Trillium* subgen. *Sessilium*, *T. subgen. Delostylium*, and the Grandiflorum Group was in the North American Atlantic region. Likewise, the Erectum Group likely originated in the North American Atlantic region.

DISCUSSION

Phylogenetic Analysis

The phylogenetic analysis based on our plastome data provided evidence to help resolve the relationships among members of Paridae (Fig. 1). With the inclusion of samples of *Pseudotrillium*, *Trillidium*, and *Kinugasa*, a comprehensive view of the relationships within *Paris s.l.* emerged. Similarly, questions about the monophyly and relationship between *Trillidium* and *Trillium* were clarified. Broader sampling of both species and markers also illuminated that there are four distinct lineages within *Trillium* and that the pedicellate species, which have been sometimes considered to form *T. subgen. Trillium*, do not form a monophyletic group. Additionally, *T. hibernsonii*, long regarded as a variety of *T. ovatum*, was shown to be distinct, and more closely related to the Erectum group than to *T. ovatum*.

Melanthiaceae tribal relationships were resolved with high support and were consistent with results of previous studies. Melanthiaceae was confirmed as sister to a clade of all the other tribes in agreement with past Melanthiaceae studies (Pellicer et al., 2014; Givnish et al., 2016; Huang et al., 2016; Kim et al., 2016b; Ji et al., 2019; Kim et al., 2019; Yang et al. 2019; Ling & Zhang 2020). Chionographidae and Heloniadae were confirmed as sister groups, and together were sister to Xerophylleae + Parideae. Xerophylleae was confirmed as the immediate sister group to Parideae (Pellicer et al., 2014; Givnish et al., 2016; Huang et al., 2016; Kim et al., 2016b; Ji et al., 2019; Kim et al., 2019; Yang et al. 2019; Ling & Zhang 2020). Xerophylleae, with its two disjunct species restricted to North America, is in contrast to sister Parideae, which has multiple species and genera and occurs widely across temperate areas of the northern hemisphere.

The phylogenetic analysis resolved generic-level relationships within Parideae. The first diverging branch of Parideae was *Pseudotrillium*, a result initially surprising (Osalo and

Kawano, 1999; Farmer and Schilling, 2002), but now well supported (Farmer, 2006; Ji et al., 2006; Pellicer et al., 2014; Kim et al., 2016a; Kim et al., 2016b; Givnish et al., 2016; Kim et al., 2019; Xu et al., 2019). Differences of *Pseudotrillium* from the Parideae lineage include its distinct morphology and geography: its spotted petals, continuously elongating pedicel, coriaceous cordate leaves, and its endemism to yellow pine or redwood groves in ultramafic derived serpentine soils in the Siskiyou Mountains of the northwestern US (Case and Case, 1997; Farmer and Schilling, 2002; Calflora, 2021). This analysis reports the first complete set of plastid coding genes for *Pseudotrillium*.

The results showed that *Paris s.l.* is monophyletic, with the two species representing *Paris s.s.* forming a clade that is sister to *Kinugasa* + *Daiswa*. This is in contrast to the phylogenetic results of Huang et al. (2016), which showed *Paris s.l.* as not monophyletic, but rather placed *Daiswa* sister to *Trillium*, based on a whole plastome dataset which omitted *Pseudotrillium*, *Kinugasa*, and *Trillidium*. Based on this result Huang et al. (2016) made a convincing argument for recognizing *Daiswa* as separate from *Paris*. With the more comprehensive sampling in the current study, however, *Daiswa* was placed sister to *Paris* rather than to the *Trillium* + *Trillidium* clade. We also tested this result by analyzing the same dataset from the Huang et al. (2016) study both before and after the addition of our *Pseudotrillium* plastome data. After inclusion of *Pseudotrillium*, that dataset also produced the same result as in our dataset, which underscores the significance of taxon sampling strategy. The current results are consistent with either a broad interpretation of *Paris* or a narrower interpretation separating *Paris s.s.* from *Daiswa*, and optionally also *Kinugasa*. Huang et al. (2016) also argued for separating *Daiswa* from *Paris s.s.* citing evidence of geographical differences as well as differences in plastid gene content and arrangement including differences of numbers of copies of the *trnI*^{CAU} gene (three copies in *Paris s.s.*, but only a single copy in *Daiswa*). Takhtajan (1983) offered strong morphological evidence for separating *Paris s.l.* into three genera. The distinguishing characteristics were presence of seed sarcotesta, loculicidal dehiscence of fruits (capsules), ovary morphology, chemistry, and rhizome thickness. The current study is in general agreement with Huang et al. (2016) in the recognition of the eastern and western clades of *Daiswa*, except for the relationship of *D. mairei*. With the inclusion of *D. thibetica* and *D. rugosa* in this study, *D. mairei* was inferred to be sister to the eastern + western *Daiswa* clade as a whole rather than part of the western lineage. In the phylogeny of the current study, *Kinugasa* was more closely related to *Daiswa*, in agreement with some past studies (Osaloo & Kawano, 1999; Farmer & Schilling, 2002; Farmer, 2006; Pellicer et al., 2014; and Kim et al., 2019; Yang et al. 2019; Xu et al., 2019; many of these used *Paris s.l.* nomenclature), but contrary to others which placed *Kinugasa* sister to *Paris s.s.* (Ji et al., 2006; Kim et al., 2016b) or *Kinugasa* sister to *Trillium* (Kato et al., 1995b).

The phylogenetic results provided supporting evidence on both the recognition of *Trillidium* and its expansion to include two species. The phylogeny placed Asian *T. govanianum* and North American *T. undulatum* together in a clade with high support (Fig. 1). Although earlier studies showed the two to be sister species (Osaloo and Kawano, 1999; Farmer and Schilling, 2002; Farmer, 2006; Kim et al., 2016b; Kim et al., 2019), this result was sometimes ascribed to long branch attraction (Farmer and Schilling, 2002; Farmer, 2006). *Trillium* and *Trillidium* were both individually and collectively monophyletic in the current phylogeny, so it is a matter of choice whether or not to recognize *Trillidium* at the level of genus. However, exclusive similarities between the two *Trillidium* species in fruit morphology (a scarlet or purple berry), pollen ornamentation, and karyotype banding patterns in conjunction with the present

molecular evidence set them apart from other *Trillium* species and lend support to recognition of *Trillidium* (Case and Case, 1997; Fukuda, 2001; Weakley et al., 2018). In addition, both species share whitish rhizomes, rather than brown, petiolate leaves, and habitat-preferences of cool-climate and acidic soil in mixed conifer and *Rhododendron* forests (Samejima and Samejima, 1987; Case and Case, 1997). Finally, the sister group status of *Trillidium* and *Trillium* was in agreement with several past studies (Kato et al., 1995a; Farmer and Schilling, 2002; Kim et al., 2016a; Kim et al., 2016b; Givnish et al., 2016; Kim et al., 2019). This is, however, in contrast to Yang et al. (2019) and Ji et al. (2019); and Ling & Zhang (2020) which placed *Trillidium* sister to *T.* subgen. *Sessilium*; and Farmer (2006) which inferred the *Trillidium* clade to be in an incompletely resolved *Trillium* clade.

Based on the phylogenetic results, *Trillium* can be separated into four major lineages (Fig. 1). Two of these have been recognized previously as subgenera, and the other two only as informal groups. The first lineage represented by *T. tschonoskii*, *T. camschatcense*, *T. cernuum*, *T. sulcatum*, and *T. rugelii* corresponds to the informally recognized Erectum group (Gleason, 1906; Barksdale, 1938; Ihara and Ihara, 1978, 1982; Farmer, 2007). The results show that *T. hibbersonii* may be considered to be part of the Erectum group, rather than the Grandiflorum group. Compared to the somewhat similar Grandiflorum group, the Erectum group has generally more separate petals, more open corollas with exposed and prominent ovaries, and stigmas that reach beyond the anthers, or nearly so, at full anthesis, and these features are also found in *T. hibbersonii*. The Erectum group clade was sister to a clade with the three other lineages. One of these, represented by *T. nivale*, *T. ovatum*, and *T. grandiflorum*, forms a clade which is partially consistent with Gleason's (1906) Grandiflorum group (except he also included the disparate *Trillidium* and *Pseudotrillium*). This Grandiflorum group clade was sister to a clade with the last two lineages. *Trillium* subgen. *Sessilium* species formed a clade which was sister to a clade of species belonging to the rarely used *T.* subgen. *Delostylium* (Rafinesque, 1830; Farmer, 2007; Schilling et al., 2017).

A four-lineage concept for *Trillium*, based on the phylogenetic results, would be in contrast to the traditional sessile vs. pedicellate subgeneric circumscription that is now widely adopted. For instance, all of the pedicellate species have been informally lumped into *T.* subgen. *Trillium* (Case, 2003). However, the current phylogeny demonstrated that *T.* subgen. *Trillium* is not monophyletic, but rather a grade, and suggested that the Erectum group and the Grandiflorum group may be recognized separately at the subgeneric rank based on molecular as well as morphological rationale (see also Gleason, 1906; Barksdale, 1938; Ihara and Ihara, 1978, 1982; Farmer, 2007). This paraphyly of *T.* subgen. *Trillium* also suggested that the pedicellate trait is a plesiomorphy.

The results emphasized the distinctiveness of the rare endemic *Trillium hibbersonii* as a species. Although it bears a superficial resemblance to *T. ovatum* and has generally been considered a variety of this species, it was placed in an entirely different clade with members of the Erectum group (Fig. 1). These results make it clear that it is not a variety of *T. ovatum* or even closely related to it and it has been recognized as a distinct species (O'Neill et al., 2020). Additionally, this result notably adds a western North American component to the distribution of the eastern Asian—eastern North American disjunct Erectum group (Fig. 5).

Divergence Timing and Historical Biogeography

In the current study, the divergence dates for Melanthiaceae were estimated within the timeframe of the Paleogene and Neogene periods (also known as Tertiary, 66 – 2.6 mya) (Fig. 2).

It is theorized that favorable climatic conditions and the subsequent spread of temperate forests in the northern hemisphere throughout the Paleogene and Neogene periods helped establish the eastern North American – eastern Asian disjunction patterns, most of which are thought to have occurred in the Miocene epoch (23 – 5.3 mya) and to eventually have left behind isolated plant distributions known as relict Tertiary flora due to global cooling and glaciation (Wen, 1999). An ancient Bering Land Bridge (pre-Pleistocene) between present day Russia and Alaska is the most likely explanation for this pattern of plant disjunction if occurring less than 30 mya, and has been hypothesized to have existed at least during five different times prior to and during the Tertiary period based on indirect evidence of fossils, geology, climate, and biogeography (Tiffney, 1985; Elias et al., 1996; Wen, 1999; Donoghue and Smith, 2004; Milne, 2006). Fossil evidence suggests that during the early Miocene, a time when the continents were otherwise much as they are today, parts of Beringia (the now underwater land between Siberia and Alaska) hosted a temperate deciduous forest and that later, during the Pleistocene epoch, the area may have served as a glacial refugia for some coastal and aquatic plant species due to the ocean-driven weather there (Wolfe, 1994; Elias et al., 1996). These conditions led to a favorable scenario for the continental-scale floristic exchanges which must have occurred and the present disjunction patterns which are characteristic of Melanthiaceae.

The time divergence analysis suggested that Parideae diversified in the Early Miocene epoch (18.84 mya). This result seems realistic as the Miocene was a relatively warm period, though in a cooling trend, in which Asia, North America and the Arctic were free of glaciation, and because it was a time when the Bering Sea Land-bridge (BSL) was available for animal migration and thus plant distribution shifts (Tiffney and Manchester, 2001). The suggested Miocene timeframe would have accommodated the many instances of Asian-North American disjunctions within Parideae, for example, the basal split of western North American *Pseudotrillium* and the successive split between *Paris s.l.*, mainly of Asia, and *Trillium s.l.* of Asia and North America.

Diversification in *Trillium s.s.* likely began during the Late Miocene epoch (12.86 mya), but continued through the Pliocene and Pleistocene epochs. This diversification commenced with the split of the Erectum group from the lineage leading to the other *Trillium* clades. Within the Erectum group, the *T. hibbersonii* lineage diverged first at 9.11 mya, suggesting a radiation centered in western North America. The Asian and the North American Erectum group clades split from each other at 5 mya during the Pliocene. Throughout all of these events, the BSL was still available for plant dispersal. The northern North American species, *T. cernuum*, which today has a distribution spanning an area which was almost entirely covered by the Laurentide ice sheet, split from two southeastern US species, *T. rugelii* + *T. sulcatum* during the early Pleistocene (2.08 mya). The Pleistocene was marked by glaciation, which likely prohibited these southeastern species from becoming more widespread. The *Delostylium* + *Sessilium* clade split from the Grandiflorum group in the Late Miocene epoch (10.91 mya) approximately before the Erectum group diversified. *Delostylium* split from *Sessilium* around 9.64 mya also during the Late Miocene epoch. *Delostylium*, which occurs only in the southeastern U.S. (Fig. 2), diversified as early as the late Miocene (6.32 mya), a time in which the climate might have allowed for more widespread dispersal. *Sessilium*, which has a majority of species in eastern North America, does have a few taxa that occur in western U.S. states (Fig. 6). The study suggested that eastern *Sessilium* species, *T. cuneatum* and *T. decumbens*, split during the Pliocene (4.61 mya), however, the inclusion of a western *Sessilium* species would be necessary to reveal more about the timing of the diversification of this clade. The earliest diverging lineage

of the Grandiflorum group, *T. nivale*, which split in the Late Miocene (8.93 mya), suggests a possible central or eastern North American origin for this group. Whereas the divergence estimation suggested that eastern North American taxon, *T. grandiflorum*, split from western North American taxon, *T. ovatum*, around 6.75 mya, while North America was unglaciated and passable between the east and the west except for the barrier imposed by the arid Rocky Mountain cordillera region. Documented plant species disjunctions between eastern North America and western North America such as are seen in the Grandiflorum group and *Sessilium* clades are not uncommon, though not as common as are the instances of eastern Asia – eastern North America disjunctions (Xiang et al., 1998; Donoghue and Smith, 2004). Furthermore, it has been suggested that a mesic corridor likely existed to the north of the Rocky Mountains across Canada through the Tertiary period which could have allowed the transcontinental pattern seen in *Trillium* (Tiffney and Manchester, 2001).

Some early lineage splits within Melanthiaceae were inferred to have occurred prior to the Miocene, the epoch in which most eastern Asia—eastern North America disjunctions are thought to have occurred (Wen, 1999). For example, Melanthiaceae diverged from the lineage leading to the remaining Melanthiaceae tribes during the Paleocene (59.04 mya), a time when the earth had a tropical climate and was recovering from a major post-Cretaceous extinction event. Chionographidae + Heloniadae diverged from Xerophylleae + Parideae (48.15 mya), Chionographidae split from Heloniadae (35.06 mya), and Xerophylleae split from Parideae (36.89 mya) all during the warm Eocene epoch, which was overall a time of radiation for plants, though in a cooling trend toward the start of the Oligocene (Wolfe, 1985; Wing et al., 2005; Utescher and Mosbrugger, 2007). Each Melanthiaceae tribe, except for Xerophylleae, displays the eastern North American—eastern Asian disjunction pattern among its species.

The current study suggested that the diversification of *Paris s.l.* began in the Miocene (14.6 mya). Subsequent to the split of *Paris s.s.*, *Kinugasa* split from *Daiswa* in the same epoch (9.92 mya). *Daiswa thibetica* diverged from a clade of remaining *Daiswa* species at 5.67 mya, during the late Miocene, but the crown age for the clade with the majority of *Daiswa* species was during the cooling Pliocene (3.27 mya). The lineage continued to diversify through the Pleistocene (2.58 mya to 11.7 kya), a time marked by advancing glaciation and interglacial cycles. This is in contrast to Kim et al. (2019) study which showed an earlier divergence for *D. thibetica* [*P. thibetica*] at approximately 8 mya and the crown age for the remaining clade of species at approximately 6.5 mya, both in the Miocene. Yang et al. (2019) suggested a slightly later Pliocene crown age (4.52 mya) for *Daiswa* (as *Paris*) compared with this study, but the former study did not include *D. thibetica*.

The time divergence estimations of the current study were more similar to the estimates of past studies which included key taxa than those that did not, regardless of starting stem age for Melanthiaceae and regardless of amount of data on which the phylogenetic inference was based (see Table 5). Givnish et al. (2016) presented a phylogeny with divergence time estimations based on 75 plastid coding genes, had a stem age for Melanthiaceae of 104 mya, and included *Pseudotrillium* and *Trillidium*. Givnish et al. (2016) estimated that the split between *Trillium s.l.* and *Paris* occurred ca. 17.3 mya, and that the crown ages of these genera were approximately 13.6 and 13.7 mya, respectively. Though the current study, which used 70 plastid coding genes, started with a much later Melanthiaceae stem age prior of 84.19 mya, it yielded similar results for the *Trillium s.l.* and *Paris s.l.* divergence at 16.36 mya, with crown ages around 14.6 mya for each. Kim et al. (2019) started with a Melanthiaceae stem age close to the one inferred in Givnish et al. (2016) (101mya) and inferred an earlier *Paris/Trillium* split at 22.5 mya and

slightly later crown ages for *Paris s.l.* (~12 mya) and for *Trillium s.l.* (~12 mya) compared with the current study. The phylogenetic inference of Kim et al. (2019) was based on a much smaller four plastid marker dataset and had some possible misidentifications in *Trillium* samples, however it used samples from key lineages: *Pseudotrillium*, *Kinugasa*, and *Trillidium*. Yang et al. (2019) used the same Melanthiaceae stem age as Givnish et al. (2016) to calibrate their analysis, however they inferred a much earlier *Paris s.l./Trillium s.l.* split of 38.21 mya and a likewise earlier *Paris s.l.* crown age of 33.71 mya. Although, Yang et al. (2019) was based on a larger whole plastome dataset and sampled from *Kinugasa*, *Pseudotrillium* and *Trillidium* were omitted. The current study showed a *Pseudotrillium* divergence (or Parideae crown age) of 18.84 mya compared with 23 – 31.2 mya in studies which included this taxon (Givnish et al., 2016; Kim et al., 2019). The current study showed a more recent crown age for *Trillium s.s.* (14.59 mya), similar to Givnish et al. (2016) (13.6 mya), compared with 19 – 26 mya in other studies (Kim et al., 2019; Ji et al., 2019; Yang et al., 2019). Moreover, incongruencies in the phylogenies themselves due to taxon omission mean some divergence estimations cannot be compared. For example, the phylogenetic topologies from the divergence time estimation studies of Kim et al. (2019), Ji et al. (2019) and Yang et al. (2019) were not totally congruent with the current study or with each other whereas Kim et al. (2019) and the current study were in general agreement with the topology inferred by Givnish et al. (2016), however, Yang et al. (2019) and Ji et al. (2019) were not. These results illustrate the importance of taxon inclusion and the possibility that unavoidable omission due to taxon extinctions of the past could lead to multiple incorrect conclusions in both phylogenetic inference and time divergence analyses.

The BioGeoBEARS range reconstruction analysis primarily suggested an eastern North American origin for most Melanthiaceae lineages. The inferred location of the Melanthiaceae origin was a combined area of eastern North America + eastern Asia, which may suggest either uncertainty in the analysis, or a widespread distribution when family diversification began during the Paleocene. This result differed from Givnish et al. (2016) in which the origin of Melanthiaceae is in eastern North America alone. The current analysis suggested that the Melanthiaceae lineage likely originated in the eastern Asian region, though it only included two members of *Veratrum*. By contrast, Kim et al. (2019), which had a more representative sampling of Melanthiaceae genera, inferred a North American origin for Melanthiaceae, albeit with an eastern Asian origin for *Veratrum* in agreement with the current study. The lineages of Chionographidae and Heloniadae likely originated in eastern North America during the late Eocene, though the inferred diversification area for each was a combined region of eastern Asia and eastern North America during the Miocene. The Xerophylleae lineage most likely originated in eastern North America, but was suggested to have become widespread throughout western North America + eastern North America before its two species split from each other. Likewise, the inferred origin of the Parideae lineage was eastern North America, but the lineage may have then spread across three regions (eastern Asia, eastern and western North America) during the Miocene by the time its diversification began. The analysis suggested that the *Pseudotrillium* lineage split off within the same region it occupies today, western North America. The overall results contradicted the possibility that Parideae originated in western North America which is suggested by the location of the basal lineage, *Pseudotrillium* (Farmer, 2006) and by Givnish et al. (2016) which suggests that the Parideae ancestor originated in western North America. The inferred origin of *Paris* + *Daiswa* + *Kinugasa* (*Paris s.l.*) was eastern Asia, in agreement with Ji et al. (2019), which concludes that *Paris s.l.* likely originated in northeastern Asia and northern China. The inferred origin of *Trillium* + *Trillidium* (*Trillium s.l.*) was in eastern North America, and subsequently,

the MRCA of *Trillidium* likely inhabited a combined range of eastern Asia + eastern North America during the Miocene. The current study suggested that the *Paris s.l.* + *Trillium s.l.* clade originated in a combined region of eastern Asia + eastern North America in disagreement with Givnish et al. (2016) which suggests the clade has eastern Asian origins.

The biogeographic reconstruction helped elucidate range origins and movements within *Trillium*. An eastern North American origin was inferred for *Trillium s.s.* as well as all four of the major *Trillium* lineages with subsequent expansion to western North America and eastern Asia. The Erectum Group lineage likely dispersed from eastern North America into a widespread distribution of eastern North America + western North America. It should be noted that if *Trillium hibernsonii* had been omitted from the study, the results might have suggested an origin from a combined region of eastern Asia + eastern North America instead, as was suggested for the clade of remaining Erectum Group species. The inferred origin for the MRCA of Asian Erectum group species was in eastern Asia + Irano-Turanian region. The MRCA of the remaining North American *Trillium* species, the *T.* subgen. *Sessilium* + *T.* subgen. *Delostylium* + Grandiflorum Group clade, was inferred to have originated in eastern North America. Within the Grandiflorum group, *T. ovatum* and *T. grandiflorum* may have later become more widespread across eastern and western North America after splitting from *T. nivale*, though the Rocky Mountain region would have been a barrier, as well as for *T.* subgen. *Sessilium* in a westward expansion.

Finally, the current analysis added more examples to the body of knowledge regarding two biogeographic hypotheses: (1) that most eastern North American—eastern Asian disjunctions occurred during the Miocene, and (2) that climate-driven diversification readily occurred during the Pleistocene. According to Wen (1999), most well-studied eastern North American—eastern Asian disjunctions occurred predominantly during the Miocene, with a full range of 2 – 25 mya stretching from the late Oligocene through the Pliocene. All eastern North American—eastern Asian disjunctions in the current analysis occurred during this timeframe. This included the splits between *Chionographis* and *Chamaelirium*, *Helonias* and *Heloniopsis* + *Ypsilandra*, *Trillidium govanianum* and *Trillidium undulatum*, and Asian and North American Erectum group species (Pliocene). Likewise, the splits which occurred from an inferred combined region of eastern North America + eastern Asia also occurred during the Miocene: *Paris s.l.* and *Trillium s.l.*, and *Trillidium* and *Trillium s.s.*

The second hypothesis invokes the Tertiary–Quaternary debate which explores Quaternary climatic vs. Tertiary geographic influences on biodiversity and speciation (Rull, 2008). Others settle for a compromise between the two arguments as some study systems have displayed continuous diversification over time rather than an accelerated diversification in the Pleistocene (Zecca et al., 2012). While the current analysis suggested that Melanthiaceae displayed a steady diversification pattern starting in the Eocene, the range reconstruction for Parideae makes a case for Pleistocene climate-driven diversification. This could be seen in the reconstruction for *Daiswa* which began diversifying in the Pliocene but had the greatest number of splits during the Pleistocene. This relatively recent diversification of *Daiswa* during the Pleistocene, a genus restricted to Asia, might have hampered its dispersal elsewhere due to Asian continental glaciation at the time. Ji et al. (2019) suggests that monsoonal weather, glaciation, and geologic changes from the Miocene through the Pleistocene drove clade diversification and range shifts in *Paris s.l.* Similarly, the Erectum group in *Trillium* displayed a relatively high number of splits during the Pleistocene, both in Asia and in eastern North America. It is possible hybridization may have also played a role in the recent increase in speciation of these two

groups. Diversification of Grandiflorum group and *T.* subgen. *Delostylium*, however, likely occurred in the hospitable Miocene, while *T.* subgen. *Sessilium* likely began diversification in the cooling Pliocene. Future studies including more samples from *T.* subgen. *Sessilium*, the most species-rich clade within *Trillium*, as well as more samples of *Paris s.l.*, would provide further data to help answer this question for the tribe.

TAXONOMIC TREATMENT

To solidify our understanding of the well supported infrageneric *Trillium* clades, and to connect the inferences made from molecular-based analyses with traditional species morphology, it seems appropriate to update the nomenclature of *Trillium* by defining one new subgenus and by redefining the paraphyletic, autonymic subgenus *Trillium*. Having an infrageneric level classification makes sense for a genus so obviously marked by differences in geography and basic morphology. A classification which reflects true evolutionary relationships could be significant for apprehending causes behind observed phenomena in other evolutionary or ecological studies of *Trillium*, e.g. seed dispersal studies such as in Miller et al. (2021). The following taxonomic treatment and dichotomous key, which characterizes four subgenera and the unifying features of each, may be useful to other researchers in *Trillium*.

Key to the Subgenera of *Trillium*

- 1 Flowers usually pedicellate; leaf color solid, usually not mottled.....2
- 1 Flowers sessile; leaf color usually mottled.....4. ***T.* subgen. *Sessilium***
- 2 Stigmas uniformly thin, united in a common style or at least basally connate; ovary at anthesis small or inconspicuous, 3- or 6-angled, typically colored white, greenish-white, or green; petals typically delicate, undulate, basally overlapping, pink or white aging to various pink shades, or remaining white (*T. nivale*); adaxial petal venation not engraved; flower funnelform or at least with basally erect petals shallowly encircling ovary; leaf shape ovate, elliptic-ovate, ovate-rhombic, ovate-lanceolate, or linear.....3
- 2 Stigmas typically fleshy, thicker basally and tapered, separate; ovary at anthesis prominent, exposed, exerted or only slightly obscured, typically strongly 6-angled or ridged, generally in a range of dark reddish hues, creamy white, or white with markings; petals typically coarse or thick-textured, margins entire, separate or only slightly overlapping, if white in color, aging to brown; adaxial petal venation typically engraved on surface; flower ringent, open, or widely agape, not funnelform; leaf shape rhombic, broadly elliptic, rhombic-ovate, or lanceolate-elliptic (*T. hibbersonii*)1. ***T.* subgen. *Trillium***
- 3 Stigmas united in a short or long style; leaf shape elliptic-ovate, ovate-lanceolate, oblong or linear..... 3. ***T.* subgen. *Delostylium***
- 3 Stigmas basally connate; leaf shape ovate, elliptic-ovate, or ovate-rhombic.....2. ***T.* subgen. *Callipetalon***

Taxonomic Synopsis

Trillium Linnaeus (1753), nom. cons. Type:—*Trillium cernuum* L. 49 species.

1. ***Trillium*** L. subgen. ***Trillium***. (autonym) Type:—*Trillium cernuum* L. 15 species.

Synonym: *Trillium* subgen. *Anthopium* Raf. (1830). Type:—*Trillium acuminatum* Raf. [= *T. erectum* L.].

Species:—*Trillium apetalon* Makino; *T. camschatcense* Ker Gawl.; *T. cernuum* L.; *T. channellii* I. Fukuda, J.D. Freeman & Itou; *T. erectum* L.; *T. hague* Miyabe & Tatewaki; *T. hibernsonii* (T.M.C.Taylor & Szczaw.) D.O'Neill & S.B.Farmer; *T. flexipes* Raf.; *T. rugelii* Rendle; *T. simile* Gleason; *T. smallii* Maxim.; *T. sulcatum* T.S. Patrick; *T. taiwanense* S.S. Ying; *T. tschonoskii* Maxim.; *T. vaseyi* Harb.

Geographic distribution:—North America and Asia. Seven species inhabit eastern and north central US into southeastern Canada (see Fig. 5). Inclusion of an eighth species in this group, *T. hibernsonii*, adds a disjunct western component to the North American distribution. At least seven species inhabit east Asia, especially Japan, eastern China and eastern Russia, though the precise number is debatable. *Trillium tschonoskii* and *T. camschatcense* are the most widespread of Asian species, however *T. tschonoskii* reaches the furthest west stretching even to the Himalayan Mountains, at elevations up to 3200 m.

Phenology:—Spring flowering, some early summer. April to May (*Trillium rugelii*, *T. simile*, *T. sulcatum*, *T. smallii*); April to June (*T. vaseyi*, *T. apetalon*, *T. erectum*, *T. flexipes*, *T. tschonoskii*); May to June (*T. hague*); May to July (*T. camschatcense*); June (*T. taiwanense*); late May (*T. channellii*). Time periods vary depending on latitude and elevation.

Habitat:—Typically inhabit rich mesic forests, deciduous or mixed, or coves, often on slopes, sometimes near riverbanks. Some species may inhabit somewhat acidic soils in *Rhododendron* thickets (*T. erectum*, *T. simile*, *T. vaseyi*). *Trillium hibernsonii* grows on rocky sea cliffs in scant, acidic soils. *Trillium channellii* is exceptional for inhabiting volcanic plains near lakeside areas.

Endemism:—Most of the Asian species may be considered endemics as well as a few of the North American species. These include *Trillium apetalon*, *T. channellii*, *T. hague*, *T. hibernsonii*, *T. simile*, *T. smallii*, *T. taiwanense*, and *T. vaseyi*.

Morphological trends:—Flowers pedicellate; leaves rhombic, rhombic-ovate, broadly elliptic or orbicular, and coloration unmottled; petals usually separate (not overlapping as in *T.* subgen. *Callipetalon*) and coarse in texture, margins entire, not typically undulate, if white, not aging to pink (except in *T. hibernsonii*), but rather brown; venation on adaxial petal surface engraved; prominent ovary deeply 6-angled; 3 separate, fleshy, stigmas, most basally thickened (without prominent style as in subgen. *Delostylium*). Flower open, agape or ringent, not funnellform, leaving ovary exposed.

Taxonomic issues:—The autonym is used because the type specimen for *Trillium*, *T. cernuum*, is included in this clade. The name *Trillium* subgen. *Anthopium* Raf. (1830) was treated as a synonym of *T.* subgen. *Trillium* by Freeman (1975) and was also avoided here because this subgenus as described by Rafinesque includes a mixture of pedicellate species from multiple groups (e.g. *T. acuminatum* Raf. (= *T. erectum*), *T. ovatum*, *T. pusillum* and *T. pictum* Pursh (= *Trillidium undulatum*), as well as other synonyms and unresolved names.)

The seven eastern North American species (excluding *T. hibernianum*) have been considered by some to be a species complex (see Millam, 2006; Stoeckel, 2010). Hybrids are common in this subgenus. Asian *Trillium* species are all polyploid except for diploid *T. camschatcense*. *Trillium hagei*, the fertile hexaploid, not the *T. x hagei* sterile triploid, is included here as in the Case and Case (1997) and Samejima and Samejima (1962 and 1987) treatments.

2. ***Trillium*** L. subgen. ***Callipetalon*** Lampl. & E.E. Schill., subgen. nov. Type:—*Trillium grandiflorum* (Michx.) Salisb. 3 species.

Species:— *Trillium grandiflorum* (Michx.) Salisb.; *T. nivale* Riddell; *T. ovatum* Pursh.

Etymology: *Callipetalon*, Greek *calli-* (beautiful) and *petalon* (petal), conveys the nature of the famously beautiful flowers with snow-white petals found in this *Trillium* clade.

Geographic distribution:—North America. Two species inhabit the eastern US: *T. nivale* which stretches from the mid-Atlantic states westward to the central Great Plains states and *T. grandiflorum* which overlaps with much of *T. nivale*'s distribution, stretching from Minnesota to Nova Scotia southward to northern Alabama (see Fig. 7). *T. ovatum* inhabits western US states northward to southern Canada.

Phenology:—Late winter to spring flowering: early March to June depending on latitude and elevation. Late April to June (*T. cernuum*); Late April to early June (*T. grandiflorum*); Early March to Early April (*T. nivale*, arriving amidst late winter snows in northern states).

Habitat:—In rich deciduous or mixed woods, fields, or floodplains (*T. grandiflorum*); limestone substrate, rocky areas or floodplain with gravel-sand soils (*T. nivale*); coniferous and mixed forests or along mountain streams (*T. ovatum*).

Endemism:—The three species are widespread, however certain infraspecific taxa are not (*T. ovatum* var. *oettingeri* occurs in the Salmon and Siskiyou Mts., *T. ovatum* fo. *maculosum* occurs in one county in California). *Trillium crassifolium* occurs in one county in Washington.

Morphological trends:—Petals overlapping or somewhat so, pink or more often white aging to various shades of pink; flower slightly to obviously funnelform, obscuring or partially concealing ovary; stigmas, 3, slender; anthers longer than filaments; leaves ovate, elliptic-ovate, or ovate-rhombic.

Taxonomic issues:—The members of this subgenus were all included in Gleason's (1906) "*T. grandiflorum* type" morphological group, though he also included in it *Trillidium* and *Pseudotrillium*. *Trillium crassifolium* Piper, *T. scouleri* Rydb. ex Gleason, *T. ovatum* var. *oettingeri*, *T. ovatum* var. *stenosepalum*, and *T. ovatum* forma *maculosum* are widely considered to be synonyms of *T. ovatum* (Case and Case, 1997; Meredith et al., 2020; NatureServe, 2021).

3. ***Trillium*** L. subgen. ***Delostylium*** Raf. (1830). Type:—*Trillium stylosum* Nuttall (= *T. catesbaei* Elliott) 5 species.

Synonym: *Delostylis* Raf. (1819). Type:—*Trillium stylosum* Nuttall.
Trillium subgen. *Delostylis* Raf. ex Farmer (2007). Type:— *T. catesbaei* Ell.

Species:—*Trillium catesbaei* Elliott; *T. georgianum* S.B. Farmer; *T. persistens* W.H. Duncan; *T. pusillum* Michx.; *T. texanum* Buckley.

Geographic distribution:—Southeastern and south central US, from Maryland westward to Texas (see Fig. 8).

Phenology:—Late winter—spring flowering, February—June depending on latitude and elevation. Late February—early March (*T. texanum*); early March—mid-April (*T. persistens*); March—early June (*T. pusillum*); late March—early April (*T. georgianum*); late March—June (*T. catesbaei*).

Habitat:—Acidic soils and rhododendron thickets, mixed woodland or mountain coves, flats along small streams (*T. catesbaei* and *T. persistens*). Low swampy woods, floodplain swamps, acidic soil (*T. pusillum* and *T. texanum*). Flatwoods over seasonally wet calcareous substrate (*T. georgianum*).

Endemism:—Federally listed as endangered, *Trillium persistens* is very rare, inhabiting only four counties in northern Georgia and South Carolina. Recently described *T. georgianum* is known from one county in Georgia.

Morphological trends:—Stigmas, 3, uniformly thin, united in a common style; petals somewhat thin-textured, margins undulate, venation not engraved, pink, or white aging to various pink shades; leaf shape trends toward more narrow (blade longer than wide; elliptic-ovate, ovate-lanceolate, oblong, or linear) than those of species from the other pedicellate-flowered subgenera; ovary 6-angled.

Taxonomic issues:—*Trillium texanum* and *T. georgianum* were formerly considered by some as varieties of *T. pusillum*. Farmer (2007) proposed seven species in addition to these be elevated from widely recognized varieties of the *Trillium pusillum* complex (*T. ozarkanum*, *T. virginianum*, *T. monticola*, *T. alabamicum*, *T. palustris*, *T. carolinianum*, and *T. telmacola*). *Trillium virginianum* is probably the most notable of these, being the only taxon of the pedicellate clades with sessile to subsessile flowers.

4. ***Trillium*** L. subgen. ***Sessilium*** Raf. (1830). Type:—*Trillium sessile* L. 26 species.

Synonyms: *Esdra* Salisb. (1866). Type:—*Trillium sessile* L.
Phyllantherum Raf. ex Nieuwl. (1913). Type:— *Phyllantherum sessile* (L.) Nieuwl. (= *Trillium sessile* L.)
Trillium subgen. *Phyllantherum* Raf. (1820). Lectotype:—*Trillium sessile* L. designated by Freeman (1975).
Trillium [unranked] *Phyllantherum* (Raf.) Schult. & Schult.f. (1830). Type:—*Trillium sessile* L.

Species:—*Trillium albidum* J.D. Freeman; *T. angustipetalum* (Torr.) J.D. Freeman; *T. chloropetalum* (Torr.) Howell; *T. cuneatum* Raf.; *T. decipiens* J.D. Freeman; *T. decumbens* Harb.; *T. delicatum* Floden & E.E. Schill.; *T. discolor* T. Wray ex Hook.; *T. foetidissimum* J.D. Freeman; *T. gracile* J.D. Freeman; *T. kurabayashii* J.D. Freeman; *T. lancifolium* Raf.; *T. ludovicianum* Harb.; *T. luteum* (Muhl.) Harb.; *T. maculatum* Raf.; *T. oostingii* Gaddy; *T. parviflorum* V.G. Soukup; *T. petiolatum* Pursh; *T. recurvatum* L.C. Beck; *T. reliquum* J.D. Freeman; *T. sessile* L.; *T. stamineum* Harb.; *T. tennesseense* E.E. Schill. & Floden; *T. underwoodii* Small; *T. viride* L.C. Beck; *T. viridescens* Nutt.

Geographic distribution:—This group has a disjunct distribution among eastern and western US states (see Fig. 6).

Phenology:—Late winter to spring flowering; primarily from March to April, though some species may flower as early as late January (*T. decipiens*) or February (*T. chloropetalum*, *T. foetidissimum*, *T. lancifolium*, *T. maculatum*, *T. underwoodii*) or as late as May (*T. discolor*, *T. kurabayashii*, *T. luteum*, *T. parviflorum*, *T. petiolatum*, *T. recurvatum*, *T. sessile*, *T. stamineum*, *T. tennesseense*, *T. viride*, *T. viridescens*) depending on latitude and elevation.

Habitat:—Variable habitats are encompassed by this group, but most species generally thrive in sloping mixed woodlands with mesic, limestone soils. Many may be found in wooded intermittent floodplain habitats.

Endemism:—Several species in *Trillium* subgen. *Sessilium* have restricted ranges: *Trillium delicatum*; *T. discolor*; *T. tennesseense*; *T. kurabayashii*; *T. oostingii*; *T. parviflorum*. Still others in this group have somewhat wider distributions, but may be restricted to a single river watershed. *Trillium lancifolium* is rare and endangered in its wide but scattered distribution. *Trillium reliquum* is a federally listed species, however it is abundant enough to be classified NT (not threatened) and stable by the IUCN Red List.

Morphological trends:—Sessile flowers; leaf color variously mottled; petals mostly erect, thick-textured, margins entire; ovary 3- or 6-angled or winged; stigmas, 3, separate, linear or subulate.

Taxonomic issues:—Freeman refined our understanding of *Trillium* subgen. *Sessilium* (as *T.* subgen. *Phyllantherum*) in his significant taxonomic revisions, describing or elevating in rank 7 species and naming 8 color forms (Freeman 1969; Freeman 1975; Freeman and Heineke 1987). He conceptualized three species groups within the subgenus based on morphology. Subsequently, Murray (1983, 1984) devised a lengthy classification scheme of the subgenus complete with a host of sections, subsections, series, and subseries names. *Trillium cuneatum* and *T. lancifolium* appear to be species complexes and are under study by the present author.

References

- Adams J.M. 1997. Global land environments since the last interglacial. Oak Ridge National Laboratory, TN, USA. <http://www.esd.ornl.gov/ern/qen/nerc.html>. <accessed 28 November 2016>.
- APG (Angiosperm Phylogeny Group). 1998. An ordinal classification for the families of flowering plants. *Annals of the Missouri Botanical Garden*. 85: 531–553.
- Barksdale, L. 1938. The pedicellate species of *Trillium* found in the southern Appalachians. *Journal of the Elisha Mitchell Scientific Society*. 54(2): 271-296.
- Baurain, D., Brinkmann, H., and H. Philippe. 2007. Lack of Resolution in the Animal Phylogeny: Closely Spaced Cladogeneses or Undetected Systematic Errors? *Molecular Biology and Evolution*. 24(1): 6–9.
- Bouckaert, R.R., Heled, J., Kuehnert, D., Vaughan, T.G., Wu, C.-H., Xie, D., Suchard, M.A., Rambaut, A., and A.J. Drummond. 2014. BEAST 2: A software platform for Bayesian evolutionary analysis. *PLoS Computational Biology*. 10(4): e1003537.
- Calflora: Information on California plants for education, research and conservation, with data contributed by public and private institutions and individuals, including the Consortium of California Herbaria. [web application]. 2021. Berkeley, California: The Calflora Database [a non-profit organization]. Available: <https://www.calflora.org/> (Accessed: 02/28/2021).
- Case Jr., F.W., 2003. *Trillium*. In: Flora of North America Editorial Committee (eds.). Flora of North America North of Mexico, Vol. 26. Oxford: Oxford University Press.
- Case Jr., F.W., and R.B. Case. 1997. Trilliums. Portland, OR: Timber Press.
- Chionographis* Maxim. in GBIF Secretariat. 2019. GBIF Backbone Taxonomy. Checklist dataset <https://doi.org/10.15468/39omei>. (Accessed via GBIF.org on 10 September, 2019).
- Darriba D, Taboada GL, Doallo R, Posada D. 2012. jModelTest 2: more models, new heuristics and parallel computing. *Nature Methods*. 9(8): 772.
- Dong, W., Xu, C., Li, C. Sun, J., Zuo, Y., Shi, S., Cheng, T., Guo, J., and S. Zhou. 2015. *Ycf1*, the most promising plastid DNA barcode of land plants. *Scientific Reports*. 5: 8348.
- Donoghue, M.J. and S.A. Smith. 2004. Patterns in the assembly of temperate forests around the Northern Hemisphere. *Philosophical Transactions of the Royal Society Biological Sciences*. 359(1450): 1633–1644.
- Douglas, G.W. and J. Pojar. 2001. *Trillium ovatum* Pursh variety *hibbersonii* (Taylor et Szczawinski) Douglas et Pojar, variety nova. *The Canadian Field-Naturalist*. 115: 343.
- Drummond A.J., Suchard M.A., Xie D. & A. Rambaut. 2012. Bayesian phylogenetics with BEAUti and the BEAST 1.7. *Molecular Biology and Evolution*. 29: 1969-1973.
- eFloras. 2019. Published on the Internet <http://www.efloras.org> (Accessed 10 September 2019). Missouri Botanical Garden, St. Louis, MO & Harvard University Herbaria, Cambridge, MA.
- Elias, S. A., Short, S. K., Nelson, C.H., and H.H. Birks. 1996. Life and times of the Bering land bridge. *Nature*. 382(6586): 60-63.
- Farmer, S.B. 2006. Phylogenetic analyses and biogeography of Trilliaceae. *Aliso*. 22: 579-592.
- Farmer, S.B. 2007. A Systematic Study of *Trillium* subgenus *Delostylis*. Ph.D. Diss. University of Tennessee, Knoxville.
- Farmer, S., and E. Schilling. 2002. Phylogenetic Analyses of Trilliaceae Based on Morphological and Molecular Data. *Systematic Botany*. 27(4): 674-692.

- Freeman, J.D. 1969. A revisionary study of sessile-flowered *Trillium* L. (Liliaceae). Ph.D. Diss. Vanderbilt University, Nashville, TN.
- Freeman, J.D. 1975. Revision of *Trillium* subgenus *Phyllantherum* (Liliaceae). *Brittonia*. 27: 1-62.
- Freeman, J.D. and T.E. Heineke. 1987. *Trillium recurvatum* Beck forma *esepalum* f. nov., a new sepalless form of the prairie wakerobin. *Rhodora*. 89(857): 17-20.
- Fukuda, I. 2001. The origin and evolution in *Trillium*. 2. Chromosome variation of *Trillium undulatum* in North America. *Cytologia*. 66: 319-327.
- Fuse, S. and M.N. Tamura. 2016. Biosystematic studies on the genus *Heloniopsis* (Melanthiaceae) I. Phylogeny inferred from plastid DNA sequences and taxonomic implications. *Nordic Journal of Botany*. 34: 584-595.
- Givnish, T.J., Zuluaga, A., Marques, I., et al. 2016. Phylogenomics and historical biogeography of the monocot order Liliales: out of Australia and through Antarctica. *Cladistics*. 32 (6): 1-25.
- Gleason, H.A. 1906. The pedunculate species of *Trillium*. *Bulletin of the Torrey Botanical Club*. 33(7): 387-396.
- Graybeal, A. 1998. Is it better to add taxa or characters to a difficult phylogenetic problem? *Systematic Biology*. 47(1): 9-17.
- Guindon, S. and O. Gascuel. 2003. A simple, fast and accurate method to estimate large phylogenies by maximum-likelihood. *Systematic Biology*. 52: 696-704.
- Heath, T.A., Zwickl, D.J., Kim, J., and D.M. Hillis. 2008. Taxon sampling affects inferences of macroevolutionary processes from phylogenetic trees. *Systematic Biology*. 57(1): 160-166.
- Hedtke, S.M., Townsend, T.M. and D.M. Hillis. 2006. Resolution of Phylogenetic Conflict in Large Data Sets by Increased Taxon Sampling. *Systematic Biology*. 55(3): 522-529.
- Hewitt, G. 2000. The genetic legacy of the Quaternary ice ages. *Nature*. 405 (6789): 907-913.
- Hillis, D.M. 1998. Taxonomic sampling, phylogenetic accuracy, and investigator bias. *Systematic Biology*. 47: 3-8.
- Huang, Y., Xiaojuan, L., Yang, Z., Yang, C., Yang, J., and Y. Ji. 2016. Analysis of complete chloroplast genome sequences improves phylogenetic resolution in *Paris* (Melanthiaceae). *Frontiers in Plant Science*. 7: 1797.
- Ihara, K. and M. Ihara. 1982. A biosystematic study on the pedicellate-flowered species of North American *Trillium* (2) An interspecific relationship shown by karyotypes and mode of microsporogenesis. *Journal of Phytogeography and Taxonomy*. 30: 74-82.
- Ihara, M. and K. Ihara. 1978. A biosystematic study of the pedicellate-flowered species of North American *Trillium* (1) Geographical distribution of major groups and their gynoecium norms. *Journal of Geobotany*. 25: 139-172.
- Iles, W.J.D., Smith, S.Y., Gandolfo, M.A., and S.W. Graham. 2015. Monocot fossils suitable for molecular dating analyses. *Botanical Journal of the Linnean Society*. 178: 346-374.
- Janssen, T. and K. Bremer. 2004. The age of major monocot groups inferred from 800+ *rbcL* sequences. *Botanical Journal of the Linnean Society*. 146(4): 385-398.
- Ji, Y., Fritsch, P.W., Li, H., Xiao, T. and Z. Zhou. 2006. Phylogeny and Classification of *Paris* (Melanthiaceae) Inferred from DNA Sequence Data. *Annals of Botany*. 98: 245-256.
- Ji, Y., Yang, L., Chase, M.W., Liu, C., Yang, Z., Yang, J., Yang, J.-B. and T.-S. Yi. 2019. Plastome phylogenomics, biogeography, and clade diversification of *Paris* (Melanthiaceae). *BMC Plant Biology*. 19: 543.

- Kartesz, J.T., The Biota of North America Program (BONAP). 2015. Taxonomic Data Center. Chapel Hill, N.C. [maps generated from Kartesz, J.T. 2015. Floristic Synthesis of North America, Version 1.0. Biota of North America Program (BONAP)]. Available at <http://www.bonap.net/tdc>.
- Kato, H., Kawano, S., Terauchi, R., Ohara, M., F.H. Utech. 1995a. Evolutionary Biology of *Trillium* and Related Genera (Trilliaceae) I. Restriction Site Mapping and Variation of Chloroplast DNA and its Systematic Implications. *Plant Species Biology*. 10: 17-29.
- Kato, H., Terauchi, R., Utech, F.H., and S. Kawano. 1995b. Molecular systematics of the Trilliaceae *sensu lato* as inferred from *rbcL* sequence data. *Molecular Phylogenetics and Evolution*. 4(2): 184-193.
- Katoh, K. and D.M. Standley. 2013. MAFFT Multiple Sequence Alignment Software Version 7: Improvements in Performance and Usability. *Molecular Biology and Evolution*. 30(4): 772–780.
- Kim, C., Kim, S.-C., and J.-H. Kim. 2019. Historical biogeography of Melanthiaceae: A case of out-of-North America through the Bering Land Bridge. *Frontiers in Plant Science*. 10: 396.
- Kim, S.-C., Kim, J.S. and J.-H. Kim. 2016a. Insight into infrageneric circumscription through complete chloroplast genome sequences of two *Trillium* species. *Annals of Botany Plants*. 8: plw015.
- Kim, S.-C., Kim, J.S., Chase, M.W., Fay, M.F., and J.-H. Kim. 2016b. Molecular phylogenetic relationships of Melanthiaceae (Liliales) based on plastid DNA sequences. *Botanical Journal of the Linnean Society*. 181(4): 567-584.
- Ling, L.-Z., and S.-D. Zhang. 2020. The complete chloroplast genome and phylogenetic analysis of *Paris delavayi* (Melanthiaceae). *Mitochondrial DNA Part B*. 5(3): 2126-2128.
- Lomolino, M.V., Riddle, B.R., and R.J. Whittaker. 2017. Biogeography: Biological Diversity across Space and Time, 5th ed. Sunderland, MA: Sinauer Associates.
- Magallón, S., Gómez-Acevedo, S., Sánchez-Reyes, L.L., and T. Hernández-Hernández. 2015. A metacalibrated time-tree documents the early rise of flowering plant phylogenetic diversity. *New Phytologist*. 207(2): 437-453.
- Matzke, N.J. 2018. BioGeoBEARS: BioGeography with Bayesian (and likelihood) Evolutionary Analysis with R Scripts. version 1.1.1, published on GitHub on November 6, 2018. DOI: <http://dx.doi.org/10.5281/zenodo.1478250>
- Meades, S.J., Schnare, D., Lawrence, K. and C. Faulkner. 2019. Northern Ontario Plant Database Website. Version 1, January 2004. Algoma University College and Great Lakes Forestry Centre, Sault Ste. Marie, Ontario, Canada. Available at www.northernontarioflora.ca/.
- Melton, A.E., Chen, S., Zhao, Y., Fu, C., Xiang, Q.-Y., Cheng, S., Wong, G. K.-S., Soltis, P.S., Soltis, D.E., and M.A. Gitzendanner. 2020. Genetic insights into the evolution of genera with the eastern Asia–eastern North America floristic disjunction: a transcriptomics analysis. *American Journal of Botany*. 107(11): 736-1748.
- Meng, R., Meng, Y., Yang, Y.-P., and Z.-L. Nie. 2021. Phylogeny and biogeography of *Maianthemum* (Asparagaceae: Nolinoideae) revisited with emphasis on its divergence pattern in SW China. *Plant Diversity*. 43(2): 93-101.
- Mennes, C.B., Lam, V.K., Rudall, P.J., Lyon, S.P., Graham, S.W., Smets, E.F., and V.S.F.T. Merckx. 2015. Ancient Gondwana break-up explains the distribution of the mycoheterotrophic family Corsiaceae (Liliales). *Journal of Biogeography*. 42: 1123–1136.

- Meredith, C.R., Schilling, E. & *Trillium* Working Group 2019. 2020. *Trillium ovatum*. The IUCN Red List of Threatened Species 2020: e.T116996352A135833545. <https://dx.doi.org/10.2305/IUCN.UK.2020-2.RLTS.T116996352A135833545.en>. (Accessed 27 February 2021).
- Millam, K.C. 2006. The *Trillium erectum* species-complex (Melanthiaceae): Insights from molecular systematics and biogeography. Ph.D. Diss. University of Wisconsin-Madison.
- Miller, C.N., Papeş, M., Schilling, E.E., and C. Kwit. 2021. Reproductive traits explain occupancy of predicted distributions in a genus of eastern North American understory herbs. *Diversity and Distributions*. 00: 1–18.
- Milne, R.I. 2006. Northern hemisphere plant disjunctions: a window on tertiary land bridges and climate change? *Annals of Botany*. 98: 465–472.
- Murray, A.E.. 1983. Notae spermatophytae No. 2. *Kalmia*. 13: 3-32.
- Murray, A.E. 1984. Notae spermatophytae No. 4. *Kalmia*. 14: 2-24.
- NatureServe. 2021. NatureServe Explorer [web application]. NatureServe, Arlington, VA. Available <https://explorer.natureserve.org/>. (Accessed June 08, 2021).
- O'Neill, D.M. 1995. Taxonomic study of *Trillium ovatum* forma *hibbersonii*. Masters Thesis. The University of British Columbia.
- O'Neill, D.M., Farmer, S.B., Floden, A., Lampley, J. and E.E. Schilling. 2020. *Trillium hibbersonii* (Melanthiaceae), a phylogenetically distinct species from western North America. *Phytotaxa*. 436(2): 193–195.
- Osaloo, S.K., Utech, F.H., Ohara, M., and S. Kawano. 1999. Molecular systematics of Trilliaceae I. Phylogenetic analyses of *Trillium* Using *matK* gene sequences. *Journal of Plant Res.* 112: 35-49.
- Osaloo, S.K. and S. Kawano. 1999. Molecular systematics of Trilliaceae II. Phylogenetic analyses of *Trillium* and its allies using sequences of *rbcL* and *matK* genes of cpDNA and internal transcribed spacers of 18s-28s nrDNA. *Plant Species Biology*. 14: 75–94.
- Pellicer, J., Kelly, L.J., Leitch, I.J., Zomlefer, W.B. and M.F. Fay. 2014. A universe of dwarfs and giants: genome size and chromosome evolution in the monocot family Melanthiaceae. *New Phytologist*. 201(4): 1484–1497.
- Philippe, H., Brinkmann, H., Lavrov, D.V., Littlewood, D.T.J., Manuel, M., Wörheide, G., et al. 2011. Resolving Difficult Phylogenetic Questions: Why More Sequences Are Not Enough. *PLoS Biology*. 9(3): e1000602.
- Poe, S. 1998. Sensitivity of phylogeny estimation to taxonomic sampling. *Systematic Biology*. 47(1): 18-31.
- Pollock, D.D., Zwickl, D.J., McGuire, J.A., and D.M. Hillis. 2002. Increased taxon sampling is advantageous for phylogenetic inference. *Systematic Biology*. 51(4): 664-71.
- POWO. 2021. Plants of the World Online. Facilitated by the Royal Botanic Gardens, Kew. Published on the Internet, <http://www.plantsoftheworldonline.org/>. (Accessed 10 September 2019).
- Rannala, B., Huelsenbeck, J. P., Yang, Z. and R. Nielson. 1998. Taxon sampling and the accuracy of large phylogenies. *Systematic Biology*. 47(4): 702-710.
- Rambaut, A., Drummond, A.J., Xie, D., Baele, G. and M.A. Suchard. 2018. Posterior summarisation in Bayesian phylogenetics using Tracer 1.7. *Systematic Biology*. 67(5): 901–904.
- Ree, R.H. and S.A. Smith. 2008. Maximum likelihood inference of geographic range evolution by dispersal, local extinction, and cladogenesis. *Systematic Biology*. 57(1): 4–14.

- Ree, R.H. and I. Sanmartín. 2018. Conceptual and statistical problems with the DEC+J model of founder-event speciation and its comparison with DEC via model selection. *Journal of Biogeography*. 45(4): 741-749.
- Rendle, A.B. 1901. Notes on *Trillium*. *Journal of Botany, British and Foreign*. 39: 321-335.
- Reveal, J.L. and K.N. Gandhi. 2014. *Trillium* subg. *Sessilium* Raf. (1830), an earlier name for *Trillium* subg. *Phyllantherum* (Schult. & Schult.f) J.D. Freeman (Melanthiaceae: Parideae). *Phytoneuron*. 2014-62: 1–3.
- Rokas, A. and S.B. Carroll. 2005. More Genes or More Taxa? The Relative Contribution of Gene Number and Taxon Number to Phylogenetic Accuracy. *Molecular Biology and Evolution*. 22(5): 1337–1344.
- Rull, V. 2008. Speciation timing and neotropical biodiversity: the Tertiary–Quaternary debate in the light of molecular phylogenetic evidence. *Molecular Ecology*. 17: 2722–2729.
- Samejima, J. and K. Samejima. 1962. Studies on the eastern Asiatic *Trillium* (Liliaceae). *Acta Horti Gothoburgensis*. 25: 157-257.
- Samejima, K. and J. Samejima. 1987. *Trillium* genus illustrated. Sapporo, Japan: University of Hokkaido Press. p. 237.
- Schilling, E.E., Floden, A., Lampley, J.A., Patrick, T.S. & S.B. Farmer. 2017. A New Species in *Trillium* subgen. *Delostylium* (Melanthiaceae, Parideae). *Phytotaxa*. 296(3): 287–291.
- Schultes, J.A. and J.H. Schultes. 1830. Caroli a Linné, equitis, Systema vegetabilium: secundum classes, ordines, genera, species. Cum characteribus differentiis et synonymiis. Stuttgartiae: J.G. Cotta. p. 1497.
- Soltis, D.E. and P.S. Soltis. 2004. *Amborella* not a “basal angiosperm”? Not so fast. *American Journal of Botany*. 91: 997–1001.
- Stamatakis, A. 2014. RAxML Version 8: A tool for Phylogenetic Analysis and Post-Analysis of Large Phylogenies. *Bioinformatics*. 30(9): 1312–1313.
- Stevens, P.F. 2021. Angiosperm Phylogeny Website. Version 14, July 2017-2021. Available at <http://www.mobot.org/MOBOT/research/APweb/>.
- Stoehrel, C.P. 2010. A study of the systematic relationships between members of the *Trillium erectum* complex. Masters Thesis. Western Carolina University, Cullowhee, NC.
- Sytsma, K.J., Spalink, D., and B. Berger. 2014. Calibrated chronograms, fossils, outgroup relationships, and root priors: re-examining the historical biogeography of Geraniales. *Biological Journal of the Linnean Society*. 113: 29–49.
- Takhtajan, A. 1983. A revision of *Daiswa* (Trilliaceae). *Brittonia*. 35: 255-270.
- Takhtajan, A. 1986. Floristic Regions of the World. (translated by T.J. Crovello & A. Cronquist). Berkeley, CA: University of California Press.
- Tamura, M. 2016. *Kinugasa japonica* Melanthiaceae. *Curtis's Botanical Magazine*. Royal Botanic Gardens Kew. 33(3): 261-267.
- Taylor, T.M.C. and A.F. Szczawinski. 1974. *Trillium ovatum* Pursh forma *hibbersonii* Taylor et Szczawinski f. nov. *Syesis*. 7: 250.
- Tiffney, B.H. 1985. Perspectives on the origin of the floristic similarity between Eastern Asia and eastern North America. *Journal of Arnold Arboretum*. 66: 73–94.
- Tiffney, B. and S. Manchester. 2001. The use of geological and paleontological evidence in evaluating plant phylogeographic hypotheses in the northern hemisphere tertiary. *International Journal of Plant Sciences*. 162(S6): S3-S17.
- USDA, NRCS. 2021. The PLANTS Database National Plant Data Team, Greensboro, NC. Available at <http://plants.usda.gov>. (Accessed 02 January 2021).

- Utescher, T. and V. Mosbrugger. 2007. Eocene vegetation patterns reconstructed from plant diversity — A global perspective. *Palaeogeography, Palaeoclimatology, Palaeoecology*. 247(3–4): 243–271.
- Veratrum* L. in GBIF Secretariat. 2019. GBIF Backbone Taxonomy. Checklist dataset <https://doi.org/10.15468/39omei>. (Accessed via GBIF.org on 10 September 2019).
- Vinnersten, A. and K. Bremer. 2001. Age and biogeography of major clades in Liliales. *American Journal of Botany*. 88(9): 1695–1703.
- Weakley, A.S., Sorrie, B.A., LeBlond, R.J., Poindexter, D.B., Floden, A.J., Schilling, E.E., Franck, A.R., and J.C. Kees. 2018. New combinations, rank changes, and nomenclatural and taxonomic comments in the vascular flora of the southeastern United States, IV. *Journal of the Botanical Research Institute of Texas*. 12(2): 461 – 480.
- Wen, J. 1999. Evolution of Eastern Asian and Eastern North American Disjunct Distributions in Flowering Plants. *Annual Review of Ecology and Systematics*. 30: 421–455.
- WFO. 2021. Melanthiaceae Batsch ex Borkh. Published on the Internet, <http://www.worldfloraonline.org/taxon/wfo-70000000371>. (Accessed 10 Jul 2021).
- Wiley, L. 1969. Rare Wild Flowers of North America. Portland, Oregon: published by the author.
- Wing, S.L., Harrington, G.J., Smith, F.A., Bloch, J.I., Boyer, D.M. and K.H. Freeman. 2005. Transient Floral Change and Rapid Global Warming at the Paleocene-Eocene Boundary. *Science*. 310: 993–996.
- Wolfe, J.A.. 1985. Distribution of major vegetational types during the Tertiary. E.T. Sundquist, W.S. Broecker (eds.). The carbon cycle and atmospheric CO₂; Natural variations Archean to Present. Geophysical Monograph Series, vol. 32. Washington D.C.: American Geophysical Union. pp. 357–375.
- Wolfe, J.A. 1994. An analysis of Neogene climates in Beringia. *Palaeogeography, Palaeoclimatology, Palaeoecology*. 108: 207–216.
- Xiang Q.-Y., Soltis D.E., and P.S. Soltis. 1998. The eastern Asian and eastern and western North American floristic disjunction: congruent phylogenetic patterns in seven diverse genera. *Molecular Phylogenetics and Evolution*. 10(2): 178–90.
- Xiang, Q.-Y. and D.E. Soltis. 2001. Dispersal-Vicariance Analyses of Intercontinental Disjuncts: Historical Biogeographical Implications for Angiosperms in the Northern Hemisphere. *International Journal of Plant Sciences*. 162(6 Suppl.): S29–S39.
- Xu, Z., Wei, N., Tan, Y., Peng, S., Ngumbau, V.M., Hu, G.-W., and Q.-F. Wang. 2019. *Paris lihengiana* (Melanthiaceae: Parideae), a new species from Yunnan, China. *Phytotaxa*. 392(1): 045–053.
- Yang, L., Yang, Z., Liu, C., He, Z., Zhang, Z., Yang, J., Liu, H., Yang, J., and Y. Ji. 2019. Chloroplast phylogenomic analysis provides insights into the evolution of the largest eukaryotic genome holder, *Paris japonica* (Melanthiaceae). *BMC Plant Biology*. 19: 293.
- Zecca, G., Abbott, J.R., Sun, W.-B., Spada, A., Sala, F., and F. Grassi. 2012. The timing and the mode of evolution of wild grapes (*Vitis*). *Molecular Phylogenetics and Evolution*. 62(2): 736–747.
- Zhang, J., Li, R., Xiang, X., Manchester, S.R., Lin, L., Wang, W., Wen, J., and Z. Chen. 2013. Integrated Fossil and Molecular Data Reveal the Biogeographic Diversification of the Eastern Asian - Eastern North American Disjunct Hickory Genus (*Carya* Nutt.). *PLOSone*. 8(7): n. p.

- Zomlefer, W.B., Williams, N.H., Whitten, W.M., and W.S. Judd. 2001. Generic circumscription and relationships in the tribe Melanthieae (Liliales, Melanthiaceae), with emphasis on *Zigadenus*: evidence from ITS and *trnL-F* sequence data. *American Journal of Botany*. 88(9): 1657–1669.
- Zomlefer, W.B., Whitten, W.M., Williams, N.H. and W.S. Judd. 2006. Infrageneric Phylogeny of *Schoenocaulon* (Liliales: Melanthiaceae) with Clarification of Cryptic Species Based on Its Sequence Data and Geographical Distribution. *American Journal of Botany*. 93(8): 1178-1192.
- Zwickl, D.J. and D.M. Hillis. 2002. Increased taxon sampling greatly reduces phylogenetic error. *Systematic Biology*. 51(4): 588-98.

Appendix

Appendix A

Table 1.1. A summary of molecular studies of Parideae (Trilliaceae) or *Trillium*. (Key: T=Incomplete taxon sampling; C=simple cladistics methods; M=possible sample misidentification; I=chloroplast markers only; P= few markers or little resolution due to conservative markers).

<u>Publication</u>	<u>Focal Taxon</u>	<u>Markers used</u>	<u>Potential study limitations</u>
Kato et al., 1995b	Trilliaceae s.l. (<i>Trillium</i> n=2)	<i>rbcL</i>	T; C; I; P
Kato et al., 1995a	Trilliaceae (<i>Trillium</i> s.l. n=25)	RFLP of chloroplast	T; C; I
Osaloo et al., 1999	Trilliaceae (<i>Trillium</i> n=41)	<i>matK</i>	C; M; I; P
Osaloo & Kawano, 1999	Trilliaceae (<i>Trillium</i> n=18)	<i>rbcL</i> , <i>matK</i> , <i>ITS</i>	C; P
Farmer & Schilling, 2002	Trilliaceae (<i>Trillium</i> n=16)	<i>ITS</i> , <i>matK</i> , + morphology	T; C; P
Farmer 2006	Trilliaceae (<i>Trillium</i> n=54+)	<i>ITS</i> , <i>matK</i>	C; P
Ji et al., 2006	<i>Paris</i> (<i>Trillium</i> n=10)	<i>ITS</i> , <i>psbA-trnH</i> , <i>trnL-trnF</i>	T; C; M; P
Pellicer et al., 2014	Melanthiaceae (<i>Trillium</i> , n=23)	<i>trnL-trnF</i> , <i>matK</i>	T; I; P
Kim et al., 2016b	Melanthiaceae (<i>Trillium</i> s.l. n=29)	<i>atpB</i> , <i>matK</i> , <i>rbcL</i> , <i>ndhF</i> , <i>trnL-trnF</i>	I; M; P
Kim et al., 2016a	<i>Trillium</i> (<i>Trillium</i> s.l. n=29)	Same as Kim et al., 2016b. Two plastomes examined.	Same as Kim et al., 2016b.
Givnish et al., 2016	Liliales (<i>Trillium</i> n=1)	75 plastid genes	T; I
Huang et al., 2016	<i>Paris</i> (<i>Trillium</i> n=4)	complete chloroplast genomes	T; I
Kim, Kim, & Kim, 2019	Melanthiaceae (<i>Trillium</i> s.l. n=27)	<i>atpB</i> , <i>rbcL</i> , <i>matK</i> , and <i>ndhF</i>	I; M; P
Yang et al., 2019	<i>Paris</i> (<i>Trillium</i> s.l. n=5)	complete chloroplast genomes	T; I; M
Ji et al., 2019	<i>Paris</i> (<i>Trillium</i> s.l. n=6)	complete chloroplast genomes and nrDNA (<i>18S</i> , <i>ITS1</i> , <i>5.8S</i> , <i>ITS2</i> and <i>26S</i>)	T; M

Table 1.2. Table of study taxa. Genbank accession numbers are given where appropriate. * denotes NGS data generated in the current study; § denotes SRA accessions.

<u>Taxon-ID</u>	<u>Species</u>	<u>DNA#</u>	<u>Voucher (TENN)</u>	<u>Location</u>	<u>Plastome</u>
Chm_luteum	<i>Chamaelirium luteum</i>	4122*	A. Floden et al. 2595	Campbell Co., TN, USA	
Chio_japon	<i>Chionographis japonica</i>				KF951065.1
D_chinensi	<i>Daiswa chinensis</i>				KX784048
D_cronquis	<i>Daiswa cronquistii</i> [Paris <i>cronquistii</i>]				KX784041
D_dunniana	<i>Daiswa dunniana</i>				KX784042
D_fargesii	<i>Daiswa fargesii</i> [D. <i>forrestii</i>]				KX784043
D_forresti	<i>Daiswa forrestii</i>				KX784044
D_luquanen	<i>Daiswa luquanensis</i> [Paris <i>luquanensis</i>]				KX784045
D_mairei	<i>Daiswa mairei</i> [Paris <i>mairei</i>]				KX784046
D_marmorat	<i>Daiswa marmorata</i> [Paris <i>marmorata</i>]				KX784047
D_rugosa	<i>Daiswa rugosa</i> [Paris <i>rugosa</i>]				KY247142
D_thibetic	<i>Daiswa thibetica</i> [Paris <i>thibetica</i>]				KY247143
D_vietname	<i>Daiswa vietnamensis</i> [Paris <i>vietnamensis</i>]				KX784050
D_yunnanen	<i>Daiswa yunnanensis</i>				KX784049
Fr_hupehen	<i>Fritillaria hupehensis</i>				KF712486.1
H_bullata	<i>Helonias bullata</i>				ERX2099772 §
Hp_tubiflo	<i>Heloniopsis tubiflora</i>				NC_027159
K_japonica	<i>Kinugasa japonica</i> [Paris <i>japonica</i>]				MH796668
Lm_longifl	<i>Lilium longiflorum</i>				KC968977
P_quadrifo	<i>Paris quadrifolia</i>				KX784051

Table 1.2. Continued.

P_verticil	<i>Paris verticillata</i>				KJ433485
Ps_rivale	<i>Pseudotrillium rivale</i>	4498*	E.E. Schilling s.n. 2016	Salt Spring Island, B.C., cult.	
Td_govania	<i>Trillidium govanianum</i>	4627*	E.E. Schilling and Susie Egan s.n.	Woodinville, WA, cult.	
Td_undulat	<i>Trillidium undulatum</i>	4182*	E.E. Schilling 14-DNA4182 with A. Floden	Greene Co., TN	
T_camschat	<i>Trillium camschatcense</i>				NC_046451
T_catesbae	<i>Trillium catesbaei</i>	4363*	J. Lampley 269 et al.	Clay Co., NC	
T_cernuum	<i>Trillium cernuum</i>	5136*	Welby Smith #36088	Washington Co., MN	
T_cuneatum	<i>Trillium cuneatum</i>	3397*	Floden 462	Campbell Co., TN, USA	NC_027185
T_decumben	<i>Trillium decumbens</i>	3689*	Floden et al. 2343	Murray Co., GA	KR534612
T_georgian	<i>Trillium georgianum</i>	4322*	JA Lampley 252 et al.	Dalton, GA	
T_grandifl	<i>Trillium grandiflorum</i>	4324*	Floden et al. s.n. DNA4324 April 2015	Overton Co., TN	
T_nivale	<i>Trillium nivale</i>	789*	Steven s.n. April 2003	Putnam Co., IN	
T_ovat_hib	<i>Trillium ovatum hibernsonii</i>	4630*	E.E. Schilling s.n. 2016	Salt Spring Island B.C., cult.	
T_ovat_sco	<i>Trillium ovatum scouleri</i>	4201*	Floden and Schilling s.n. Aug. 2014	Idaho	
T_rugelii	<i>Trillium rugelii</i>	4341*	Floden, Schilling and Lampley s.n. April 2015	Hawkins Co., TN	
T_sulcatum	<i>Trillium sulcatum</i>	4267*	Floden s.n. April 2015	Bucks Pocket AL	
T_tschonok	<i>Trillium tschonokii</i>				KR780076
Ver_nigrum	<i>Veratrum nigrum</i>				SRP071777 §
Ver_oxysp	<i>Veratrum oxyspalum [patulum]</i>				NC_022715
Vb_aristat	<i>Verbesina aristata</i>	4021*	McNeilus 90-726	Santa Rosa Co FL	
X_asphodel	<i>Xerophyllum asphodeloides</i>				ERX2099771 §

Table 1.2. Continued.

X_tenax	<i>Xerophyllum tenax</i>	NC_027158
Yp_thibeti	<i>Ypsilandra thibetica</i>	NC_044639.1

Table 1.3. Table of geographic categories used in the BioGeoBEARS historical range reconstruction analysis. Individual taxa were coded A-G based on current distribution. Also listed are the corresponding areas from Takhtajan's classification of floristic provinces.

<u>Takh. No.</u>	<u>BioGeoBEARS Coding</u>	<u>Takhtajan's Floristic Provinces</u>
1	A	Circumboreal region - Old World
1	H	Circumboreal region - New World
2	B	Eastern Asiatic region
3	C	North American Atlantic region
4	D	Rocky Mountain Floristic region
6	E	Mediterranean region
8	F	Irano-Turanian region
17	G	Indochinese region

<u>TaxonID</u>	<u>Taxon Takh. No.</u>	<u>Taxon BioGeoBEARS Coding</u>
Chio_japon	2	B
Chm_luteum	3	C
D_chinensi	2	B
D_cronquis	2	B
D_dunniana	2,17	B,G
D_fargesii	2	B
D_forresti	2,8	B,F
D_luquanen	2	B
D_mairei	2	B
D_marmorat	2,8	B,F
D_rugosa	2	B
D_thibetic	2,8	B,F
D_vietname	2	B
D_yunnanen	2,8	B,F
H_bullata	3	C
Hp_tubiflo	2	B
K_japonica	2	B
P_quadrifo	1,2,6,8	A,B,E,F
P_verticil	2,8	B,F
Ps_rivale	4	D
T_camschat	1,2	A,B
T_catesbae	3	C
T_cernuum	1,3	H,C
T_cuneatum	3	C
T_decumben	3	C
T_georgian	3	C
T_grandifl	3	H,C
T_nivale	3	C

Table 1.3. Continued.

T_ovat_hib	4	D
T_ovat_sco	4	D
T_rugelii	3	C
T_sulcatum	3	C
T_tschonos	2,8	B,F
Td_govania	2,8	B,F
Td_undulat	3	C
Ver_nigrum	1,2,6,8	A,B,E,F
Ver_oxysep	1,1,2	A,H,B
X_asphodel	3	C
X_tenax	4	D
Yp_thibeti	2	B

Table 1.4. Listing of 70 plastid coding genes used in the plastome matrix dataset. Genes are listed alphabetically, not sequentially by order in chloroplast.

accD, atpA, atpB, atpE, atpF, atpH, atpI, ccsA, matK, ndhA, ndhB, ndhC, ndhD, ndhE, ndhF, ndhG, ndhH, ndhI, ndhJ, ndhK, petA, petG, petL, petN, psaA, psaB, psaC, psaI, psaJ, psbA, psbB, psbC, psbD, psbE, psbF, psbH, psbI, psbJ, psbK, psbL, psbM, psbN, psbT, psbZ, rbcL, rpl14, rpl2, rpl20, rpl22, rpl23, rpl32, rpl33, rpl36, rpoA, rpoB, rpoC1, rpoC2, rps11, rps14, rps15, rps18, rps19, rps2, rps3, rps4, rps7, rps8, ycf2, ycf3, ycf4

Table 1.5. A comparison of divergence time estimation studies for events in the evolutionary history of Parideae (Melanthiaceae). Mean ages shown, in millions of years (mya). (Abbrev. for included features of each study: Pla= plastid gene, Pme= whole plastome, Ps= *Pseudotrillium*, Td= *Trillidium*, Kg= Kinugasa.).

Timeline Events	Current Study (70 Pla, Ps, Td, Kg)	Givnish et al. 2016 (75 Pla, Ps, Td)	Kim3 2019 (4 Pla, Ps, Td, Kg)	Ji et al. 2019 (Pme, Kg,; Td incongruent)	Yang et al. 2019 (Pme, Kg,; Td incongruent)
Melanthiaceae Age Stem /Crown	84.19/ 59.07	103.9/84.8	~101/92.1	~114/84.8	103.9/ 89.18
<i>Pseudotrillium</i> diverges	18.84	~23	31.2	--	--
<i>Trillium s.l.</i> , <i>Paris s.l.</i> diverge	16.36	17.3	22.5	33.94	38.21
<i>Trillium</i> , <i>Trillidium</i> diverge	14.59	13.6	~19	~24, in part	~26, in part
<i>Trillidium govanianum</i> , <i>T. undulatum</i> diverge	10.29	--	8.2	--	--
<i>Trillium</i> Erectum group diverges	12.86	--	~11, in part	~29	32.11
<i>Trillium</i> Grandiflorum group diverges	10.91	--	Mixed groups	--	--
T. subgen. <i>Delostylium</i> , <i>Sessilium</i> diverge	9.64	--	Mixed groups	--	--
<i>Paris s.l.</i> crown age	14.61	13.7	~12	28.66	33.71

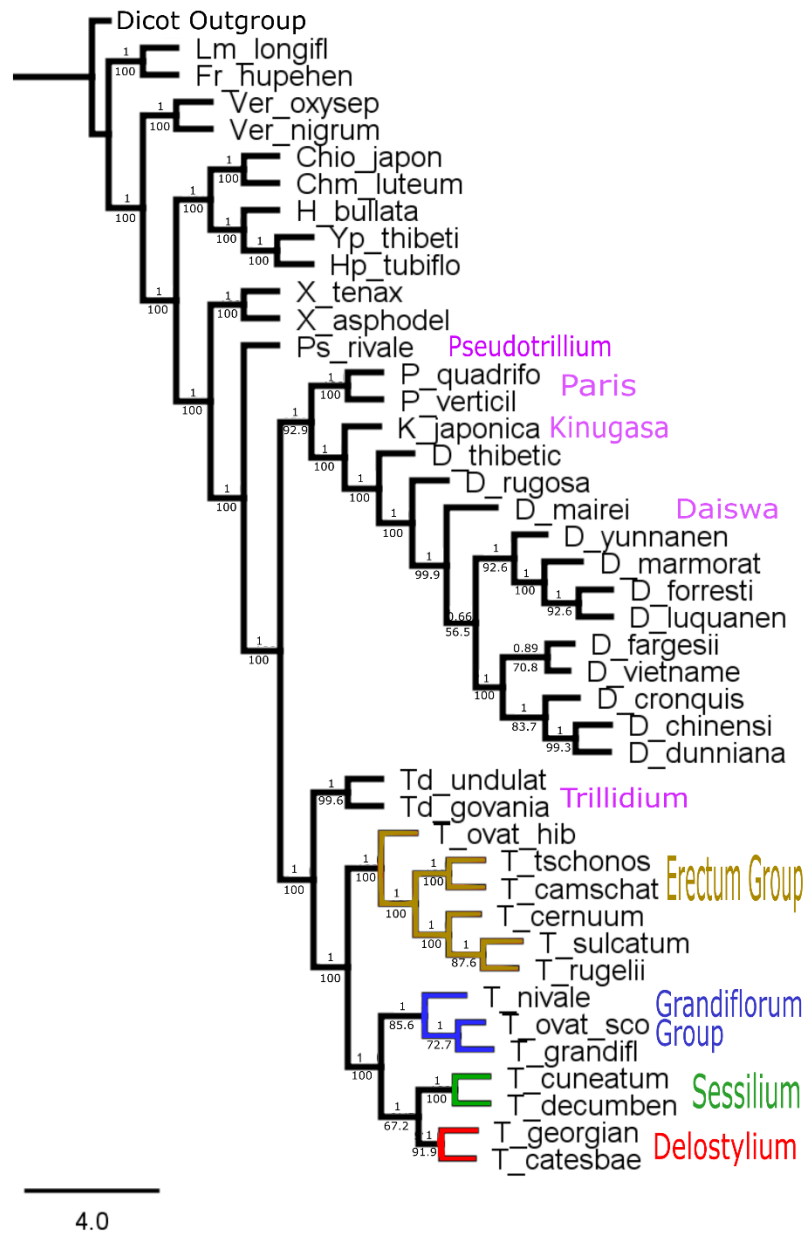
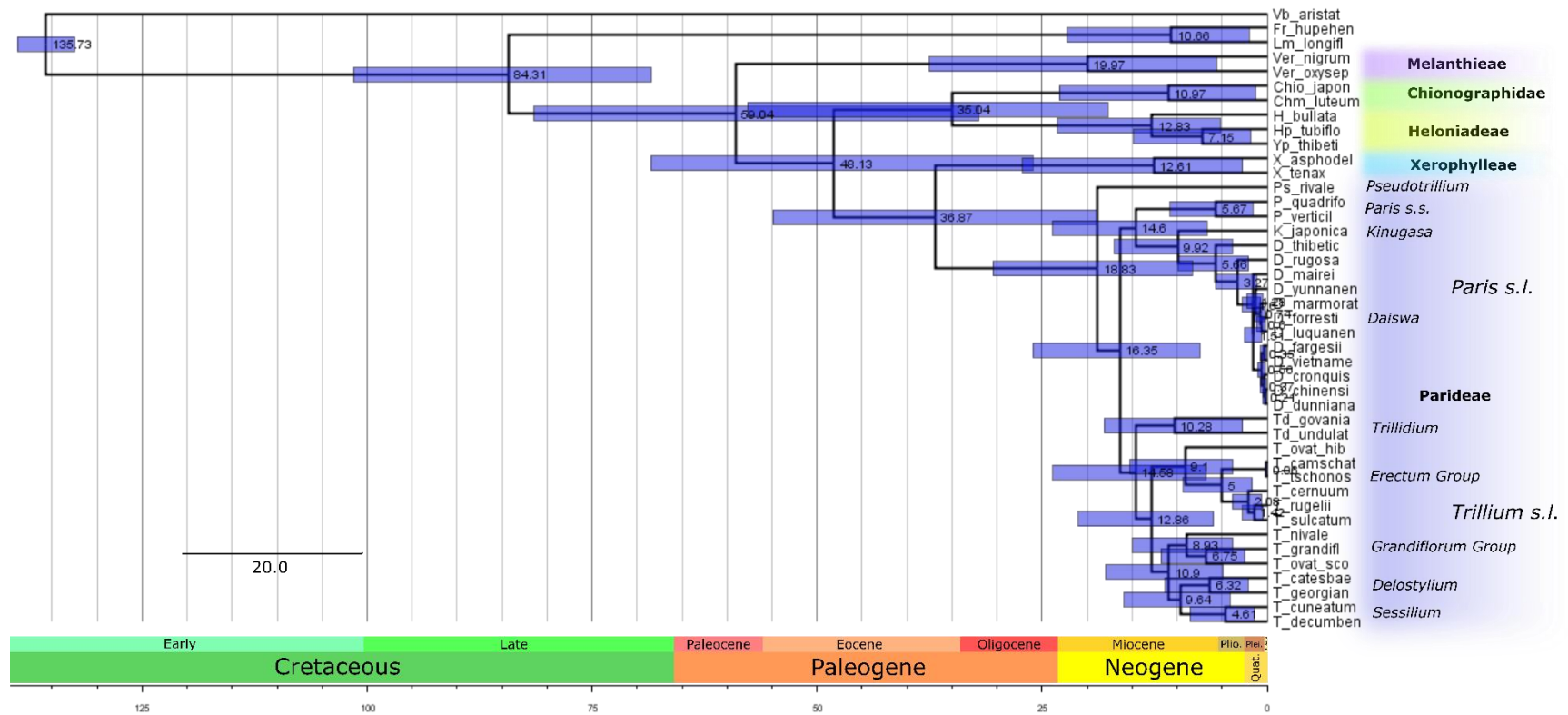


Figure 1.1. Majority rule consensus tree. Phylogenetic tree using RAxML showing relationships of Melanthiaceae tribes highlighting Parideae and *Trillium*. Bayesian posterior probabilities are listed above the branches and maximum likelihood bootstrap scores are listed beneath.



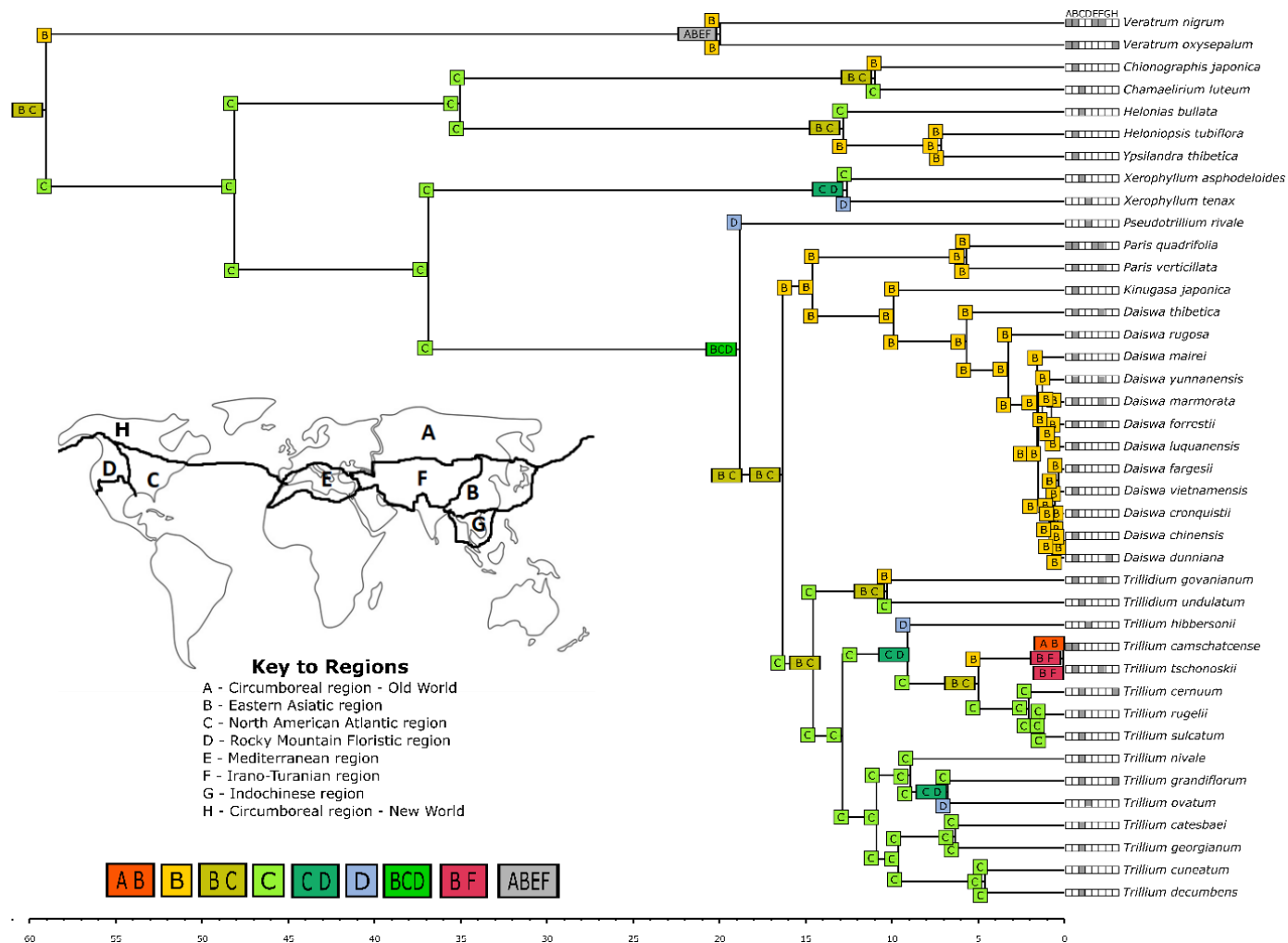


Figure 1.3. Historical biogeographic range reconstruction for Melanthiaceae based on BioGeoBEARS. Colored boxes labeled with letters A – G represent regions most likely occupied by ancestral taxa. Multiple letter combinations convey that a taxon was inferred to have inhabited a multiple region distribution. Map inset depicts world regions which were used to code samples in the study based on floristic provinces delineated by Takhtajan. Geographic coding of each sample is represented by gray boxes to the left of species names.

Figure 1.4. Historical biogeographic range reconstruction for Melanthiaceae showing degree of probability for each inference. Size of largest pie wedge represents the probability of the inferred ancestral range of each ancestral taxon. Different colors represent one of the 256 possible historical ranges and combinations of ranges and are too numerous to define other than that of the most likely ranges and combinations provided in Fig. 3.

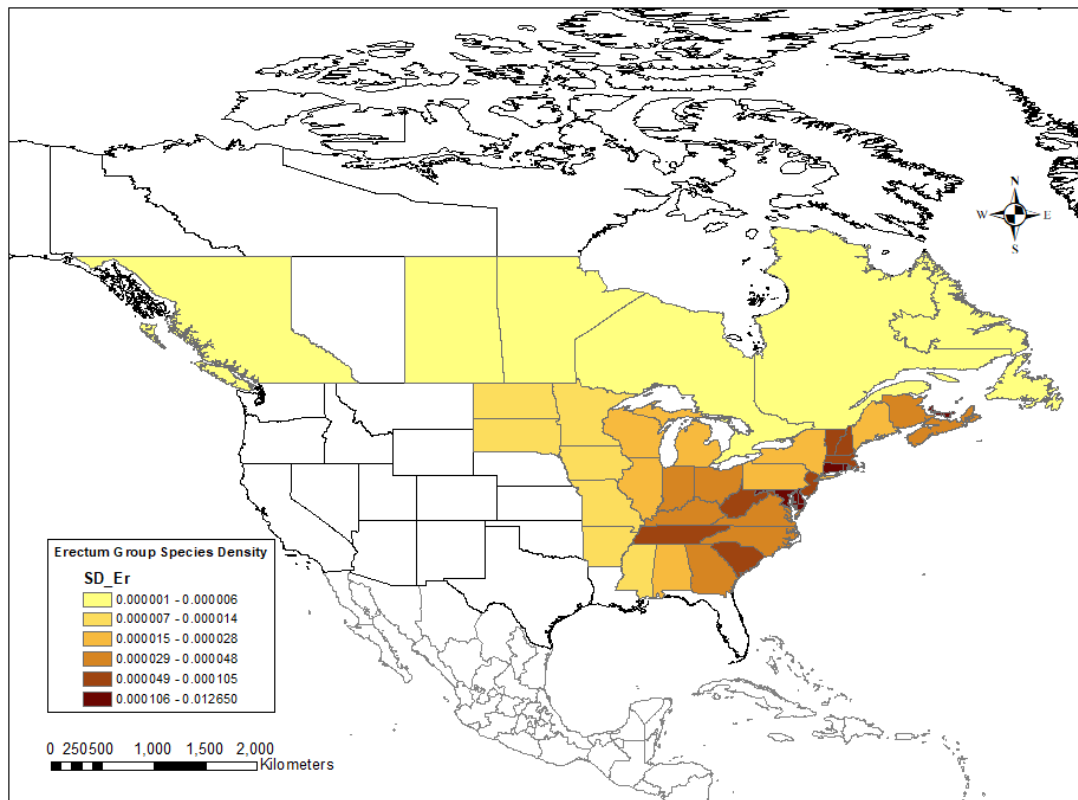


Figure 1.5. *Trillium* Erectum group species density. Map depicting North American Erectum group species density by state or province. Highest density is represented by dark brown and lowest density by yellow. Land area data does not include bodies of water and was obtained from the United States Census Bureau (www.census.gov) and Statistics Canada (www.statcan.gc.ca).

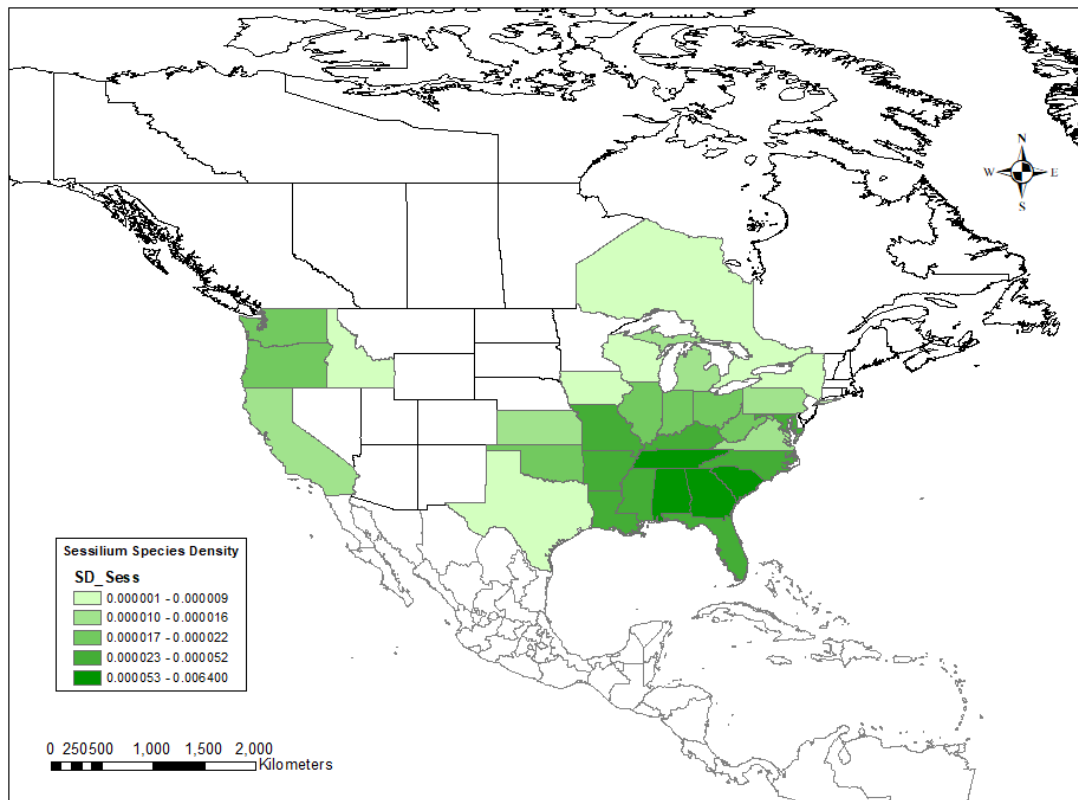


Figure 1.6. *Trillium* subgen. *Sessilium* species density. Map depicting *T.* subgen. *Sessilium* species density by state or province. Highest density is represented by dark green and lowest density by pale green.

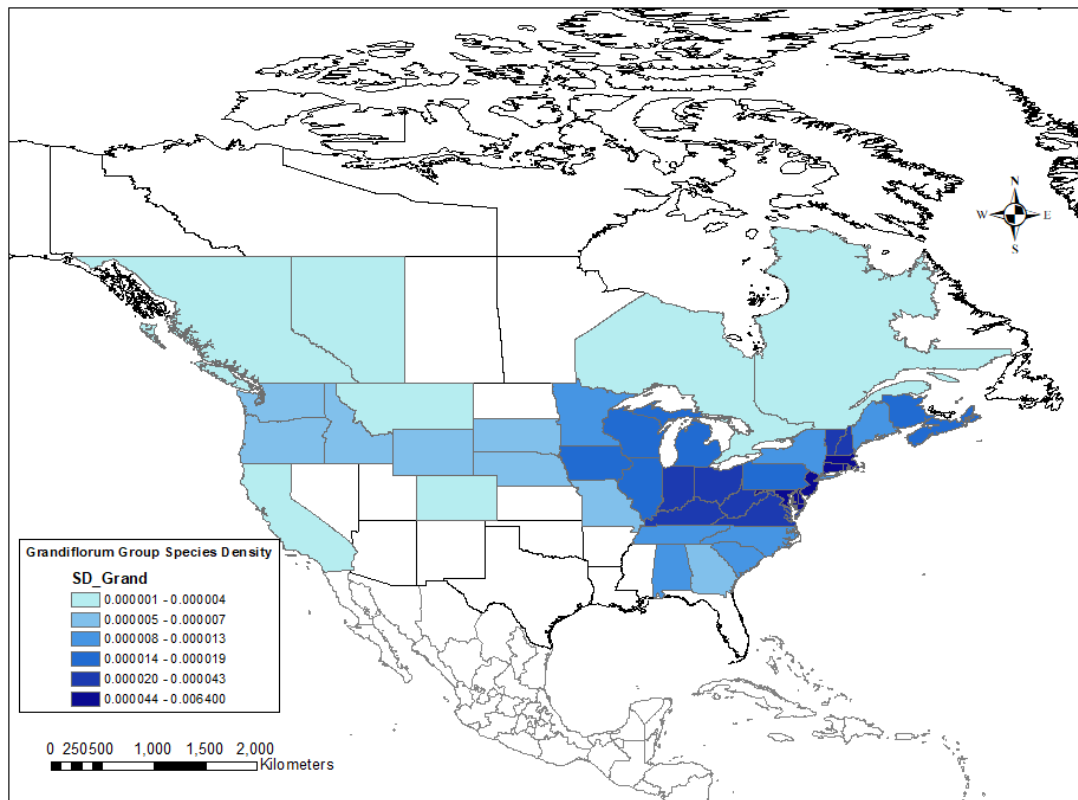


Figure 1.7. *Trillium* Grandiflorum group species density. Map depicting Grandiflorum group species density by state or province. Highest density is represented by dark blue and lowest density by pale blue.

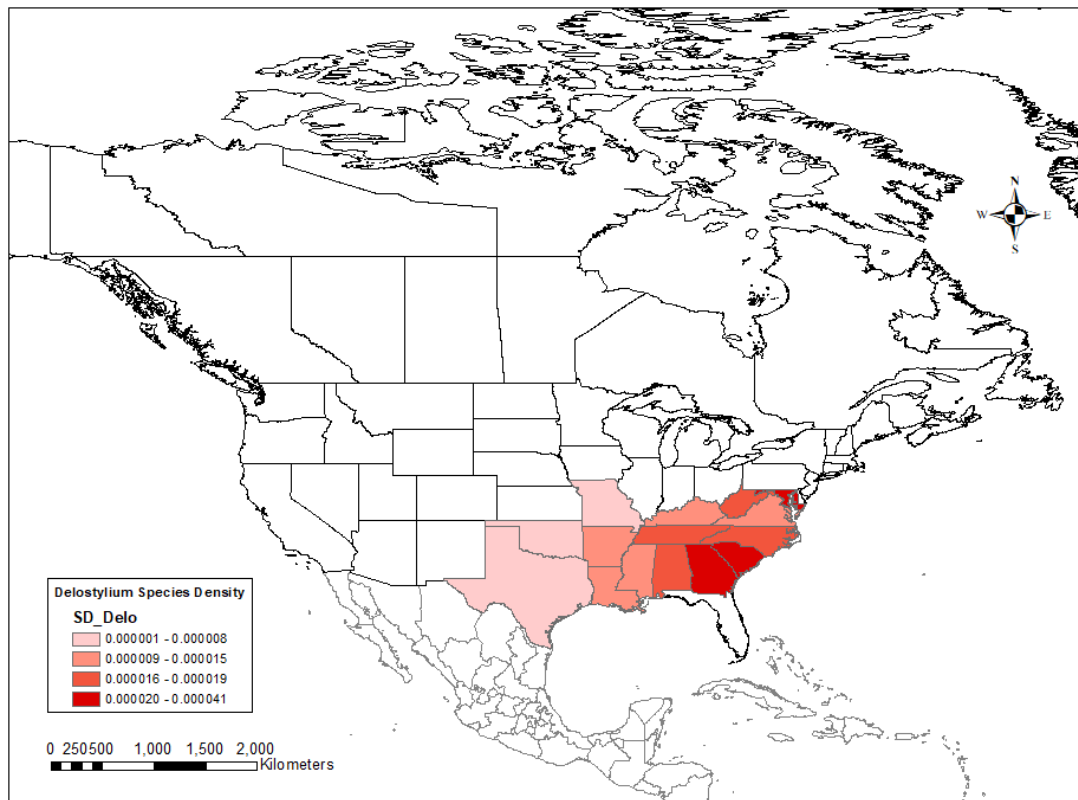


Figure 1.8. *Trillium* subgen. *Delostylium* species density. Map depicting *T.* subgen. *Delostylium* species density by state or province. Highest density is represented by bright red and lowest density by pale pink.

Chapter 2

Taxonomic relationships among the Toadshades:
A phylogenetic study of the *Trillium cuneatum* complex

INTRODUCTION

Trillium cuneatum species complex and allies

Recent phylogenetic analyses have indicated that *Trillium cuneatum* Raf. (Melanthiaceae) is paraphyletic, suggesting that it may harbor cryptic species within its circumscription (Schilling et al., 2013, Schilling et al. 2019). *Trillium cuneatum*, as currently circumscribed, spans seven states of the southeastern US (KY, TN, MS, AL, GA, NC, and SC) and has the third largest estimated Extent of Occurrence (EOO) of all *T.* subgen *Sessilium* Raf. [=*T.* subgen. *Phyllantherum* Raf.] species (Freeman, 1975; Meredith et al., 2020a). Also the largest-sized of the eastern sessile species (Case and Case, 1997), *T. cuneatum* inhabits rich, upland woods with limestone soils at lower elevations (< 400m) (Case, 2003; Weakley, 2020). While generally recognizable, *T. cuneatum* is highly variable and inconsistent morphologically across its distribution (Freeman, 1975; Case, 2003). Freeman (1975) suggested that the variability may be due in part to the variety of habitats and physiographic regions *T. cuneatum* occupies, or in some instances, that it may be due to hybridization in localized areas where it overlaps with *T. maculatum* Raf., *T. luteum* (Muhl.) Harb., or *T. ludovicianum* Harb. Population studies using maternally inherited plastid data for *T. cuneatum* found that there appeared to be genetic structure among several populations across the southeastern US (Gonzales et al., 2008; Wallace and Doffitt, 2013). Schilling et al. (2013; 2019) showed the paraphyly of *T. cuneatum* in that it formed a clade with *T. luteum* and *T. maculatum*. Together with cryptic morphological variability and genetic differentiation across populations, this suggests that *T. cuneatum* may be a species complex. Clarifying species delimitation in species complexes is crucial for avoiding errors in studies of biodiversity and speciation processes (Pinheiro et al., 2018) as well as in application of conservation efforts.

Phylogenetic analysis can sometimes reveal the existence of cryptic species, as demonstrated by the case of *Trillium delicatum* Floden & E.E. Schill. (Schilling et al., 2019). One example of an apparently distinct morphotype of *T. cuneatum* was found in certain populations at the northern terminus of Lookout Mountain near Chattanooga, TN. These populations, though bearing similarities to *T. cuneatum*, were noticed by specialist gardeners for their subtle differences from that species in aspects of the flower habit, petal shape, floral scent, and flowering time (Fig. 2.1D, cf. 2.1B; T. Avent, pers. comm., 2021). Representatives from these populations were used in two previous phylogenetic studies which placed them in a clade with *T. maculatum* rather than *T. cuneatum* (Schilling et al., 2013; Schilling et al., 2019). From the outset of the current study, another set of populations which were distinctive were noticed by the authors thanks in part to preliminary phylogenetic analyses. These populations occur in northeastern Alabama and western Georgia.

Further complicating the taxonomy of *Trillium cuneatum* is the existence of another similar species, *T. luteum*. Though widely recognized as a species, *T. luteum* has been regarded by some as a form of *T. cuneatum* because it is mainly distinguished from that species based on its flower color and consistent lemon floral scent (Radford et al., 1968; Serota, 1969; Case, 2003; Freeman, 1975; and Weakley, 2020). Recent phylogenetic studies placed *T. luteum* in a poorly resolved clade with some samples of *T. cuneatum* (Schilling et al., 2013; Schilling et al., 2019). Further, it has been suggested that the two species may hybridize locally when populations overlap, however their ranges are mostly allopatric and they are syntopic in only a few places (Freeman, 1975; Case and Case, 1997; Case, 2003). *Trillium luteum* occurs in moist coves over mafic or calcareous substrates of the Ridge and Valley province and southern Appalachian Mountains across four states (TN, KY, GA, and NC). It has been reported as adventive as far

north as Ontario (Case and Case, 1997; Case, 2003; Kartesz, 2015; Weakley, 2020; Meredith et al., 2020c). The petals of *T. luteum* are greenish yellow to bright yellow and the ovary and stamens are greenish white to pale yellow. Freeman observed that *T. luteum* has little to no color variation in populations and that even the oldest herbarium specimens could be identified due to the complete lack of purple pigmentation as seen in the pale appearance of petal bases and reproductive parts (1975). However, it is often confused with the yellow color morphs which frequently occur in populations of maroon-flowered *Trillium* species, besides being mistaken for other pale or green-flowered species. In summary, the combination of few solid identification features for *T. luteum*, the propensity of *T. cuneatum* populations to produce yellow flower morphs, and an apparent lack of genetic differentiation from some populations of *T. cuneatum*, highlights the need for further taxonomic clarification of *T. luteum*.

Another species which is part of the broader *Trillium cuneatum* clade, and similar in appearance, is *T. maculatum*. Schilling et al. (2019) showed that the phylogenetic placement of *T. maculatum* renders *T. cuneatum* paraphyletic. It is distinguished from *T. cuneatum* notably in floral scent (spicy, banana-like), long clawed narrowly spatulate petals, broad anther connectives, strong leaf mottling, and more southerly distribution (Case 2003; Weakley, 2020). *Trillium maculatum* is frequently confused with many other maroon-flowered *Trillium* species and it is possible that some records have been misidentified (Freeman, 1975; Meredith et al., 2020d). *Trillium maculatum* occurs along and south of the Fall Line, inhabiting the upper Coastal Plain and portions of the Piedmont Province across AL, GA, FL, and SC. It occurs in rich forests over calcareous substrates, differing from *T. cuneatum* in that it does not occur in cove forests and is more likely to occur near rivers and in rich alluvial floodplains (Freeman, 1975; Kartesz, 2015; Meredith et al., 2020d). The two species are not known to occur in sympatry with each other, reducing the potential for hybridization (Freeman, 1975).

Recent studies using population genetics methods lent support to the hypothesis that *Trillium cuneatum*, as currently circumscribed, includes multiple lineages. Two complementary population genetic studies examined *T. cuneatum* and each presented evidence of genetic substructuring, collectively finding eight distinct plastid haplotypes across the species distribution (Gonzales et al., 2008; Wallace and Doffitt, 2013). However, these studies did not directly address speciation or the paraphyly of *T. cuneatum* and were limited by plastid datasets with relatively low variability: the *trnL* intron and the *trnL-trnF* intergenic spacer were used in each study, yielding six variable characters from a combined sequence length of 812 bp.

Taxonomy of Trillium subgen. Sessilium

Although *Trillium* subgen. *Sessilium* is endemic to North America and is a diverse assemblage well-known for distinctive spring displays, the taxonomy of the subgenus is under-explored for multiple reasons. Molecular phylogenetic studies could not thoroughly settle questions of evolutionary relationships due to limited gene or taxon sampling (see discussion regarding sampling in Lampley, Dissertation Ch. 1). Additionally, no molecular-based study has fully tested the accuracy or relevance of morphology-based classifications in the group (Schilling et al., 2013). Molecular studies have supported the monophyly of the subgenus, but have left issues of taxonomic delimitation of species unclear (Kawano and Kato, 1995; Kato et al., 1995; Osaloo et al., 1999; Osaloo and Kawano, 1999; Farmer and Schilling, 2002; Farmer, 2006; Schilling et al., 2013; Schilling et al., 2019). Furthermore, morphological variability within species and confusing similarities between species have led to identification and circumscription challenges in *T.* subgen. *Sessilium*. Further complicating this is that *Trillium* species display a

simplicity of form which in many cases make them only subtly distinguishable by a few key characteristics (Gleason, 1906; Barksdale, 1938; Freeman, 1975). Freeman's (1975) definitive taxonomic revision of *T. subgen. Sessilium* still informs our understanding of the group today, though it was necessarily based on morphology and geography alone. Freeman (1975) recognized and clarified the taxonomy and nomenclature of 22 species, including six new species, as well as defining three informal morphological species alliances, ending long-standing disorder in the group (Case and Case, 1997). However, a resolved and thoroughly sampled *T. subgen. Sessilium* phylogeny based on molecular evidence, including recently described taxa, is needed to fully understand relationships among species and putative species complexes, thus providing a solid foundation for future studies.

A testament to the incomplete knowledge of *Trillium* subgen. *Sessilium* is that four novel species have been recognized in the seemingly well-explored USA just in the time since Freeman's revision (1975). Thus over 15% of the recognized species total in the subgenus has been discovered in the last 40 years, with two of these species only collected for the first time in 2007 (Schilling et al., 2019) and 2012 (Schilling et al., 2013). The first of these relatively recent discoveries, distributed in the Puget Trough and Willamette Valley of Washington and Oregon, white-flowered *T. parviflorum* V.G. Soukup had been lumped by Freeman (1975) with the only known white-flowered species in *T. subgen. Sessilium*, *T. albidum* J.D. Freeman. Despite the superficial resemblance and adjacent distributions, Soukup (1980) distinguished the two species by differences in overall size, petal shape, fragrance, and fruit coloration. Recognition of *T. parviflorum*, which is listed as vulnerable by the IUCN Red List, has allowed for careful assessment of the species which would have been otherwise overlooked (Wayman and Meredith, 2020). Second, *T. oostingii* Gaddy, endemic to the Wateree River in the Coastal Plain region of South Carolina, was recognized and distinguished from *T. lancifolium* Raf. which it closely resembles (Gaddy, 2008). The IUCN Red List considers this species endangered: it has the smallest EOO of any *T. subgen. Sessilium* species and needs further research and conservation actions (Meredith et al., 2020e). Third, an endemic species, *T. tennesseense* E.E. Schill. & Floden, native to two counties in northeastern Tennessee, was shown to be distinct from close allies *T. oostingii*, *T. lancifolium*, and *T. recurvatum* L.C. Beck through molecular and morphological analysis, but is synonymized with *T. lancifolium* by some (Schilling et al., 2013; Meredith et al., 2020b, USDA, NRCS, 2021). If it is widely accepted as a species, IUCN Red List recommends that *T. tennesseense* be listed as Critically Endangered and that conservation measures be put in place to prevent extinction of the taxon (Meredith et al., 2020b). Lastly, *T. delicatum* of central Georgia was recently described having been differentiated from *T. decumbens* Harb., both genetically and morphologically, with which it shares a decumbent habit (Schilling et al., 2019). This species is also narrowly endemic and considered Endangered by the IUCN Red List and in need of conservation action especially to protect against destruction by feral hogs (Trillium Working Group 2019 and Meredith, 2020). Recent studies have hinted at species complexes and paraphyly in *T. subgen. Sessilium* which strongly suggests that further investigation is warranted to reduce the likelihood of cryptic species being overlooked and possibly threatened (Schilling et al., 2013; Schilling et al., 2019).

Trillium subgenus Sessilium biogeography

Trillium subgenus *Sessilium* displays a disjunct geographic distribution between the eastern and western US and contains a relatively high number of endemic species (Weakley, 2008). The center of diversity for *T. subgen. Sessilium* appears to be in the Piedmont and

Coastal Plain regions of the eastern US, south of the maximum extent of Pleistocene glaciation (see Lampley Dissertation Ch. 1, Fig. 2.6; Freeman, 1975; Case and Case, 1997; Weakley, 2008). Diversification began approximately 4.6 mya (stem age 9.6 mya) during the Pliocene epoch, well before the Pleistocene glaciation (2.6 mya—11.7 kya) of North America (Lampley, unpub.). Most US states which were entirely or partially covered by the Laurentide Ice Sheet have no *T.* subgen. *Sessilium* species extant (IUCN, 2021). Those states, as well as the Canadian province Ontario, that have gained *T.* subgen. *Sessilium* species since glaciation only have *T. recurvatum* or *T. sessile* L. or both (Illinois is an exception with three species, including *T. viride* L.C. Beck in the southwestern portion of the state along the Mississippi and Missouri Rivers). *Trillium* subgen. *Sessilium* likely retreated to multiple glacial refugia including areas of the relatively warm Coastal Plain Province and was slow to spread north post-Pleistocene except for a few successful species (Gonzales et al., 2008). One impediment was likely limited seed dispersal capability in many sessile *Trillium* species which could also contribute to genetic isolation and diversification (Wallace and Doffitt, 2013; Miller and Kwit, 2018). Additionally, natural herbivory may have been a significant limitation for *Trillium* species spread in terms of delayed reproduction and seed count (Knight, 2003; Auberson-Lavoie and Vellend, 2020). Over 46% of *T.* subgen. *Sessilium* species (12 of 26) have an EOO of 43,000 km² or less and occur in no more than three states (IUCN, 2021). One species is listed as Critically Endangered, three are listed as Endangered, two are listed as Vulnerable, and three are listed as Near Threatened by the IUCN Red List (IUCN, 2021). In addition, six sessile *Trillium* species are variously distributed in western mountain ranges covering parts of Washington, Oregon, California, and Idaho (Freeman, 1975; Case and Case, 1997; Case, 2003). This suggests that a common ancestor of *T.* subgen. *Sessilium* was more widespread in the late Miocene and early Pliocene epochs and that the western and eastern states were once connected by some corridor of mesic forests (Tiffney and Manchester, 2001).

Morphology-based concepts of *Trillium* subgen. *Sessilium*

Freeman (1975) used his observations of geographic and morphological similarities among species of *Trillium* subgen. *Sessilium* to align species into three groups (see Table 2.1). Group I included *T. recurvatum* and *T. lancifolium* which were united by recurved sepals, incurved anthers, thin rhizomes and an eastern to central US distribution. Group II, the *Trillium sessile* group, included seven species of the eastern United States. Morphological similarities of the group include anther connectives extended beyond the anther sacs, sharply angled ovaries, thick compact rhizomes, and typically linear stigmas (except in *T. sessile* and *T. decumbens*). Freeman (1975) placed 13 species in a “residual” Group III, the *Trillium maculatum* group, which also included five western species. Species of Group III have in common lateral anther dehiscence, anther connectives which do not or only slightly extend beyond anther sacs, and subulate stigmas. *Trillium cuneatum* was placed in Group III and was thought to be most closely related to *T. luteum*, based on intermediate forms observed where the two species overlap in distribution, and to *T. maculatum*, based on intermediate forms in individuals although the distributions of the two species are separate and distinct (Freeman, 1975). The geographic disjunction pattern of Group III between the western and eastern US is not seen in the other two groups. Murray (1983; 1984) subsequently named three sections in *Trillium* subgen. *Sessilium* (as *Phyllantherum*): *T.* sect. *Recurvata* A.E. Murray, *T.* sect. *Sessilia* A.E. Murray, and *T.* sect. *Maculata* A.E. Murray along with subsections, series, and subseries (see Table 2.1). This classification scheme was wholly based on Freeman’s (1975) previous treatment – recognizing

his three groups without alteration – and for the most part offered no new information except in the combining of some species together into subsections without clear explanation of rationale.

Recent molecular-based phylogenetic studies have shown that morphological classifications have not fully reflected relationships in subgenus *Sessilium*. Schilling et al. (2013) pointed out a few instances where Freeman's three group concept failed. For example, *T. sessile* (Group II) was placed in a clade with members of Group III. The remainder of Group II members formed a paraphyletic grade. Group III was monophyletic except for the inclusion of *T. sessile* and the exclusion of *T. albidum* (Group III). Though it was not expressly discussed, the phylogenetic analysis of Schilling et al. (2019) provided evidence that at least some members of Group II formed a clade and should share a common morphological group, namely, *T. discolor* T. Wray ex Hook., *T. decumbens*, *T. underwoodii* Small, *T. decipiens* J.D. Freeman, and *T. reliquum* J.D. Freeman. Likewise, Group III members *T. foetidissimum* J.D. Freeman, *T. ludovicianum*, *T. gracile* J.D. Freeman, *T. viride*, and *T. viridescens* Nutt. formed a clade. This, however, excluded the remaining Group III members which themselves formed two separate clades: one clade with *T. cuneatum*, *T. maculatum*, and *T. luteum* samples, and a second clade with only the western US species. Similar to Schilling et al. (2013), *Trillium sessile* (Group II) samples formed a clade with two Group III clades (Schilling et al., 2019). Group I members formed a clade in both analyses.

Phylogenetic analyses have also suggested in which morphological group the most recently described *T.* subgen. *Sessilium* species might have been placed had they been recognized by Freeman or Murray. One phylogenetic analysis included *T. parviflorum* in a western US-only clade with Group III members and placed it as sister with *T. albidum*, the only other white-flowered species (Schilling et al., 2019). This result is not surprising because *T. parviflorum* also possesses Group III morphological traits and only occurs in the western US. Likewise, *T. tennesseense* and *T. oostingii* both share Group I morphological traits and phylogenetic placement (Schilling et al., 2013; Schilling et al., 2019). The case of *T. delicatum* was less clear: it formed a clade with only *T. stamineum* Harb. of Group II (Schilling et al., 2019). The *T. delicatum* + *T. stamineum* clade stood alone in the phylogeny, apart from other Group II representatives (Schilling et al., 2019). While *T. delicatum* shares the Group II characteristics of prolonged anther connectives and angled ovaries, it has slender, *T. lancifolium* like rhizomes, rather than thick, compact rhizomes.

Objectives

The objectives of this study are 1) to obtain a comprehensive species level phylogeny of *T.* subgen. *Sessilium* with an emphasis on sampling multiple populations of *T. cuneatum*, especially where variability had been observed in populations, employing a broad set of variable plastid and nuclear markers (*ITS*, *trnH-psbA*, *rpl32-trnL*^(UAG), *ycf1*, and *ycf2*); 2) to analyze the putative *T. cuneatum* complex to evaluate its putative paraphyly and cryptic lineages and to clarify its evolutionary history within *T.* subgen. *Sessilium*; 3) to assess the taxonomy of *T. cuneatum* while examining morphological and geographical correlates to clades delineated in the *T. cuneatum* complex phylogeny; 4) to examine previous morphological classifications and sections of *T.* subgen. *Sessilium* in light of the results of the phylogenetic analysis. A result of the molecular and morphological assessments is that we recognized and described two novel species, *T. freemanii* sp. nov. and *T. radiatum* sp. nov.

MATERIALS AND METHODS

Taxon sampling

In all, 150 *Trillium* samples were used in the study including 28 *T.* subgen. *Sessilium* species (see Table 2.2). One or two populations of each species within *T.* subgen. *Sessilium*, including central and western US species, were analyzed to produce a broad taxonomic sampling of the subgenus. Additionally, individuals from multiple populations of *T. cuneatum* (74 pops.), *T. maculatum* (9 pops.), and *T. luteum* (13 pops.) were sampled across the geographic distribution to allow a detailed assessment of the variability in these three species. Additional samples of *T. ludovicianum* were used (4 pops.) because that species has been suggested to hybridize with *T. cuneatum* and has been suggested to resemble the *T. cuneatum* populations located near Chattanooga, TN. Representatives of all other major lineages of *Trillium* were also included as outgroups. DNA extractions from herbarium specimens older than about a year failed, making it necessary to use freshly collected material. New samples were collected in the field and leaf material was frozen at -80°C or dried in silica gel and stored until DNA extracts could be prepared.

Morphologic examination and microscopic imaging

In addition to herbarium voucher specimens, living cultivated samples and fresh field collections of several populations of *Trillium cuneatum*, *T. luteum*, and *T. maculatum* were used in this study to observe and compare morphologic traits and three-dimensional features that could not be otherwise observed in dried specimens. Populations of *T. cuneatum* were grouped into lineages based on DNA sequences of samples. These observations were made over a few successive years during the course of this research. Microscopic imaging and Z-stack imaging were performed using a Leica stereo microscope with Leica Application Suite X software (Leica Microsystems, Wetzlar, Germany) at the University of Tennessee Herbarium (TENN).

Molecular procedures

Whole genomic DNA extracts were prepared by flash freezing and pulverizing approximately 0.1 g of each leaf sample in liquid nitrogen, and then by processing with the DNEasy Plant Minikit and its default protocols (Qiagen, Inc., Hilden, Germany). Also processed were eight DNA extractions from a previous study that had been stored at the University of Tennessee Knoxville and kept at -20°C continuously since that time (DNA# 718 – 2139, Table 2.2, Farmer, 2007).

PCR amplification was performed in 20 µL reactions which included <0.5µg/50µL DNA template and master mix (10X PCR buffer, 1.5 mM MgCl₂, 0.2 mM each dNTP, 1.25 u GoTaq DNA polymerase (Promega, Madison, WI, USA), and 0.2 µM each of forward and reverse primers (Eurofins Scientific, Luxembourg)). Amplification protocol of the nuclear ribosomal *ITS* gene region was performed as in Schilling et al. (2007). Amplification protocols of chloroplast intergenic spacer regions *trnH-psbA* and *rpl32-trnL*^(UAG) were performed as described in Shaw et al. (2005) and as in Shaw et al. (2007), respectively. Partial regions of both the *ycf1* and *ycf2* plastid coding genes were used in the study. *Ycf1* has been shown to be a gene with excellent phylogenetic utility in Orchidaceae, and an excellent DNA barcode candidate for land plants, in general (Neubig et al. 2009, Dong et al. 2015). Similarly, *ycf2* has been shown to provide well-resolved phylogenies in angiosperms which are consistent with phylogenies generated from much larger whole plastome datasets (Huang et al. 2010). We found *ycf1* and *ycf2* to be relatively divergent in *Trillium* and therefore used portions of them in this study to

reveal phylogenetic signal at an infraspecific level. Novel primers were designed for region *ycf1* (Forward primer “TY1F2” 5’ to 3’: AGCTCCTTGCCAAGGTTTC and Reverse primer “TY1R2” 5’ to 3’: AAAAGGATCCATGCGTGCTC). Amplification protocol for region *ycf1* was similar to the “slow and cold” *rpl16* protocol described in Shaw et al. (2007) and performed as follows: starting template 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, ramp 0.2°C/s to 65°C, primer extension at 65°C for 4 min., followed by a final extension at 65°C for 10 min. Novel primers were designed for region *ycf2* (Forward primer “97F” 5’ to 3’: GATCCAATAGCGTTCCGTTAG and Reverse primer “1164R” 5’ to 3’: CTCATTATCAGACAATCACTTATT). Amplification protocol for *ycf2* was performed as for *ycf1*.

PCR products were checked using gel electrophoresis on 1% agarose gels and subsequently purified using ExoSAP-IT PCR Product Cleanup Reagent (Affymetrix, Inc., USB, Cleveland, Ohio, USA). A NanoDrop 2000 spectrophotometer was used to measure PCR product concentration prior to Sanger sequencing and DNA extract concentration prior to library prep for next-generation sequencing (NGS) (Thermo Scientific, Waltham, MA, USA). Sanger sequencing was performed at the University of Tennessee Genomics Core (Knoxville, Tennessee, USA) using the Applied Biosystems 3730 Genetic Analyzer electrophoresis instrument and the BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Waltham, MA, USA). Library prep and NGS was performed by Global Biologics, LLC (Columbia, MO, USA) on the Illumina Hi-Seq platform to produce 100 bp reads for 17 *Trillium* subgen. *Sessilium* taxa, and one other *Trillium* taxon used in this study (see Table 2.2).

Electropherograms of initial Sanger sequence data were examined and manually edited either in the Geneious v. 8.1.9 software platform (<https://www.geneious.com>) or MEGA v.5.2.2 (Tamura et al., 2011) application prior to phylogenetic analyses. Sequence data obtained from NGS reads for selected samples were assembled in Geneious using the protocols as described in Lampley (Dissertation Ch. 1).

Phylogenetic analyses

Sanger and NGS generated sequence data were aligned using the program MAFFT v7.017 (Katoh and Standley, 2013) with default settings, and further adjusted by eye within Geneious. The datasets for the gene regions *ITS*, *trnH-psbA*, *rpl32-trnL*^(UAG), *ycf1*, and *ycf2* were each aligned separately and then concatenated in Geneious. The partial gene region *ycf2* was treated as two separate partitions, *97F* and *1164R*, making sure to remove overlap, and aligned separately. The program FastGap v.1.2 (Borchsenius, 2009) was then used to code indels as characters using the method of Simmons and Ochoterena (2000). Coded characters were added to the end of the aligned matrix.

Partition homogeneity tests (or, incongruence length difference (ILD) test, Farris et al., 1995a, 1995b) implemented in PAUP* (Swofford, 2003) were run to check among the gene alignment partitions for character incongruence. The program ran a heuristic search using the tree bisection and re-connection (TBR) branch swapping method with simple taxon addition in 500 iterations.

Phylogenies were inferred via Maximum Likelihood (ML) and Bayesian statistical methods for the final concatenated dataset of 150 *Trillium* samples. The ML analysis was implemented in RAxML v.7.2.8 (Stamatakis, 2006) within the Geneious program. The Bayesian analysis was implemented in MrBayes v.3.2.6 (Ronquist and Huelsenbeck, 2003) also within Geneious. The General Time Reversible model with gamma-distributed rate variation and a

proportion of invariant sites (GTR + I + G; Lampley, Dissertation Ch. 1) was used following results of Guindon and Gascuel (2003) and Darriba et al. (2012). For the Bayesian analysis, a total chain length of 1,100,000 generations was produced on four heated chains (temperature = 0.2) with a subsampling frequency of once every 500 generations. The effective sample size (ESS) value, calculated in Geneious, was used as a convergence diagnostic and the first 9% of trees (100,000) was discarded as “burn-in.” A bootstrapping analysis in RAxML, starting with a complete random tree, produced 500 replicates which were used to calculate a majority rule consensus tree with bootstrap support scores for each branch.

RESULTS

An aligned matrix of concatenated nuclear ribosomal (*ITS*) and chloroplast gene regions (*trnH-psbA*, *rpl32-trnL* partial *ycf1*, and partial *ycf2*) was used in the phylogenetic analyses. The aligned matrix had a total of 5,764 characters, including 219 indel coded characters. A total of 3,785 sites were variable, excluding indel coded characters. Pairwise comparisons from partition homogeneity tests resulted in test statistics that were considered non-significant ($\alpha > 0.05$) except for comparisons between *ITS* + *trnH-psbA* and also *ITS* + *ycf1* which were only slightly lower ($\alpha = 0.04$ for each), but deemed reasonably robust ($\alpha > 0.01$) to support the inclusion of all gene regions with the goal of improving phylogenetic accuracy (Cunningham, 1997). Of 156 samples analyzed in the matrix, 44 were removed due to identity with other samples across the combined gene regions. The 44 taxa were included in the tree figure by displaying names to the right of corresponding identical sequences (Fig. 2.2).

The results of the Bayesian Inference (BI) and Maximum Likelihood (ML) analyses were in approximate agreement and therefore support scores for both are presented on the Bayesian tree (Fig. 2.2). Trees in both the BI and ML analyses were rooted on *T. erectum* of *T. subgen. Trillium*. The first split above *T. catesbaei* separated two clades. One with moderate support values (66.4 bootstrap support (BS) and 0.75 posterior probability (PP)) was composed of sessile species of the lower southeastern US; the other included the remaining members of *T. subgen. Sessilium*. Within the first clade, the ‘Southeastern Clade,’ *T. discolor* was sister to a strongly supported subclade (100 BS, 1.0 PP), within which *T. decumbens* was placed sister to a group that had strong support (99 BS, 1.0 PP) consisting of *T. reliquum*, *T. underwoodii*, and *T. decipiens*. Samples of *T. underwoodii* and *T. decipiens* formed a strongly supported clade (100 BS, 1.0 PP). *Trillium stamineum* was placed in a moderately supported clade with *T. delicatum* (71 BS, 0.99 PP); this clade was sister to a moderately supported clade (86 BS, 1.0 PP) consisting of the remainder of sessile species. At the next level, a split separated *T. petiolatum* Pursh from the rest of the sessile species; support for the latter clade was strong (100 BS, 1.0 PP). The remainder of the western US sessile species formed a strongly supported clade, the ‘Western Clade,’ with successive splits of *T. angustipetalum* (Torr.) J.D. Freeman, *T. chloropetalum* (Torr.) Howell, *T. kurabayashii* J.D. Freeman, and *T. albidum* + *T. parviflorum*.

Within the weakly supported clade (0.59 PP) placed sister to the Western Clade, a split separated two clades. One with strong support (100 BS, 1.0 PP) included *T. cuneatum* and allies (referred to as the ‘Cuneatum Complex’ clade) and the other moderately supported clade (79 BS, 1.0 PP) split into two subclades. The first subclade had moderate support (70 BS, 1.0 PP). Within it, a strongly supported clade (100 BS, 1.0 PP) of *T. sessile* samples was placed sister to a strongly supported clade (100 BS, 1.0 PP) (referred to as the ‘Thin Rhizome Clade’) which had successive splits of *T. oostingii*, *T. tennesseeense*, *T. lancifolium* and *T. lancifolium* + *T. recurvatum* samples. The second subclade was strongly supported (96 BS, 1.0 PP) and included

sessile species from the central southern US. This ‘Central Clade’ had successive splits of *T. viride*, *T. ludovicianum*, *T. foetidissimum*, *T. gracile* and *T. viridescens*. In contrast to the BI analysis, the results of the ML analysis placed the Western Clade as sister to the Cuneatum Complex clade.

The Cuneatum complex clade included samples of *T. cuneatum*, *T. luteum*, and *T. maculatum* and separated into seven subclades. Through the inclusion of *T. luteum* and *T. maculatum*, *T. cuneatum* was paraphyletic. Clade 1 (labeled in Fig. 2.2) was strongly supported (97 BS, 1.0 PP) and included all *T. luteum* samples used in the study as well as some *T. cuneatum* samples. Clade 1 was sister to a lineage containing Clades 2–7. Clade 2, consisting of *T. cuneatum* samples, was highly supported (100 BS, 1 PP) and was placed as sister to the moderately supported Clade 3 (73 BS, 1 PP). Clade 3 (or the ‘Radiatum Clade’) was also composed of *T. cuneatum* samples. Clades 2 and 3 formed a common clade with moderate support (0.89 PP) according to the BI analysis, however this clade was not resolved in the ML analysis. Clade 4 was well supported (85 BS, 1.0 PP) in both analyses and placed sister to Clades 2 + 3 in the BI analysis. Clade 4 (or the ‘Freemanii Clade’) also consisted of samples currently recognized as *T. cuneatum*. Sister to Clade 2 + 3 + 4 was the well-supported (86 BS, 1 PP) Clade 5 + 6 + 7. Clade 5 was strongly supported (99 BS, 1.0 PP) and included samples of *T. cuneatum*. Clade 5 was placed sister to Clade 6 + 7 in the BI analysis. Clade 6 was resolved in the BI analysis (0.99 PP) and consisted of two branches of *T. cuneatum* samples. Clade 7 was highly supported (100 BS, 1.0 PP) and it contained all of the of *T. maculatum* representatives used in the study.

The entities from Clade 3 (‘Radiatum Clade’) and Clade 4 (‘Freemanii Clade’) are described here (taxonomic treatment below) as distinct species: *T. freemanii* sp. nov. and *T. radiatum* sp. nov., respectively.

DISCUSSION

The current phylogenetic investigation, based on chloroplast and nuclear ribosomal sequence data, employing comprehensive sampling of the *Trillium cuneatum* complex across much of its distribution, supported previous indications that this taxon is not monophyletic. Rather, six genetically and geographically distinct lineages emerged providing evidence that *T. cuneatum* is a species complex with cryptic sister species. The phylogenetic analysis also offered a surprising interpretation of the evolutionary history of two recognized species, *T. maculatum* and *T. luteum*. A broad sampling of *Trillium maculatum* populations formed a robustly supported clade which was subsumed within the *T. cuneatum* s.l. clade, while *T. luteum* formed a weakly supported cluster within a clade containing samples of *T. cuneatum* s.s. These results informed a morphological examination and taxonomic revision. Two novel species are proposed and described herewith, while there remains the prospect that more species may yet be elucidated from the more cryptic lineages. Finally, the analysis provided further support for our understanding of the biogeography and evolutionary relationships within *T.* subgen. *Sessilium* based on molecular data, in contrast to morphological classifications proposed in the past.

***Trillium cuneatum* s.l., a species complex**

The results of the phylogenetic analysis revealed that *T. cuneatum* is paraphyletic and should be considered a species complex. *Trillium luteum* and *T. maculatum* were included in the Cuneatum Complex with strong support and showed that *T. cuneatum* is not monophyletic. The phylogenetic inference of the Cuneatum Complex clade suggested that there are at least five

other lineages which are currently identified as *T. cuneatum* s.l. besides the *T. cuneatum* s.s. + *T. luteum* and *T. maculatum* lineages. This result supports an eight species concept within the Cuneatum Complex or, alternatively, the circumscription of *T. cuneatum* could be expanded to include *T. maculatum* and *T. luteum*. The latter may not be a universally acceptable solution, however, because these are two widely recognized species. Additionally, the confirmation that multiple distinct genetic lineages can be detected is not surprising: *T. cuneatum* has long been noted for its morphological variability within and among populations (Freeman, 1975), and the reasons for the variability may be the presence of cryptic taxa.

The results of the phylogenetic inference did not support the separation of *T. luteum* and *T. cuneatum* s.s. Clade 1 (labeled in Fig. 2.2) was strongly supported (97 BS, 1.0 PP) and included all *T. luteum* samples used in the study as well as *T. cuneatum* s.s. samples, so-called because the clade included a *T. cuneatum* sample from North Carolina near the location of the type specimen of *T. cuneatum* and included other samples from adjoining eastern and northern parts of its recognized distribution (see map, Fig. 2.3). The *T. luteum* samples of this study were designated as such based on sampling location (occurring in the Ridge and Valley province or the southern Appalachians) and by only choosing populations with yellow-flowered individuals with no sign of maroon color morphs at the site. Yellow or green-flowered individuals were considered as color morphs of *T. cuneatum* in mixed populations. All *T. luteum* representatives, but one (4339_luteu), formed a weakly supported clade (0.57 PP) within Clade 1 in the Bayesian analysis likely based on a 1 nucleotide synapomorphy in the ITS alignment. In the ML analysis, *T. luteum* samples joined most *T. cuneatum* samples in a less resolved clade with mixed support (64 BS, 0.99 PP). A possible reason for the inconclusive result is that *T. luteum* is in the process of speciation, or alternatively, that more informative molecular markers are needed to clarify this relationship. Evidence to support the species status of *T. luteum* can be found in its consistent flower color, complete lack of pigmentation, consistent lemon floral scent, its Ridge and Valley or southern Appalachians distribution and its irregular and less-angled ovary shape, discussed in more detail below.

Clade 2 may represent a cryptic species within the Cuneatum Complex. Clade 2 received strong support and was also consistently supported by individual gene alignments. It contained samples of *Trillium cuneatum* s.l. from Walker Co., AL, Giles Co., TN, and Pontotoc Co., MS. Sample 4655_WalAL of this clade was a yellow-flowered individual from a noteworthy population purported to be one of the few instances of *T. luteum* in the state of Alabama. A few disjunct populations of yellow-flowered *Trillium* occur in central Alabama and have been called *T. luteum* by some, though botanists disagree on its identification (T.W. Barger, pers. comm., 2019; B. Keener, pers. comm., 2019; A. Schotz, pers. comm., 2019; D. Spaulding, pers. comm., 2019; Meredith et al., 2020c; Weakley, 2020). However, the current analysis suggests that this is neither *T. luteum* nor *T. cuneatum* s.s., but rather a separate lineage in its own right. Time constraints precluded detailed morphological analysis of this lineage, which will be a topic of a future study.

Clades 3 and 4 each received moderately strong support (73 BS/1 PP and 85 BS/1 PP, respectively) and consisted of samples which showed distinct geographic patterns. Clade 3 (the Radiatum clade) was composed of several *T. cuneatum* samples which had in common a distribution throughout the southern portion of the Ridge and Valley physiographic province and areas around Lookout Mtn. in northwestern Georgia, northeastern Alabama and southeastern Tennessee. Clade 4 (the Freemanii clade) consisted of samples currently recognized as *T. cuneatum* from populations in Alabama and Tennessee bounded by the middle course of the

Tennessee River and the Elk River to the northwest. This included the noted populations from the Chattanooga, TN area, mentioned above. Samples from each of these clades were examined closely in a morphological study, discussed in further detail below. The clades also form the basis of two suggested new species, also discussed below.

Clades 5 and 6 were strongly supported but comprised widespread or disjunct samples. Clade 5 was strongly supported (99 BS, 1.0 PP) and included widespread samples from *Trillium cuneatum* populations occurring in the southern distribution of that species across Georgia and Alabama (see map, Fig. 2.3). Three groups were resolved within Clade 5 with mixed support: the first branch (1.0 PP) consisted of samples from central Georgia and eastern Alabama, the second branch (88 BS, 1.0 PP) samples were from central Alabama, and the third branch (0.97 PP), sister to the second branch, contained samples from northeastern Alabama and northwestern Georgia. One of these samples, 3684_DadGA, occurred in sympatry with a sample from Clade 3 on the western slopes of Lookout Mountain in the vicinity of Cloudland Canyon and was in bud at the time of collection. Clade 6 consisted of two branches of disjunct *T. cuneatum* s.l. samples. The upper branch (99 BS, 1.0 PP) included *T. cuneatum* s.l. samples from populations along Guntersville Lake (Tennessee River) and North Sauty Creek in Marshall and Jackson counties of northeastern Alabama, while the lower branch contained samples from *T. cuneatum* s.l. populations in Hardin, Wayne, Lewis, and Maury counties, distributed in central, southern Tennessee, east of the lower course of the Tennessee River. This clade was resolved in the Bayesian analysis (0.99 PP), but formed two separate clades in the Maximum Likelihood analysis.

The current study confirmed the monophyly of *Trillium maculatum* and provided support for its recognition as a species. Clade 7 was highly supported (100 BS, 1.0 PP) and contained all of the *T. maculatum* representatives used in the study. Although, *T. maculatum* has often been misidentified in the past, there are convincing morphologic traits which make it distinct in addition to its distribution in the Coastal Plain province (Freeman, 1975; Case and Case, 1997). Clade 6 was sister to the *T. maculatum* clade in the Bayesian analysis (0.99 PP) and suggests that Clade 6 representatives could be included in the circumscription of *T. maculatum*. However, these plants were from a different geographic province than *T. maculatum* and did not share many of its distinctive traits such as strongly mottled bracts, oblanceolate-spatulate petals, 3-angled ovary, spatulate anther connectives, late winter flowering phenology, and banana-like floral fragrance (Case, 2003; J. Lampley, pers. obs.).

Relationships in Trillium subgen. Sessilium

The results based on the dataset of the current study showed some differences from a past study regarding relationships within other groups of *Trillium* subgen. *Sessilium* and provided greater resolution. The members of the Central clade in the current study were the same species as in a clade in Schilling et al. (2019), however, the results revealed slightly different relationships. For example, *T. viridescens* was sister to *T. gracile* rather than *T. viride*. Both green-flowered species, *T. viride* and *T. viridescens* Nutt., are often mistaken for one another and are best differentiated by the presence or absence of adaxial leaf stomata. *Trillium viride* occurs between Illinois and Missouri, *T. viridescens* occurs in Arkansas, Missouri and Oklahoma, and *T. gracile* occurs in southeastern Texas; their distributions do not overlap. In addition, *T. ludovicianum* was sister to a clade with *T. foetidissimum* and *T. gracile* + *T. viridescens* rather than only sister to *T. gracile*. *Trillium ludovicianum* is centrally located in Louisiana among the other three species. In the current study, *T. sessile* was sister to the Thin Rhizome clade rather

than in an unresolved clade with the Central and Cuneatum Complex clades. *Trillium sessile* has thick fleshy rhizomes rather than thin and does not have incurved anthers, but does have in common with *T. lancifolium* a six-angled ovary and anther connectives which extend beyond the anther sacs. Within the Thin Rhizome clade, *T. oostingii* was sister to a clade of *T. tennesseense*, *T. lancifolium*, and *T. recurvatum* rather than sister to *T. tennesseense*, which it most closely resembles. *Trillium lancifolium* was paraphyletic within this clade, however. A *T. lancifolium* sample (4410) from Dallas Co. in southwestern Alabama was sister to a highly supported clade (100 BS, 1.0 PP) containing another *T. lancifolium* sample (4400) from Calhoun Co. in northeastern Alabama, which was in turn sister to a highly supported monophyletic clade (100 BS, 1.0 PP) of *T. recurvatum* samples. *Trillium lancifolium*, which is variable in morphology and flowering phenology across its distribution, was shown as paraphyletic in previous studies (Schilling et al., 2013; Schilling et al., 2019). By contrast, the position of *T. petiolatum* and the position, topology, and constituents of the Southeastern Clade and the *T. stamineum* + *T. delicatum* clade were in agreement with Schilling et al. (2019).

The current phylogenetic analysis provided further support for an eastern North American origin for *Trillium* subgen. *Sessilium* with subsequent expansions to the west. Lampley (Dissertation Ch. 1) inferred that *T.* subgen. *Sessilium* had a mean stem age of 9.6 mya and originated in eastern North America. Schilling et al. (2019) suggested that *T.* subgen. *Sessilium* originated in the southeastern US due to a basal-branching clade of southeastern species and suggested that *T. petiolatum* and the clade of western US species made the shift westward in two separate dispersal events. *Trillium petiolatum*, a species from west of the Rocky Mountains and Columbia Plateau was the only western US sessile *Trillium* excluded from the Western Clade in that and the current study. The current phylogenetic results were more resolved for the Western clade, differing from Schilling et al. (2019) in that the clade was sister to the clade including *T. sessile*, the Thin Rhizome Clade, the Central Clade, and the Cuneatum Complex rather than the Western Clade forming a less resolved group with the same. The order of branching in this portion of the tree suggested an alternative hypothesis may be possible: *T.* subgen. *Sessilium* may have expanded westward after an eastern origin, *T. petiolatum* subsequently split and remained in the west, then a following split led to the Western Clade lineage which also remained in the west, followed by an expansion back to the central US and a split which led to *T. sessile* (partly of the central US) and the Central Clade. The *T. sessile* lineage may have expanded further east, as its current distribution spreads to the eastern most US states, where the Thin Rhizome clade split and expanded further south. In this scenario, the Cuneatum Complex clade could have also split off from a centrally located lineage and expanded back to the southeast.

A modification for Trillium sections and morphological-based group classification

The current study showed that the three sections of *Trillium* subgen. *Sessilium* based on the morphological group concept of Freeman (1975) do not give the full picture of evolutionary relationships and should be modified to include five new sections. Group I was monophyletic in the current phylogeny and may be retained as Sect. *Recurvata*, and include *T. recurvatum*, *T. lancifolium*, *T. oostingii*, and *T. tennesseense* (i.e., the Thin Rhizome group). The Group II morphological concept was accurate for the Southeastern Clade, but as a whole may be broken into three groups. For example, *T. sessile* did not form a clade with other Group II members, but was sister to Group I, and *T. stamineum* formed a clade with newly described *T. delicatum*. Therefore, I suggest that Sect. *Sessilia* only include *T. sessile* and that one new section be created

for the Southeastern Clade, which includes *T. decipiens*, *T. decumbens*, *T. discolor*, *T. reliquum*, and *T. underwoodii*, and one new section to include *T. stamineum* and *T. delicatum*. The Group III morphological concept was maintained in three separate clades: the Cuneatum Complex, the Central Clade, and the Western Clade. In addition, *T. petiolatum* of Group III formed a clade by itself. Therefore Group III may be broken into four sections: first, Sect. *Maculata* which would include *T. maculatum*, *T. cuneatum*, *T. luteum* and new species of this complex, second, a new section for the Western Clade, which would include *T. albidum*, *T. angustipetalum*, *T. chloropetalum*, *T. kurabayashii*, and *T. parviflorum*, third, a new section for the Central Clade, which would include *T. foetidissimum*, *T. gracile*, *T. ludovicianum*, *T. viride*, and *T. viridescens*, and finally one new section for *T. petiolatum*.

Morphological observations of the Cuneatum Complex

The results of the phylogenetic analysis helped guide a morphological examination which revealed subtle morphological differences of two putative cryptic species of the *T. cuneatum* complex. Representatives of *T. luteum*, *T. cuneatum* s.s., *T. maculatum*, Clade 4 ('*T. freemanii*') and Clade 3 ('*T. radiatum*') were compared for morphological differences that could aid in identification among the cryptic taxa (Fig. 2.1). The following general trends were observed though it must be noted that individuals within species and populations of *Trillium* are highly variable. In relative ovary position to androecium, stamens tended to be taller or slightly taller than ovary height in the group, however '*T. freemanii*' representatives often had stigmatic branches which extended above the anthers. '*Trillium radiatum*' representatives often had a much shorter ovary and stigma compared with stamen height (Fig. 2.4). A striking distinction was found in the anthers: '*T. radiatum*' had the most robust stamens of the group (Fig. 2.5). In cross-section it can be seen that most of the anthers were relatively flat abaxially with a slight convex aspect on the adaxial side, however, '*T. radiatum*' anther connectives tended to have rounded abaxial sides with prominent introrse anther sacs. The slightly extended anther connective apex was often slightly incurved which together with the relatively large anther sacs created a channeled appearance to the adaxial side of the anther. *Trillium maculatum* had distinctive anther connectives which were broad and somewhat spatulate in form. A comparison of ovaries in cross-section also revealed some differences across the group (Fig. 2.6). *Trillium cuneatum* ovaries typically had a hexagonal shape in cross-section, as did those of *T. luteum*, however the latter had much smoother angles and was typically more irregular in shape than the others. *Trillium maculatum* ovaries were observed to be nearly triangular in cross-section. '*Trillium freemanii*' ovaries were strongly six angled in cross-section with the angles coming to sharp points, whereas '*T. radiatum*' ovaries typically had deep longitudinal furrows and strong angles. The stigmatic branches of *T. cuneatum* and '*T. freemanii*' were typically more deeply divided and divergent and recurved than the others in the group. The stigmatic surfaces were also compared at low magnification (Fig. 2.7). *Trillium* is known to have dry stigmas with distinct zones of papillate stigmatic surfaces with unicellular papillae, traits correlated with self-incompatibility in plants (Heslop-Harrison and Shivanna, 1977). Distinct, translucent papillae were observed in *T. cuneatum*, *T. luteum*, and '*T. freemanii*' and had an appearance suggestive of quartz crystals. These clear papillae were most difficult to view in *T. luteum* due to the lack of contrasting pigmentation in the stigmas of that species. The papillae in *T. maculatum* appeared more irregular compared with the others in the group, but were also difficult to distinguish due to the dark purple pigmentation of both stigma and stigmatic surface. By contrast, '*T. radiatum*' had relatively short, dense, and more uniform papillae. Though this seemed to be a useful

diagnostic character in fresh specimens, the trait was difficult to observe in dried specimens and would even be an inconvenient character in the field without magnification.

TAXONOMIC TREATMENT

Trillium freemanii Lampley, Floden, & E.E. Schill, sp. nov.

TYPE: North America. United States. **TENNESSEE:** **Marion Co.:** Fishtrap Road, rocky woods above Battle Creek, Ca. 0.5 mile from I-24, 35°4'18.42"N, 85°44'36.41"W, 1 Mar 2013, Aaron Floden with E.E. Schilling 2340 (holotype: TENN; isotypes: to be distributed later).

Similar to *T. cuneatum*, but differing in earlier flowering time – blooming as early as February 28th and as late as April 3rd, whereas *T. cuneatum* s.s., of the lineage in the northern and eastern part of its range, blooms ca. March through mid-April. Also, the narrower petals of *T. freemanii* which it holds erect but basally spreading away from the flower center, are separate exposing the flower center. In contrast, the wider petals of *T. cuneatum* typically touch as they and the sepals surround the center of the flower, obscuring it from view. Also, differing by its leaves which are separate and typically draping downward and not overlapping and held variously as in *T. cuneatum*. *Trillium freemanii* also has anthers with a relatively thinner, narrower appearance than *T. cuneatum* which has thicker anther connectives and larger pollen sacs. *Trillium freemanii* differs from *T. ludovicianum* by geography, the latter inhabits Louisiana, eastern Texas and southwestern Mississippi. *Trillium ludovicianum* has a smaller stature, with stems up to 26 cm whereas *T. freemanii* has been observed with stems up to 49 cm tall. The lanceolate-ovate leaves of *T. ludovicianum* are somewhat narrower than *T. freemanii* leaves (> 2x longer than wide vs. an average 1.7x long as wide in *T. freemanii*) and tend to have a pale stripe or stripes whereas *T. freemanii* is pale with random dark mottling and not striped. *Trillium freemanii* also differs from these by fragrance: it smells like fermented fruit or vinegar whereas *T. cuneatum* flowers typically smell spicy sweet, *T. ludovicianum* smells of carrion.

Description

Perennial rhizomatous herb. **Rhizomes** compact, not slender, creamy-tan in color. **Scapes** erect, glabrous, often maroon near ground surface, pale green above, 0.3 – 0.9 cm in diameter, ca. 20 – 44 cm tall. **Leaves** sessile, glabrous, broadly ovate or rotund, with broadest point at middle or below the middle (toward the leaf base), ca. 1.7x long as wide, 6.7 – 12.2 cm long, 4.1 – 10.4 cm wide; color subtly 2-3 toned, mottled with varying shades of green; with three primary veins, acuminate or somewhat acute apices, tapering or rounded bases, and slightly undulate margins; leaves separate or barely touching and in mature plants, held aloft yet draping downward at a ca. 45° angle or less relative to the stem. **Sepals** spreading perpendicular to stem typically with a gentle arc, the distal half sometimes recurved or projecting subtly upward; color green, green with maroon streaking in the veins, or greenish-maroon; narrowly lanceolate with acute tips, ca. 4x long as wide, 2.5 – 5.3 cm long, 0.7 – 1.3 cm wide. **Petals** narrow, on average 6x long as wide, 3.8 – 6.7 cm long, 0.8 – 1.4 cm width at widest point, though may be much wider in some individuals; narrowly elliptic, oblanceolate, or narrowly rhombic with slight claw and limb, apices narrowly acute; color variable in a population but generally maroon or greenish-maroon with maroon proximally; petals held spreading-erect, extending from attachment point at a 45° angle relative to sepals and collection of stamens, often curving upward or slightly incurved; petals not remotely touching one another, giving a rather open, candelabra appearance to the

center of the flower. **Stamens** six, in a tight upright cluster, 1.1 – 1.9 cm long; anthers with relatively tall, narrow, and frontally flattened appearance; anther connective dark maroon with two pale yellow pollen sacs on either side with latrorse dehiscence; anther connective barely extends beyond pollen sacs short, filament short, maroon. **Ovary** ovoid in shape, dark maroon in color, with three stigma lobes, typically somewhat spreading and recurved, suggestive of a jester's hat, lobes ca. 0.5x as long as entire ovary; narrow in anthesis, ca. 1.0 – 1.5 cm long including stigmas, nearly equal to or slightly longer than stamens. **Fruit** ovoid, but more rounded than during anthesis, with softened angles. **Seeds** smooth, light tan in color, somewhat ovoid to reniform in shape, with fleshy elaiosome attached; ca. 17 – 59 seeds per fruit. **Floral fragrance** of somewhat pleasant fermented fruit or fruity vinegar. Fig. 2.8.

Phenology

Flowering from late February through early April. Fruit maturing as late as early July.

Distribution and habitat

Trillium freemanii populations are found in rich habitats of mixed mesophytic forests with other spring ephemerals, often on slopes with boulder fields. The species inhabits lower slopes of a southerly portion of the Cumberland Plateau where Tennessee, Georgia, and Alabama intersect, typically characterized by underlying early Carboniferous limestone, shale, and dolomite strata, on either side of the Sequatchie Valley and along the Tennessee River Gorge (Neilson, 2016; Soil Survey Staff, 2021). The known distribution of *T. freemanii* is in five counties of southeastern Tennessee and four counties in northeastern Alabama (Fig. 2.9). This distribution particularly includes the numerous surrounding hollows and coves of the dissected plateau which were formed over time by erosion and water drainages. Populations can be found as far north as Falling Water, Tennessee on slopes below Walden Ridge at the western boundary of the Valley and Ridge physiographic province. In Alabama, the distribution continues southwestward down the Cumberland Plateau on either side of the Sequatchie Valley and Tennessee River among the slopes of the Jackson County Mountains to the west and Sand Mountain to the east. Populations of *T. freemanii* are distributed as far south as Lake Guntersville reservoir, the point where the Tennessee River changes course to head northwest through Alabama. Notably, *T. freemanii* can be found on wooded slopes of the Lookout Mountain escarpment at approximately 335 m elevation outside the Ruby Falls tourist attraction in Chattanooga, Tennessee. The species is not known to occur in the Valley and Ridge except where this region meets the Cumberland Plateau along the Tennessee River just north of Chattanooga. Populations have been found at elevations ranging from 176 m to 511 m above sea level.

Conservation

Trillium freemanii appears to be an endemic species bounded by the middle course of the Tennessee River and known to occur in at least nine counties of southern Tennessee and northeastern Alabama. Populations are known from locations in Coffee, Lincoln, Franklin, Hamilton, and Marion counties in Tennessee and Morgan, Madison, Marshall and Jackson counties in Alabama. Its Extent of Occurrence (EOO) was approximated at 9,042.262 km² using the GeoCAT software application (Visualization and Analysis Systems Technologies, 2021). GeoCAT was also used to perform the Area of Occupancy (AOO) calculation for *T. freemanii* on the basis of 2 km cells surrounding the precise data points of DNA barcoded populations (n=21). The estimated AOO of 84.000 km² suggests that an IUCN ranking of Vulnerable (VU) may be

appropriate. Many populations were observed between 2015-2019 and appeared to be typically moderately sized populations with little herbivore damage. Some poaching of plants is suspected at the type locality in recent years.

Etymology

The species epithet “*freemanii*” honors Auburn University professor and herbarium curator John Daniel Freeman (1942–1997) who wrote the monograph of *Trillium* subgenus *Sessilium* (as *Phyllantherum*) referenced in this study (Freeman, 1975). Freeman was also a *Trillium* expert in the field and described eight species and seven forms of *Trillium*.

Suggested common name

The common name, “Ruby Falls Trillium,” is suggested for *T. freemanii* in consideration of the large population of it outside the Ruby Falls attraction on Lookout Mountain in which it was first observed by gardeners as distinct.

Specimens examined

Tennessee: Coffee Co.: Short Springs Natural Area N of Tullahoma, Steep bank off of trail near Bobo Creek, Steep bank in mesic, deciduous woods, 30 May 2015, *JA Lampley with EE Schilling and B Crain* 290, 291 (TENN). **Franklin Co.:** 1.8 mi S of Huntland along CR-97, Near 35.026820, -86.253344, Mixed woods along roadside near Mathias Branch with ... Sinkhole and rich loam soil at the site, Abundant, vigorous plants, 03 April 2016, *JA Lampley and EE Schilling* 316 (TENN); On US-41A across from mountain overlook, 1/2 mi SW of Sewanee University of the South campus, Near 35.181904, -85.935683, Deciduous forest with boulders near intermittent waterfall/stream..., 03 April 2016, *JA Lampley and EE Schilling* 320, 321 (TENN); 1.8 mi S of Huntland along CR-97, Near 35.026820, -86.253344..., Mixed woods along roadside near Mathias Branch with..., Sinkhole and rich loam soil at the site..., 03 April 2016, *JA Lampley and EE Schilling* 322, 323, 324 (TENN); **Hamilton Co.:** Lookout Mt. Ruby Falls, surrounding parking area, 1 Mar 2013, *Aaron Floden & E.E. Schilling* 2337 (TENN). In mesic woodland beside Ruby Falls visitor center on Lookout Mountain, Chattanooga, 35.019319, -85.339701, Abundant, 26 March 2017, *JA Lampley and EE Schilling* 358, 359 (TENN); **Lincoln Co.:** Slopes along W. side of Teal Hollow Road and Creek, 1.0 air mi. S. of Kelso community, 35.111983, -86.469988, Deciduous mesic woodland, gravelly silt loam, Ca. 793' elev., 23 March 2019, *JA Lampley and EE Schilling* 496 (TENN); **Marion Co.:** Along Little Sequatchie River, abundant in rocky, wooded floodplain on west side of river, starting from where Coppinger Cove Rd makes a deep ford across the river, and thus ends the currently drivable portion, Near 35.182963 N, -85.597457 W, 15 Mar 2014, *E.E. Schilling with D.E. Schilling* 14-01 (TENN); Mesic drainage site on east side of Cedar Mtn. North, Herb with maroon petals, 2004, *John Beck* 6187, 6190 (TENN); Ca. 1 mi S of Exit 158 of Interstate 24, lower west slopes of Little Cedar Mountain near northern terminus of inlet that borders southwest side of Little Cedar Mountain, Growing in rich woods on large mossy limestone outcrops, 35-01-08 N/ 85-35-07 W, 25 May 2005, *Dwayne Estes & John Beck* 07521 (TENN); Steep wooded slope along stream, Sweeton's Cove [sic], Green to purple flowers, 11 Apr 1964, *A.J. & Evelyn Sharp* 32545 with *Ailsie Baker and Jane Jay* (TENN); In cedar-hardwood woods on south end of Little Cedar Mountain, South of I-24, 22 Mar 1988, *H.R. DeSelm s.n.*; Moist slope of sinkhole, Devil's Den, South Pittsburg, Green petals with purplish bases, flowers, 21 Apr 1962, *A.J. Sharp* 26325 with *Mrs. D.J. Jay and Gwynn Ramsey* (TENN); Moist wooded

bank of creek, valley above Orme, Greenish purplish flowers, 21 Apr 1962, *A.J. Sharp 26311 with Gwynn Ramsey and Mrs. John Jay* (TENN); Growing among limestone boulders back of bluff along the Tennessee River near the north end of Marion Memorial Bridge on U.S. 41-64-72, southeast of Jasper, Corolla deep maroon before drying, 5 Apr 1959, *Harry L. Sherman & Richard Houk 25364* (TENN); Crossing of Left Sequatchie River, north of Sequatchie, 22 Apr 1951, *R.E. Shanks L5600 with J.W. Hardin, F.H. Barclay and F.W. Woods*; Vicinity of Cedar Mountain off Hwy 41, N 35° 01.757' W 085° 32.897', 27 Feb 2011, *E. Blyveis 159* (TENN); West bluff of Tennessee River near roadside park along U.S. Hwy. 41, just W. of Guild, Some plants with pure yellow flowers but no lemon odor of these plants could be detected, 8 Apr 1966, *J.D. Freeman 449 with Ruth F. Freeman* (TENN); Between Hales Bar and Chattanooga, Riverside slope, 2 Apr 1949, *A.J. Sharp 10639 with C.J. Felix and B. Adams* (TENN); Growing on a moist north-facing limestone slope near bend of the Sequatchie River immediately east of bridge on U.S. 41-64-72, ca. 1 mile southeast of Jasper, Corolla dark maroon before drying, 5 Apr 1959, *Harry L. Sherman 25376 with Richard Houk & A.J. Sharp* (TENN); Fish Trap Road above Battle Creek, Ca. 0.5 mile from I-24, Near 35.071, -85.744, Frequent, growing with poison ivy, Rocky woods, 17 April 2015, *JA Lampley with EE Schilling & Fabiola Soto 234, 235, 236* (TENN); Raccoon Mountain bluffs above Nickajack Lake, S. side of US-41 (Dixie Hwy.), 35.068449, -85.505371, Roadside bank and limestone boulder field..., 02 April 2017, *JA Lampley and EE Schilling 360, 361, 362* (TENN); Along Fiery Gizzard Rd. 6.0 air mi. NW of Kimball, 35.119085, -85.738091, Steep woodland and limestone outcropping with ..., 655' elev..., 03 April 2019, *JA Lampley with HL Lampley 498* (TENN); Along Fiery Gizzard Rd. and tributary of Big Fiery Gizzard Ck. 6.0 air mi. NW of Kimball, Cumberland Plateau region, 35.119085, -85.738091..., Steep woodland with limestone outcropping, and low areas beside creek, with ... 655' elev., 18 April 2019, *JA Lampley and EE Schilling 510a-d* (TENN); Raccoon Mountain bluffs above Nickajack Lake, S. side of US-41 (Dixie Hwy.), 3.2 air mi. NE of Haletown, 35.068449, -85.505371..., Roadside bank and limestone boulder field..., 1083' elev., 18 April 2019, *JA Lampley and EE Schilling 511a,b* (TENN); **Alabama: Jackson Co.:** Camp Jackson Rd. (CR 24) along Guntersville Lake outside Scottsboro, Near 34.644009, -85.959271, In wooded, lakeside recreational area, Scattered, 25 March 2016, *JA Lampley and EE Schilling 294* (TENN); Near entrance to former Buck's Pocket SP off CR 452, Near 34.475942, -86.054958, Deciduous woods near roadside, Abundant, 25 March 2016, *JA Lampley and EE Schilling 295, 296, 297, 298* (TENN); Opossum Hollow floodplain along Larkin Fork off of AL-65. NW of Estillfork, AL, Near 34.984745, -86.241743 ..., Creek floodplain in rich deciduous woods mixed with ..., Scattered, 03 April 2016, *JA Lampley and EE Schilling 317* (TENN); Low Gap, Along CR-55, 3.9 mi N of Fackler, Near 34.848642, -85.922337, Rocky roadside bank, Mixed cedar and deciduous woods, Scattered, 03 April 2016, *JA Lampley and EE Schilling 318* (TENN); Bluffs above Tennessee River and Guntersville Lake, along CR-24 near Camp Jackson, Ca. 5.2 air mi. E. of Scottsboro, 34.654215, -85.944688, Steep woodland slopes, 696' elev., 18 April 2019, *JA Lampley and EE Schilling 512* (TENN); **Madison Co.:** Mills Hollow, along Dug Hill Road, 5.4 air mi. E. of Huntsville, 34.729057, -86.488235, Remnant mixed woodland beside Hambrick Branch creek..., 02 April 2017, *JA Lampley and EE Schilling 368* (TENN); 6.4 air mi. SE of Huntsville at Blevins Gap, S. of Cecil Ashburn Dr SE, Near 34.655902, -86.515543, In mesic mixed forest and limestone boulder field with ..., Flowers with sharp spicy fruit fragrance, 02 April 2017, *JA Lampley and EE Schilling 375* (TENN); **Marshall Co.:** Forever Wild land in Village Ford Gap off of AL-227, N 34 25.829', W 86 10.152', Beech-hickory mixed hardwood forest with intermittent stream and sandy clay loam, Elev. 837', Abundant, 07 May 2015, *JA*

Lampley with Kate Gribbin 278 (TENN); Snow Point Rd. near Guntersville Dam, 34.417494, -86.410122, Roadside in mixed forest..., Plants variable, Flower fragrance spicy, fruity, 02 April 2017, *JA Lampley and EE Schilling 363, 364* (TENN); **Morgan Co.:** Along Newsome Sinks Road, Near 34.438056, -86.593833, Steep roadside in mixed, mesic forest, Flowers with spicy fruit fragrance, 02 April 2017, *JA Lampley and EE Schilling 367* (TENN); Truck Trail E. at base of Turkey Mt. near Lemon Slough of Tennessee River, Ca. 3.5 air mi. NE of Priceville, 34.546378, -86.835110, Low woodland with fine sandy loam soil, 579' elev., 09 May 2017, *JA Lampley and HL Lampley 441* (TENN).

Trillium radiatum Lampley sp. nov.

TYPE: North America. United States. **GEORGIA: Dade Co.:** GA-136 near Rising Fawn, outside Cloudland Canyon State Park, N 34° 50.812' W 85° 29.984', Along roadside and mesic, deciduous forest, 17 April 2015, *JA Lampley with EE Schilling & F Soto 240* (holotype: TENN; isotypes: to be distributed later).

Trillium radiatum differs from *T. cuneatum* and *T. freemanii* by its robust stamens with anther connectives which are typically somewhat rounded abaxially and often slightly incurved at the apex. Its prominent introrse pollen sacs together with the thick, rounded connectives create a channeled or trough-like appearance in the adaxial side of the anther. It also differs from both species by its sepals which are often diverging at a 45° angle relative to the scape and which are 0.7x as long as the petals. *Trillium radiatum* often has a sparsely pubescent stem apex and scabrous primary veins on the abaxial side of the leaves. Its bract length is ca. 0.26x the height of the plant compared with *T. freemanii* which has bract length of ca. 0.29x the height of the plant. *Trillium radiatum* is also distinctive for its slightly spreading petals which are not held as open as *T. freemanii*, but typically are more open than those of *T. cuneatum*. Finally, *T. radiatum* differs from other *Trillium* species by its pungent floral fragrance which ranges from acetone-like to unpleasant spicy fruit.

Description

Perennial rhizomatous herb. **Scapes** erect, 0.4 – 0.9 cm in diameter, ca. 24 – 40 cm tall; puberulent scape apices with scabrous primary leaf veins on abaxial side are common. **Leaves** sessile, ovate to broadly ovate, with broadest point at middle or below the middle (toward the leaf base), with broadly acute or slightly acuminate apices and mostly rounded bases; ca. 1.3x long as wide, 5.6 – 14.8 cm long, 3.9 – 12 cm wide; color 2-toned, mottled with medium green and light to silvery green shades; leaves separate to just overlapping and often spreading almost horizontally or at a 45° angle relative to the stem. **Sepals** green; narrow lanceolate with acute apices; clearly shorter than petals, ca. 0.7x the petal length, ca. 3.7x long as wide, 2.4 – 4.9 cm long, 0.6 – 1.1 cm wide; typically spreading at a 45° angle relative to the stem and resembling a stiff, up-turned shirt collar. **Petals** elliptic, oblanceolate, or subtly rhombic and often concavo-convex; not clawed, apices acute; 3.3x longer than wide, 3 – 7.2 cm long, 0.9 – 2.3 cm wide, ca. 0.5x as long as bracts; color typically maroon, though greenish-yellow has been observed; petals held erect and separate or barely touching, allowing the center of the flower to be at least partially visible when viewed from the side. **Stamens** relatively substantial; 1 – 2 cm long, 1.3x longer than the ovary height; filaments 0.1x the length of the entire stamen, increasing in width near the base; anther connectives dark maroon, with abaxial sides often noticeably convex;

connective apices extending slightly beyond the pollen sacs and often incurved; relatively large pale-yellow pollen sacs with introrse dehiscence; the adaxial side of the anthers has a longitudinally channeled appearance due to the pollen sacs and rounded anther connectives.

Ovary ovoid in shape, often a light green or greenish-maroon at the base gradually becoming purple at the apex; with three stigma lobes held relatively closely together compared with *T. freemanii*, lobes ca. 0.4x as long as entire ovary; 0.8 – 1.4 cm in height including stigmas; with six angles and three deep longitudinal grooves. **Fruit** rounded ovoid without the noticeable angles observed in anthesis. **Floral fragrance** *Trillium radiatum* has been noted to have a strong floral fragrance similar to acetone or nail polish remover. Some observations mention the flowers having an unpleasant spicy fruit fragrance. It is likely that the odors change over the course of anthesis and vary in strength depending on environmental temperature. Fig. 2.10.

Phenology

In flower from early to mid-April, though it may bloom as early as late March in some locations. Fruiting can be seen in June and early July.

Distribution and habitat

The known distribution of *Trillium radiatum* is within six counties of northeastern Alabama, northwestern Georgia, and southern Tennessee in a portion of the southern Ridge and Valley region in the vicinity of Lookout, Pigeon, Walker, Johns, Moragne, and Chandler Mountains, as well as Wills Valley (Fig. 2.11). It has been collected from the sides of these mountains and adjacent valleys, occurring at elevations ranging from 170 m to 448 m above sea level. It occurs in mesic, oak-hickory-maple upland forests or woodlands along with associates such as *Aesculus*, *Liquidambar*, *Cornus*, *Cercis*, *Hydrangea*, *Podophyllum*, *Viola*, *Geranium*, *Thalictrum*, *Uvularia*, *Dioscorea*, *Toxicodendron*, *Arisaema*, *Asarum*, *Cardamine*, *Phlox*, *Polygonatum*, *Maianthemum*, *Sanguinaria*, *Polemonium*, *Tiarella*, *Claytonia*, and *Sanicula*. *Trillium radiatum* typically occurs on mountainsides, ridges, escarpments, gorges, or hillslopes, though some populations may be found in floodplains (Soil Survey Staff, 2021). It can be found on moderate or steep slopes ranging from 5 to 60 percent inclines, and is typically found in rocky, well drained loam soils of various types including silty-clay, stony-fine sandy, channery, flaggy, cobbly-sandy, and gravelly loam (Soil Survey Staff, 2021).

Conservation

Trillium radiatum is an endemic species currently known from only six counties of the southeastern US in an area known for being a relictual area with relatively high biodiversity and endemism (NatureServe, 2013; A. Floden, pers. comm., 2021). Some examples of other rare, endemic species known from this region include *Allium speculae* Ownbey & Aase, *Clematis socialis* Kral, *Clinopodium talladeganum* B.R. Keener & Floden, *Lysimachia graminea* (Greene) Hand.-Mazz., *Monarda austroappalachiana* Floden, and *Sagittaria secundifolia* Kral.

GeoCAT software was used to estimate the size of the distribution of *Trillium radiatum*. The EOO (EOO=5,144.208 km²) suggests that an IUCN ranking of Vulnerable (VU) may be appropriate. The AOO was also calculated using 2 km cell width surrounding known location points (AOO=40.000 km²; n=12).

Etymology

The species epithet “radiatum” was chosen to reflect the radiate arrangement of the perianth and the ray like positions of the sepals and spreading bracts. The use of “radiatum” also alludes to the pungent fragrance which radiates from the plant during full anthesis.

Suggested common name

The common name “Radiant Trillium” is suggested due to the robust and healthy habit of the plant, its radiate appearance, and its noticeable, radiating fragrance.

Specimens examined

Alabama: Cherokee Co.: Along Lumpkin Mill Ck. and CR-809, off US-411 just W. of AL-GA state line, 34.084460, -85.432530, Mixed woodland floodplain, with *Aesculus*..., 691' elev., Trichomes on stems, 14 April 2018, *JA Lampley and EE Schilling 474* (TENN); Steep E-facing slope S of US Hwy 411, 0.6 mi. W of the Georgia State Line, Very rocky soil in open (cut-over) woods, Most of the plants here are pubescent, 15 April 1967, *JD Freeman with JT Murrell 550* (AUA); 1 mi. w. Gaylesville on Ala 35; loam under hardwoods of mountainside, 21 April 1968, *R Kral 30321* (AUA); Sandy loam of slopes by Little River, Canyon Mouth State Park, 12 April 1969, *R Kral 34267* (AUA); **Etowah Co.:** Attalla, Wills Valley physiographic region, Along Simmons Rd, 34.034520, -86.106727, Rich woodland slopes above Big Wills Ck. in silt and gravelly sandy loam ..., 564' elev., Scabrous stems, 14 April 2018, *JA Lampley and EE Schilling 478* (TENN); Rocky woods ca. 5 mi. NNE Reeseville by I-59, 15 April 1973, *R Kral 49598* (AUA); Rich woods along Clear Creek at the Rock Bridge near Gallant, 09 April 1994, *LM Hodge 1157 and DD Spaulding 5648* (JSU); Rich woods, sandy alluvium, common, corolla maroon, 2.5 air mi. NE of Steele, Along Little Canoe Creek off of Rocky Hollow Road about 0.5 miles NW with jct of US 11, 33°58'18"N 86° 10'56"W, 29 March 2003, *Brian R Keener 2496 with Shane Umphrey* (UNA); Rich woods, 1.20 air mi. SE of Norton, NW face of Big Ridge, Along S side of Simmons Rd. ca. 1.3 mi. E of jct. with US Hwy 431, 34° 02'07"N 86°05'33"W, 11 April 2009, *Brian R Keener 5157* (UWAL); **Georgia: Dade Co.:** GA-136 near Rising Fawn, outside Cloudland Canyon State Park, N 34° 50.812' W 85° 29.984', Robust plants, Along roadside and mesic, deciduous forest, 17 April 2015, *JA Lampley with EE Schilling & F Soto 237, 238, 239, 242, and 243* (Isotypes); GA-136 near Rising Fawn, outside Cloudland Canyon State Park, N 34° 50.812' W 85° 29.984', Robust plants, Individual is a yellow morph, Along roadside and mesic, deciduous forest, 17 April 2015, *JA Lampley with EE Schilling & F Soto 241* (TENN); **Floyd Co.:** Blacks Bluff, SW of Rome, SE side of Blacks Bluff Rd. SW, Along Coosa River, Near 34.215714, -85.210358, Sloping roadside; mesic deciduous woods with..., In stony fine sandy loam, Scattered. 30 April 2016, *JA Lampley with B Crain and A-M Sanchez-Cuervo, and EE Schilling 328* (TENN); N. of Rome, North River Rd NE off GA-53, in woods between road and Oostanaula River, Near 34.302369, -85.127525, In deciduous woods, Scattered, 30 April 2016, *JA Lampley with B Crain and A-M Sanchez-Cuervo, and EE Schilling 327* (TENN); **Walker Co.:** Dougherty Gap Road, approx. 34.614054, -85.455973, at bottom of slope in rich woodland, 04 April 2013, *Aaron Floden, J. Lonsdale and EE Schilling 2701* (TENN); Crockford-Pigeon Mt. WMA; along Blue Hole Rd. close to Blue Hole Spring and Waterfall Branch, Abundant, large individuals, Rich mixed woodland roadside, Scabrous stem; flowers with unpleasant fruit odor, 09 April 2017, *JA Lampley and EE Schilling 390* (TENN); Shahan Lane at Dry Creek. 34.650451, -85.106233, Sloping roadside in mesic deciduous woods with ..., 09 April 2017, *JA Lampley and EE Schilling 387* (TENN); Nick-a-Jack Rd., E. side of

Lookout Mt. 5.4 air mi. NW of Chickamauga, 34.901955, -85.382363, Forest and steep roadside with *Quercus*..., 1379' elev., Acetone-like flower fragrance, 07 April 2019, *JA Lampley and EE Schilling* 507 (TENN); **Tennessee: Hamilton Co.:** Ochs Hwy./GA-58; E. side of Lookout Mt., just N. of state line, 34.989183, -85.342477, Forest and steep roadside, with *Quercus*, 1421' elev., 07 April 2019, *JA Lampley and EE Schilling* 505, 506 (TENN).

References

- Auberson-Lavoie, D. and M. Vellend. 2020. Causes and consequences of deer browsing on red trillium (*Trillium erectum*) along an elevational gradient. *Botany*. 98(8): 469-478.
- Barksdale, L. 1938. The pedicellate species of *Trillium* found in the southern Appalachians. *Journal of the Elisha Mitchell Scientific Society*. 54(2): 271-296.
- Borchsenius, F. 2009. FastGap 1.2. Department of Biosciences, Aarhus University, Denmark. Published online at http://www.aubot.dk/FastGap_home.htm
- Case, F. 2003. "Trillium." In: Flora of North America Editorial Committee (Eds.). *Flora of North America North of Mexico*. Volume 26. Oxford: Oxford University Press.
- Case, F. and R. Case. 1997. *Trilliums*. Portland (OR): Timber Press.
- Cunningham, C.W. 1997. Can three incongruence tests predict when data should be combined? *Molecular Biology and Evolution*. 14: 733-740.
- Darriba, D., Taboada, G.L., Doallo, R., and D. Posada. 2012. jModelTest 2: more models, new heuristics and parallel computing. *Nature Methods*. 9(8): 772.
- Dong, W., Xu, C., Li, C. Sun, J., Zuo, Y., Shi, S., Cheng, T., Guo, J., and S. Zhou. 2015. *Ycf1*, the most promising plastid DNA barcode of land plants. *Scientific Reports*. 5: 8348.
- Farmer, S.B. 2006. Phylogenetic analyses and biogeography of Trilliaceae. *Aliso*. 22: 579-592.
- Farmer, S.B. 2007. A Systematic Study of *Trillium* subgenus *Delostylis*. PhD Diss., University of Tennessee. Available at https://trace.tennessee.edu/utk_graddiss/4235.
- Farmer, S.B., and E. Schilling. 2002. Phylogenetic Analyses of Trilliaceae Based on Morphological and Molecular Data. *Systematic Botany*. 27(4): 674-692.
- Farris, J.S., Källersjö, M., Kluge, A.G., and C. Bult. 1995a. Constructing a significance test for incongruence. *Systematic Biology*. 44: 4: 570-572.
- Farris, J.S., Källersjö, M., Kluge, A.G., and C. Bult. 1995b. Testing significance of incongruence. *Cladistics*. 10: 315-319.
- Freeman, J. 1975. Revision of *Trillium* subgenus *Phyllantherum* (Liliaceae). *Brittonia*. 27: 1-62.
- Gaddy, L.L. 2008. A new sessile-flowered *Trillium* (Liliaceae: subgenus *Phyllantherum*) from South Carolina. *Phytologia*. 90(3): 382-390.
- Gleason, H.A. 1906. The pedunculate species of *Trillium*. *Bulletin of the Torrey Botanical Club*. 33(7): 387-396.
- Gonzales, E., Hamrick, J.L., and S.-M. Chang. 2008. Identification of glacial refugia in southeastern North America by phylogeographical analyses of a forest understory plant, *Trillium cuneatum*. *Journal of Biogeography*. 35: 844-852.
- Guindon, S. and O. Gascuel. 2003. A simple, fast and accurate method to estimate large phylogenies by maximum-likelihood. *Systematic Biology*. 52: 696-704.
- Heslop-Harrison, Y. and K.R. Shivanna. 1977. The receptive surface of the angiosperm stigma. *Annals of Botany*. 41: 1233-1258.
- Huang, J.-L., Sun, G.-L., and D.-M. Zhang. 2010. Molecular evolution and phylogeny of the angiosperm *ycf2* gene. *Journal of Systematics and Evolution*. 48(4): 240-248.
- IUCN. 2021. The IUCN Red List of Threatened Species. Version 2021-1. <https://www.iucnredlist.org>. (Accessed 4 June 2021).
- Kartesz, J.T. 2015. The Biota of North America Program (BONAP). North American Plant Atlas. Chapel Hill, N.C. Available at <http://bonap.net/napa>.

- Kato, H., Terauchi, R., Utech, F.H., and S. Kawano. 1995. Molecular systematics of the Trilliaceae sensu lato as inferred from *rbcL* sequence data. *Molecular Phylogenetics and Evolution*. 4(2): 184-193.
- Katoh, K. and D.M. Standley. 2013. MAFFT multiple sequence alignment software version 7: improvements in performance and usability. *Molecular Biology and Evolution*. 30: 772-780.
- Kawano, S. and H. Kato. 1995. Evolutionary Biology of *Trillium* and Related Genera (Trilliaceae) II. Cladistic Analyses on Gross Morphological Characters, and Phylogeny and Evolution of the Genus *Trillium*. *Plant Species Biology*. 10: 169-183.
- Knight, T.M. 2003. Effects of herbivory and its timing across populations of *Trillium grandiflorum* (Liliaceae). *American Journal of Botany*. 90(8): 1207-1214.
- Meredith, C.R. and Trillium Working Group 2019. 2020a. *Trillium cuneatum*. The IUCN Red List of Threatened Species 2020: e.T146084767A146089210. <https://dx.doi.org/10.2305/IUCN.UK.2020-2.RLTS.T146084767A146089210.en>. (Accessed 17 February 2021).
- Meredith, C.R. and Trillium Working Group 2019. 2020b. *Trillium lancifolium*. The IUCN Red List of Threatened Species 2020: e.T146086425A146089255. <https://dx.doi.org/10.2305/IUCN.UK.2020-2.RLTS.T146086425A146089255.en>. (Accessed 14 February 2021).
- Meredith, C.R. and Trillium Working Group 2019. 2020c. *Trillium luteum*. The IUCN Red List of Threatened Species 2020: e.T146086501A146089265. <https://dx.doi.org/10.2305/IUCN.UK.2020-2.RLTS.T146086501A146089265.en>. (Accessed 19 February 2021).
- Meredith, C.R. and Trillium Working Group 2019. 2020d. *Trillium maculatum*. The IUCN Red List of Threatened Species 2020: e.T146086627A146089270. <https://dx.doi.org/10.2305/IUCN.UK.2020-2.RLTS.T146086627A146089270.en>. (Accessed 04 June 2021).
- Meredith, C.R., Trillium Working Group 2019, Farmer, S.B. and L.L. Gaddy 2020e. *Trillium oostingii*. The IUCN Red List of Threatened Species 2020: e.T146086849A146089280. <https://dx.doi.org/10.2305/IUCN.UK.2020-2.RLTS.T146086849A146089280.en>. Accessed 14 February 2021.
- Miller, C.N. and C. Kwit. 2018. Overall seed dispersal effectiveness is lower in endemic *Trillium* species than in their widespread congeners. *American Journal of Botany*. 105(11): 1847–1857.
- Murray, A.E. 1983. Notae spermatophytae No. 2. *Kalmia*. 13: 3-32.
- Murray, A.E. 1984. Notae spermatophytae No. 4. *Kalmia*. 14: 2-24.
- NatureServe. 2013. "NatureServe Rarity-Weighted Richness Model of Critically Imperiled and Imperiled Species in the United States." NatureServe, Arlington, Virginia. Available at https://www.natureserve.org/sites/default/files/natureserve_rwr_hotspots_2013_final.jpg. (Accessed 30 June 2021).
- Neilson, M. "Cumberland Plateau Physiographic Section." Encyclopedia of Alabama. 30 November 2016. Available at <http://encyclopediaofalabama.org/article/h-1301>. (Accessed 16 December 2020).

- Neubig, K.M., Whitten, W.M., Carlsward, B.S., Blanco, M.A., Endara, L., Williams, N.H., and M. Moore. 2009. Phylogenetic utility of *ycf1* in orchids: a plastid gene more variable than *matK*. *Plant Systematics and Evolution*. 277(1): 75–84.
- Osaloo, S.K. and S. Kawano. 1999. Molecular systematics of Trilliaceae II. Phylogenetic analyses of *Trillium* and its allies using sequences of *rbcL* and *matK* genes of cpDNA and internal transcribed spacers of 18s-28s nrDNA. *Plant Species Biology*. 14: 75–94.
- Osaloo, S.K., Utech, F.H., Ohara, M., and S. Kawano. 1999. Molecular systematics of Trilliaceae I. Phylogenetic analyses of *Trillium* Using *matK* gene sequences. *Journal of Plant Research*. 112: 35-49.
- Pinheiro, F., Dantas-Queiroz, M.V., and C. Palma-Silva. 2018. Plant Species Complexes as Models to Understand Speciation and Evolution: A Review of South American Studies. *Critical Reviews in Plant Sciences*. 37(1): 54-80.
- Radford, A.E., Ahles, H.E., and C.R. Bell. 1968. *Manual of the Vascular Flora of the Carolinas*. Chapel Hill, NC: University of North Carolina Press.
- Ronquist, F. and J.P. Huelsenbeck. 2003. MrBayes 3: Bayesian phylogenetic inference under mixed models. *Bioinformatics*. 19(12): 1572-1574.
- Schilling, E.E., Floden, A., and S.B. Farmer. 2013. A New Sessile-Flowered *Trillium* Species from Tennessee. *Castanea* 78(2): 140–147.
- Schilling, E.E., Floden, A., Lampley, J., Patrick, T.S. and S.B. Farmer. 2019. A New Species of *Trillium* (Melanthiaceae) from Central Georgia and its Phylogenetic Position in subgenus *Sessilium*. *Systematic Botany*. 44(1): 107–114.
- Schilling, E.E., Leblond, R.J., Sorrie, B.A., and A.S. Weakley. 2007. Relationships of the New England Boneset, *Eupatorium novae-angliae* (Asteraceae). *Rhodora*. 109: 145–160.
- Serota, C.A. 1969. Karyotypes and morphological variation in the sessile-flowered trilliums of North Carolina. *Canadian Journal of Botany*. 47(8): 1223-1232.
- Shaw, J., E.B. Lickey, J.T. Beck, S.B. Farmer, W. Liu, J. Miller, K.C. Siripun, C.T. Winder, E.E. Schilling, and R.L. Small. 2005. The tortoise and the hare II: Relative utility of 21 noncoding chloroplast DNA sequences for phylogenetic analysis. *American Journal of Botany*. 92: 142–166.
- Shaw, J., E.B. Lickey, E.E. Schilling, and R.L. Small. 2007. Comparison of whole chloroplast genome sequences to choose noncoding regions for phylogenetic studies in angiosperms: The tortoise and the hare III. *American Journal of Botany*. 94: 275–288.
- Simmons, M.P. and H. Ochoterena. 2000. Gaps as characters in sequence-based phylogenetic analyses. *Systematic Biology*. 49(2): 369-381.
- Soil Survey Staff, Natural Resources Conservation Service, United States Department of Agriculture. 2021. Web Soil Survey. Available at <http://websoilsurvey.sc.egov.usda.gov/>. (Accessed 22 June 2021).
- Soukup, V.G. 1980. A New *Trillium* (Liliaceae) from the Northwestern United States. *Brittonia*. 32(3): 330–332.
- Stamatakis, A. 2006. RAxML-VI-HPC: Maximum Likelihood-based Phylogenetic Analyses with Thousands of Taxa and Mixed Models. *Bioinformatics*. 22(21): 2688–2690.
- Swofford, D.L. 2003. PAUP*. Phylogenetic Analysis Using Parsimony (*and Other Methods). Version 4. Sunderland, MA: Sinauer Associates.

- Tamura, K., Peterson, D., Peterson, N., Stecher, G., Nei, M., and S. Kumar. 2011. MEGA5: Molecular Evolutionary Genetics Analysis Using Maximum Likelihood, Evolutionary Distance, and Maximum Parsimony Methods. *Molecular Biology and Evolution*. 28(10): 2731–2739.
- Tiffney, B. and S. Manchester. 2001. The use of geological and paleontological evidence in evaluating plant phylogeographic hypotheses in the northern hemisphere tertiary. *International Journal of Plant Sciences*. 162(S6): S3-S17.
- Trillium Working Group 2019 and C.R. Meredith. 2020. *Trillium delicatum*. The IUCN Red List of Threatened Species 2020: e.T159330603A159330832. <https://dx.doi.org/10.2305/IUCN.UK.2020-2.RLTS.T159330603A159330832.en>. (Accessed 14 February 2021).
- USDA, NRCS. 2021. The PLANTS Database National Plant Data Team, Greensboro, NC. Available at <http://plants.usda.gov>. (Accessed 11 July 2021).
- Visualization and Analysis Systems Technologies. 2021. Geoscience Community Analysis Toolkit (GeoCAT-comp version 2021.04.0) [Software]. Boulder, CO: UCAR/NCAR - Computational and Informational System Lab. doi:10.5065/A8PP-4358.
- Wallace, L.E. and C.H. Doffitt. 2013. Genetic Structure of the Mesic Forest-Adapted Herbs *Trillium cuneatum* and *Trillium stamineum* (Melanthiaceae) in the South-Central United States. *Castanea*. 78(2): 154–162.
- Wayman, K.A. and C.R. Meredith. 2020. *Trillium parviflorum*. The IUCN Red List of Threatened Species 2020: e.T146086886A146089285. <https://dx.doi.org/10.2305/IUCN.UK.2020-2.RLTS.T146086886A146089285.en>. (Accessed 14 February 2021).
- Weakley, A.S. 2008. “Ecology and biogeography of *Trillium* in eastern North America: Where are the *Trilliums* and why are they there?” *Trillium Symposium*, Mt. Cuba Center, Hockessin, Delaware. Formerly available at <http://www.mtcubacenter.org/images/symposium-files/Weakley-Alan-Lecture-2.pdf>. Accessed 09 December 2014.
- Weakley, A.S. 2020. *Flora of the Southeastern United States*, edition of 20 October 2020. Chapel Hill: University of North Carolina at Chapel Hill Herbarium (NCU), North Carolina Botanical Garden, University of North Carolina at Chapel Hill.

Appendix

Appendix B

Table 2.1. Table of sessile species. Geography, and morphological classification as compared to the current phylogenetic study. (E=eastern US, W=western US, CS=central-southern US). Roman numerals, I – III, correspond to informal morphological groups of Freeman (1975).

	Species	Geography	Morphological Class	Current Study
1	<i>T. albidum</i>	W	III; <i>T. sect. Maculata</i>	Western Clade
2	<i>T. angustipetalum</i>	W	III; <i>T. sect. Maculata</i>	Western Clade
3	<i>T. chloropetalum</i>	W	III; <i>T. sect. Maculata</i>	Western Clade
4	<i>T. cuneatum</i>	E	III; <i>T. sect. Maculata</i>	Cuneatum Complex
5	<i>T. decipiens</i>	E	II; <i>T. sect. Sessilia</i>	Southeastern Clade
6	<i>T. decumbens</i>	E	II; <i>T. sect. Sessilia</i>	Southeastern Clade
7	<i>T. delicatum</i>	E	n/a	
8	<i>T. discolor</i>	E	II; <i>T. sect. Sessilia</i>	Southeastern Clade
9	<i>T. foetidissimum</i>	CS	III; <i>T. sect. Maculata</i>	Central Clade
10	<i>T. freemanii</i> sp. nov.	E	n/a	Cuneatum Complex
11	<i>T. gracile</i>	CS	III; <i>T. sect. Maculata</i>	Central Clade
12	<i>T. kurabayashii</i>	W	III; <i>T. sect. Maculata</i>	Western Clade
13	<i>T. lancifolium</i>	E	I; <i>T. sect. Recurvata</i>	Thin Rhizome Clade
14	<i>T. ludovicianum</i>	CS	III; <i>T. sect. Maculata</i>	Central Clade
15	<i>T. luteum</i>	E	III; <i>T. sect. Maculata</i>	Cuneatum Complex
16	<i>T. maculatum</i>	E	III; <i>T. sect. Maculata</i>	Cuneatum Complex
17	<i>T. oostingii</i>	E	n/a	Thin Rhizome Clade
18	<i>T. parviflorum</i>	W	n/a	Western Clade
19	<i>T. petiolatum</i>	W	III; <i>T. sect. Maculata</i>	
20	<i>T. radiatum</i> sp. nov.	E	n/a	Cuneatum Complex
21	<i>T. recurvatum</i>	E	I; <i>T. sect. Recurvata</i>	Thin Rhizome Clade
22	<i>T. reliquum</i>	E	II; <i>T. sect. Sessilia</i>	Southeastern Clade
23	<i>T. sessile</i>	E	II; <i>T. sect. Sessilia</i>	
24	<i>T. stamineum</i>	E	II; <i>T. sect. Sessilia</i>	
25	<i>T. tennesseense</i>	E	n/a	Thin Rhizome Clade
26	<i>T. underwoodii</i>	E	II; <i>T. sect. Sessilia</i>	Southeastern Clade
27	<i>T. viride</i>	CS	III; <i>T. sect. Maculata</i>	Central Clade
28	<i>T. viridescens</i>	CS	III; <i>T. sect. Maculata</i>	Central Clade

Table 2.2. A table of samples used in the analysis. Samples marked with * have some, if not all, sequences generated from NGS. Genbank accession numbers are listed where appropriate. Vouchers are deposited at TENN unless noted.

<u>Taxon-ID</u>	<u>Species</u>	<u>ITS</u> <u>-5</u>	<u>psbA-</u> <u>trnH</u>	<u>rpl32-trnL</u>	<u>TY1F</u> <u>2 vcf1</u>	<u>97F</u> <u>ycf2</u>	<u>1164R</u> <u>ycf2</u>	<u>Voucher</u>	<u>Location</u>
718_macula	<i>Trillium maculatum</i>	x	KC17091 6	MG69909 2	x	x	x	Mellard DNA718 2001	Dorchester Co., SC
721_ludovi*	<i>Trillium ludovicianum</i>	x	KC17091 3	MG69908 5	x	x	x	Mellard DNA721 2001	Vernon Parish, LA
746_macula	<i>Trillium maculatum</i>	x	x	x	x	x	x	Mellard DNA746 Mar 2001	Dorchester Co., SC
749_ludovi	<i>Trillium ludovicianum</i>	x	x	x	x	x	x	Mellard DNA749 Mar 2001	Vernon Parish, LA
750_HamiTN	<i>Trillium "freemanii"</i>	x	KC17091 8	x	x	x	x	Farmer & Mellard DNA750 2001	Hamilton Co, TN
751_HamiTN	<i>Trillium "freemanii"</i>	x	KC17091 9	MG69905 9	x	x	x	Farmer & Mellard DNA751 2001	Hamilton Co, TN
2120_ovatu	<i>Trillium ovatum</i>	x	x	x	x	x	x	Legler 288 2003 WTU	Skagit Co., WA
2139_luteu	<i>Trillium luteum</i>	x	x	x	x	x	x	Farmer & Rhinehart DNA2139 Apr 2005	Cumberland Co., TN
3343_tenne	<i>Trillium tennesseense</i>	x	KC17092 0	MG69911 2	x	x	x	Schilling & Floden 12-1	Hamblen Co., TN
3354_disco*	<i>Trillium discolor</i>	x	KC17090 6	MG69907 1	x	x	x	Floden & Lonsdale 2207	Oconee Co., SC
3355_graci	<i>Trillium gracile</i>	x	KC17090 9	MG69907 6	x	x	x	Floden & Lonsdale 2206	Sabine Co., TX
3357_oosti	<i>Trillium oostingii</i>	x	KC17092 3	MG69909 3	x	x	x	Schilling 2012-04	Kershaw Co., SC
3358_stami	<i>Trillium stamineum</i>	x	KC17092 8	MG69911 1	x	x	x	Floden DNA3358 4/30/2012	Madison Co., AL
3359_luteu	<i>Trillium luteum</i>	x	KC17091 5	MG69908 7	x	x	x	Schilling 12-6	Knox Co., TN
3361_foeti*	<i>Trillium foetidissimum</i>	x	KC17090 8	MG69907 4	x	x	x	Floden 2126	East Baton Rouge Parish, LA
3362_ludov	<i>Trillium ludovicianum</i>	x	KC17091 4	MG69908 6	x	x	x	Floden 2123	Rapides Parish, LA
3363_reliq	<i>Trillium reliquum</i>	x	KC17092 6	MG69910 3	x	x	x	Floden DNA3363	Aiken Co., SC
3390_under	<i>Trillium underwoodii</i>	x	KC17093 0	MG69911 7	x	x	x	Farmer & Patrick DNA3390	Houston Co., GA
3392_macul	<i>Trillium maculatum</i>	x	KC17091 7	MG69909 1	x	x	x	Farmer and Patrick DNA3392	Houston Co., GA

Table 2.2. Continued.

3397_CamTN *	<i>Trillium cuneatum</i>	x	KC170902	MG699061	x	x	x	Floden 462	Campbell Co., TN
3405_GilGA	<i>Trillium cuneatum</i>	x	KC170903	MG699062	x	x	x	Floden DNA3405 2012	Gilmer Co., GA
3680_HamTN	<i>Trillium "freemanii"</i>	x	x	x	x	x	x	Floden & Schilling 2337	Hamilton Co., TN
3681_MarTN	<i>Trillium "freemanii"</i>	x	x	x	x	x	x	Floden & Schilling 2339	Marion Co., TN
3682_MarTN*	<i>Trillium "freemanii"</i>	x	MG698953	MG699060	x	x	x	Floden & Schilling 2340	Marion Co., TN
3683_DadGA	<i>Trillium cuneatum</i>	x	x	x	x	x	x	Floden & Schilling 2341	Dade Co., GA
3684_DadGA	<i>Trillium cuneatum</i>	x	x	x	x	x	x	Floden & Schilling 2342	Dade Co., GA
3687_macul	<i>Trillium maculatum</i>	x	MG698970	MG699090	x	x	x	Floden et al. 2349	Wilcox Co., AL
3689_decum*	<i>Trillium decumbens</i>	x	MG698957	MG699067	x	x	x	Floden et al. 2343	Murray Co., GA
3691_macul	<i>Trillium maculatum</i>	x	x	x	x	x	x	Floden et al. 2355	Russell Co., AL
3693_macul	<i>Trillium maculatum</i>	x	x	x	x	x	x	Floden et al. 2368	Twiggs Co., GA
3698_decip	<i>Trillium decipiens</i>	x	MG698955	MG699064	x	x	x	Floden et al. 2353	Russell Co., AL
3701_under	<i>Trillium underwoodii</i>	x	MG698986	MG699116	x	x	x	Floden et al. 2364	Gadsden Co., FL
3703_graci	<i>Trillium gracile</i>	x	MG698962	MG699077	x	x	x	Floden & Lonsdale DNA 3703 2013	TX
3715_angus	<i>Trillium angustipetalum</i>	x	MG698950	MG699056	x	x	x	Graham & Dickey DNA3715 April 2013	Sierra Nevada, CA
3716_chlor	<i>Trillium chloropetalum</i>	x	MG698951	MG699057	x	x	x	Graham & Weeks DNA3716 April 2013	CA
3717_albid*	<i>Trillium albidum</i>	x	MG698948	MG699054	x	x	x	Graham DNA3717	Polk Co., OR
3718_kurab	<i>Trillium kurabayashii</i>	x	MG698963	MG699078	x	x	x	Graham DNA3718	Del Norte Co., CA
3719_albid	<i>Trillium albidum</i>	x	MG698949	MG699055	x	x	x	Graham & Esther DNA3719	Polk Co., OR
3720_chlor	<i>Trillium chloropetalum</i>	x	MG698952	MG699058	x	x	x	Graham & Weeks DNA3720 "giganteum" April 2013	CA
3721_kurab	<i>Trillium kurabayashii</i>	x	MG698964	MG699079	x	x	x	Graham DNA3721	Curry Co., OR
3722_sessi	<i>Trillium sessile</i>	x	MG698982	MG699108	x	x	x	Floden 2395	Johnson Co., MO

Table 2.2. Continued.

3723_virid	<i>Trillium viride</i>	x	MG698987	MG699118	x	x	x	Floden 2397	Franklin Co., MO
3724_oosti	<i>Trillium oostingii</i>	x	MG698971	MG699094	x	x	x	Floden 2396	Kershaw Co., SC
3725_luteu	<i>Trillium luteum</i>	x	MG698968	MG699022	x	x	x	Floden et al. 2393	Campbell Co., TN
3811_recur	<i>Trillium recurvatum</i>	x	MG698974	MG699098	x	x	x	Floden & Schilling 2467	Jackson Co., TN
4078_SteGA	<i>Trillium cuneatum</i>	x	x	x	x	x	x	Floden DNA4078 March 2014	Stephens Co., GA
4079_OcoSC	<i>Trillium cuneatum</i>	x	x	x	x	x	x	Floden 2740 March 2014	Oconee Co., SC
4080_GilGA	<i>Trillium cuneatum</i>	x	x	x	x	x	x	Floden DNA4080 March 2014	Gilmer Co., GA
4082_MarTN	<i>Trillium "freemanii"</i>	x	x	x	x	x	x	Schilling DNA4082 March 2014	Marion Co., TN
4085_GreGA	<i>Trillium cuneatum</i>	x	x	x	x	x	x	Floden et al. 2710	Greene Co., GA
4086_delic	<i>Trillium delicatum</i>	x	MG698959	MG699070	x	x	x	Floden et al. 2707	Houston Co., GA
4087_DadGA	<i>Trillium cuneatum</i>	x	x	x	x	x	x	Floden et al. 2699	Dade Co., GA
4088_WalGA	<i>Trillium "radiatum"</i>	x	x	x	x	x	x	Floden et al. 2701	Walker Co., GA
4089_decum	<i>Trillium decumbens</i>	x	MG698958	MG699068	x	x	x	Floden et al. 2700	Walker Co., GA
4090_reliq*	<i>Trillium reliquum</i>	x	MG698976	MG699101	x	x	x	Floden et al. 2703	Upson Co., GA
4091_UpsGA	<i>Trillium cuneatum</i>	x	x	x	x	x	x	Floden et al. 2704	Upson Co., GA
4092_luteu	<i>Trillium luteum</i>	x	MG698969	MG699089	x	x	x	Floden et al. 2711	Whitfield Co., GA
4095_RabGA	<i>Trillium cuneatum</i>	x	x	x	x	x	x	Floden et al. DNA4095 April 2014	Rabun Co., GA
4097_CalAL	<i>Trillium "freemanii"</i>	x	x	x	x	x	x	Lampley 112 (JSU)	Calhoun Co., AL
4103_petio*	<i>Trillium petiolatum</i>	x	MG698973	MG699096	x	x	x	Floden DNA4103 4/29/2014	Davey or Darcy of the Woods ID
4104_HamTN	<i>Trillium "freemanii"</i>	x	x	x	x	x	x	Floden and Shaw 2744	Hamilton Co., TN
4106_foeti	<i>Trillium foetidissimum</i>	x	MG698961	MG699075	x	x	x	Floden 2754	Wilkinson Co., MS
4107_disco	<i>Trillium discolor</i>	x	MG698960	MG699072	x	x	x	Floden DNA4107 April 2014	Oconee Co., SC
4139_luteu	<i>Trillium luteum</i>	x	x	x	x	x	x	Schilling DNA4139 2014	Blount Co., TN
4199_petio	<i>Trillium petiolatum</i>	x	x	x	x	x	x	Floden & Schilling 2782	Boise Co., ID
4263_CalAL	<i>Trillium cuneatum</i>	x	x	x	x	x	x	Lampley & Hutchinson 232	Calhoun Co., AL
4264_MarAL	<i>Trillium cuneatum</i>	x	x	x	x	x	x	Lampley & A. Lampley 233	Marshall Co., AL

Table 2.2. Continued.

4270_tenne*	<i>Trillium tennesseense</i>	x	x	x	x	x	x	Floden s.n. DNA4270 April 2015	Hawkins Co., TN?
4281_luteu	<i>Trillium luteum</i>	x	x	x	x	x	x	Schilling DNA4281 4/4/2015	Rhea Co., TN
4282_luteu	<i>Trillium luteum</i>	x	x	x	x	x	x	Schilling DNA4282 2015	Grundy Co., TN
4301_MarTN	<i>Trillium "freemanii"</i>	x	x	x	x	x	x	Lampley and Schilling 234	Marion Co., TN
4311_DadGA	<i>Trillium "radiatum"</i>	x	x	x	x	x	x	Lampley et al. 237	Dade Co., GA
4321_DadGA	<i>Trillium "radiatum" (yellow-form)</i>	x	x	x	x	x	x	Lampley et al. 241	Dade Co., GA
4323_delic*	<i>Trillium delicatum</i>	x	x	x	x	x	x	TS Patrick s.n. April 15, 2015	Greene Co., GA
4325_PutTN	<i>Trillium cuneatum</i>	x	x	x	x	x	x	Lampley et al. 258	Putnam Co., TN
4327_PutTN	<i>Trillium cuneatum</i>	x	x	x	x	x	x	Lampley et al. 258	Putnam Co., TN
4328_OveTN	<i>Trillium cuneatum</i>	x	x	x	x	x	x	Lampley et al. 257	Overton Co., TN
4337_JacTN	<i>Trillium cuneatum</i>	x	x	x	x	x	x	Floden et al. 2801	Jackson Co., TN
4339_luteu	<i>Trillium luteum</i>	x	x	x	x	x	x	Floden et al. 2829 4/24/2015	Hancock Co., TN
4340_parvi	<i>Trillium parviflorum</i>	x	x	x	x	x	x	Floden DNA4340 4/24/2015	Jefferson Co., WA
4346_MorTN	<i>Trillium cuneatum (yellow-form)</i>	x	x	x	x	x	x	Schilling DNA4346 April 2015	Morgan Co., TN
4348_MorTN	<i>Trillium cuneatum</i>	x	x	x	x	x	x	Schilling DNA4348 April 2015	Morgan Co., TN
4355_MurGA	<i>Trillium cuneatum</i>	x	x	x	x	x	x	Lampley et al. 259	Murray Co., GA
4358_PolTN	<i>Trillium cuneatum</i>	x	x	x	x	x	x	Lampley et al. 262	Polk Co., TN
4361_CheNC	<i>Trillium cuneatum (yellow-form)</i>	x	x	x	x	x	x	Lampley et al. 265	Cherokee Co., NC
4362_CheNC	<i>Trillium cuneatum</i>	x	x	x	x	x	x	Lampley et al. 266	Cherokee Co., NC
4363_cates*	<i>Trillium catesbaei</i>	x	x	x	x	x	x	Lampley et al. 269	Clay Co., NC
4365_erect	<i>Trillium erectum</i>	x	x	x	x	x	x	Lampley et al. 274	Clay Co., NC
4367_GraNC	<i>Trillium cuneatum</i>	x	x	x	x	x	x	Lampley et al. 276	Graham Co., NC
4384_recur*	<i>Trillium recurvatum</i>	x	x	x	x	x	x	Floden DNA4384	St. Louis Co., MO
4397_MarAL	<i>Trillium "freemanii"</i>	x	x	x	x	x	x	Lampley & Gribbin 278	Marshall Co., AL
4398_MarAL	<i>Trillium cuneatum</i>	x	x	x	x	x	x	Lampley 279	Marshall Co., AL
4400_lanci*	<i>Trillium lancifolium</i>	x	x	x	x	x	x	Lampley & H. Lampley 282	Calhoun Co., AL
4404_CalAL	<i>Trillium cuneatum</i>	x	x	x	x	x	x	Lampley & H. Lampley 286	Calhoun Co., AL

Table 2.2. Continued.

4410_lanci*	<i>Trillium lancifolium</i>	x	x	x	x	x	x	Barger & Holt OC-171 (AMAL)	Dallas Co., AL
4411_sessi*	<i>Trillium sessile</i>	x	x	x	x	x	x	Schilling s.n. DNA4411 2015	Pickaway County, OH
4413_CofTN	<i>Trillium "freemanii"</i>	x	x	x	x	x	x	Lamley et al. 290	Coffee Co., TN
4425_luteu	<i>Trillium luteum</i>	x	x	x	x	x	x	Floden 2832 4/28/2015	Union Co., TN
4495_MurGA	<i>Trillium cuneatum</i>	x	x	x	x	x	x	Lamley et al. 292	Murray Co., GA
4497_luteu	<i>Trillium luteum</i>	x	x	x	x	x	x	Floden et al. 2882	Murray Co., GA
4503_JacAL	<i>Trillium "freemanii"</i>	x	x	x	x	x	x	Lamley & Schilling 294	Jackson Co., AL
4506_JacAL	<i>Trillium "freemanii"</i>	x	x	x	x	x	x	Lamley & Schilling 295	Jackson Co., AL
4517_StcAL	<i>Trillium cuneatum</i>	x	x	x	x	x	x	Lamley & Schilling DNA4517 March 2016	St. Clair Co., AL
4520_StcAL	<i>Trillium cuneatum</i>	x	x	x	x	x	x	Lamley & Schilling DNA4520 March 2016	St. Clair Co., AL
4522_SheAL	<i>Trillium cuneatum</i>	x	x	x	x	x	x	Lamley & Schilling 303	Shelby Co., AL
4531_stami*	<i>Trillium stamineum</i>	x	x	x	x	x	x	Lamley and Schilling s.n. DNA4531	Sumter Co., AL
4552_ludov	<i>Trillium ludovicianum</i>	x	x	x	x	x	x	Floden DNA4552 March 2016	Attala Co., MS
4554_luteu	<i>Trillium luteum</i>	x	x	x	x	x	x	Lamley and Schilling 319	Bledsoe Co., TN
4555_FraTN	<i>Trillium "freemanii"</i>	x	x	x	x	x	x	Lamley and Schilling 320	Franklin Co., TN
4557_FraTN	<i>Trillium "freemanii"</i>	x	x	x	x	x	x	Lamley and Schilling 323	Franklin Co., TN
4561_FraTN	<i>Trillium "freemanii"</i>	x	x	x	x	x	x	Lamley and Schilling 322	Franklin Co., TN
4562_JacAL	<i>Trillium "freemanii"</i>	x	x	x	x	x	x	Lamley and Schilling 318	Jackson Co., AL
4564_JacAL	<i>Trillium "freemanii"</i>	x	x	x	x	x	x	Lamley & Schilling 317	Jackson Co., AL
4566_OcoSC	<i>Trillium cuneatum</i>	x	x	x	x	x	x	Lamley et al. 332	Oconee Co., SC
4567_SteGA	<i>Trillium cuneatum</i>	x	x	x	x	x	x	Lamley et al. 333	Stephens Co., GA
4569_macul	<i>Trillium maculatum</i>	x	x	x	x	x	x	Lamley et al. 335	Burke Co., GA
4575_macul	<i>Trillium maculatum</i>	x	x	x	x	x	x	Lamley et al. 342	Edgefield Co., SC
4576_PolNC	<i>Trillium cuneatum</i>	x	x	x	x	x	x	Lamley et al. 343	Polk Co., NC
4578_virsc*	<i>Trillium viridescens</i>	x	x	x	x	x	x	Witsell DNA4578 April 2016	AR
4579_virsc	<i>Trillium viridescens</i>	x	x	x	x	x	x	Witsell DNA4579 April 2016	Miller Co., AR
4589_FloGA	<i>Trillium "radiatum"</i>	x	x	x	x	x	x	Lamley et al. 327	Floyd Co., GA

Table 2.2. Continued.

4591_FloGA	<i>Trillium "radiatum"</i>	x	x	x	x	x	x	Lampley et al. 328	Floyd Co., GA
4593_luteu	<i>Trillium luteum</i>	x	x	x	x	x	x	Floden and Schilling DNA4593 April 2016	Hamblen Co., TN
4655_WalAL	<i>Trillium cuneatum</i> (yellow-form)	x	x	x	x	x	x	Lampley & H. Lampley 345	Walker Co., AL
4688_MarTN	<i>Trillium "freemanii"</i>	x	x	x	x	x	x	Lampley and Schilling 360	Marion Co., TN
4689_MarAL	<i>Trillium "freemanii"</i>	x	x	x	x	x	x	Lampley & Schilling 363	Marshall Co., AL
4690_MorAL	<i>Trillium "freemanii"</i>	x	x	x	x	x	x	Lampley & Schilling 367	Morgan Co., AL
4692_MadAL	<i>Trillium "freemanii"</i>	x	x	x	x	x	x	Lampley & Schilling 368	Madison Co., AL
4693_MadAL	<i>Trillium "freemanii"</i>	x	x	x	x	x	x	Lampley & Schilling 375	Madison Co., AL
4694_JacAL	<i>Trillium cuneatum</i>	x	x	x	x	x	x	Lampley and Schilling 376	Jackson Co., AL
4702_WalGA	<i>Trillium "radiatum"</i>	x	x	x	x	x	x	Lampley & Schilling 387	Walker Co., GA
4705_WalGA	<i>Trillium "radiatum"</i>	x	x	x	x	x	x	Lampley & Schilling 390	Walker Co., GA
4708_PonMS	<i>Trillium cuneatum</i>	x	x	x	x	x	x	Lampley & Schilling 407	Pontotoc Co., MS
4710_TusAL	<i>Trillium cuneatum</i>	x	x	x	x	x	x	Lampley & Schilling 420	Tuscaloosa Co., AL
4755_MccKY	<i>Trillium cuneatum</i>	x	x	x	x	x	x	Schilling DNA4755 May 2017	McCreary Co., KY
4757_MorAL	<i>Trillium "freemanii"</i>	x	x	x	x	x	x	Lampley & H. Lampley 441	Morgan Co., AL
4900_macul	<i>Trillium maculatum</i>	x	x	x	x	x	x	C. Hunter DNA4900 2018	Alachua Co., FL
4972_decip	<i>Trillium decipiens</i>	x	x	x	x	x	x	Lampley & Schilling 469	Lee Co., AL
5016_EtoAL	<i>Trillium "radiatum"</i>	x	x	x	x	x	x	Lampley & Schilling 478	Etowah Co., AL
5017_CheAL	<i>Trillium "radiatum"</i>	x	x	x	x	x	x	Lampley & Schilling 474	Cherokee Co., AL
5031_virid	<i>Trillium viride</i>	x	x	x	x	x	x	Floden et al. DNA5031 April 2018	Washington Co., MO
5295_MauTN	<i>Trillium cuneatum</i>	x	x	x	x	x	x	Lampley & Schilling 483	Maury Co., TN
5296_LewTN	<i>Trillium cuneatum</i>	x	x	x	x	x	x	Lampley & Schilling 484	Lewis Co., TN
5297_WayTN	<i>Trillium cuneatum</i>	x	x	x	x	x	x	Lampley & Schilling 486	Wayne Co., TN
5300_HarTN	<i>Trillium cuneatum</i>	x	x	x	x	x	x	Lampley & Schilling 493	Hardin Co., TN
5301_GilTN	<i>Trillium cuneatum</i>	x	x	x	x	x	x	Lampley & Schilling 495	Giles Co., TN
5302_LinTN	<i>Trillium "freemanii"</i>	x	x	x	x	x	x	Lampley & Schilling 496	Lincoln Co., TN
5322_DadGA	<i>Trillium cuneatum</i>	x	x	x	x	x	x	Lampley 500	Dade Co., GA
5331_HamTN	<i>Trillium "radiatum"</i>	x	x	x	x	x	x	Lampley & Schilling 505	Hamilton Co., TN

Table 2.2. Continued.

5332_luteu	<i>Trillium luteum</i>	x	x	x	x	x	x	Lampley et al. 502	Hamilton Co., TN
------------	------------------------	---	---	---	---	---	---	--------------------	------------------

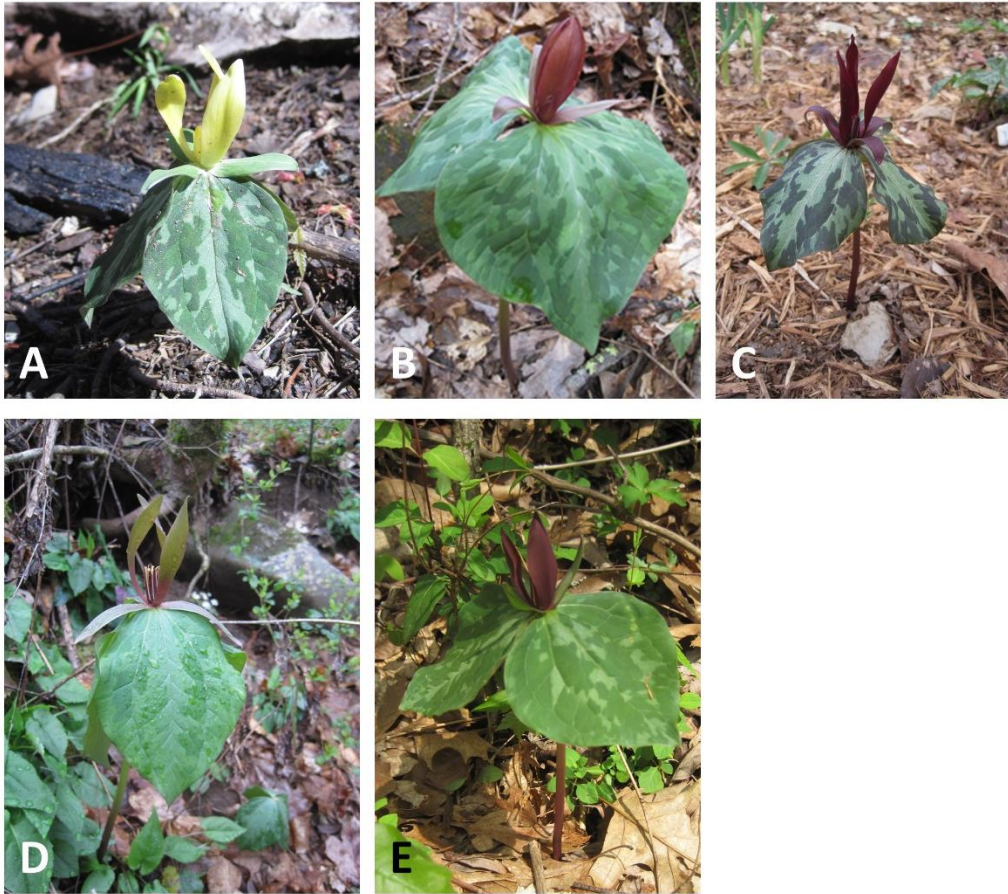


Figure 2.1. Images of the overall appearance of the species of the Cuneatum Complex. A. *Trillium luteum*. B. *T. cuneatum* s.s. C. *T. maculatum*. D. *T. freemanii*. E. *T. radiatum*. Photos by J. Lampley.

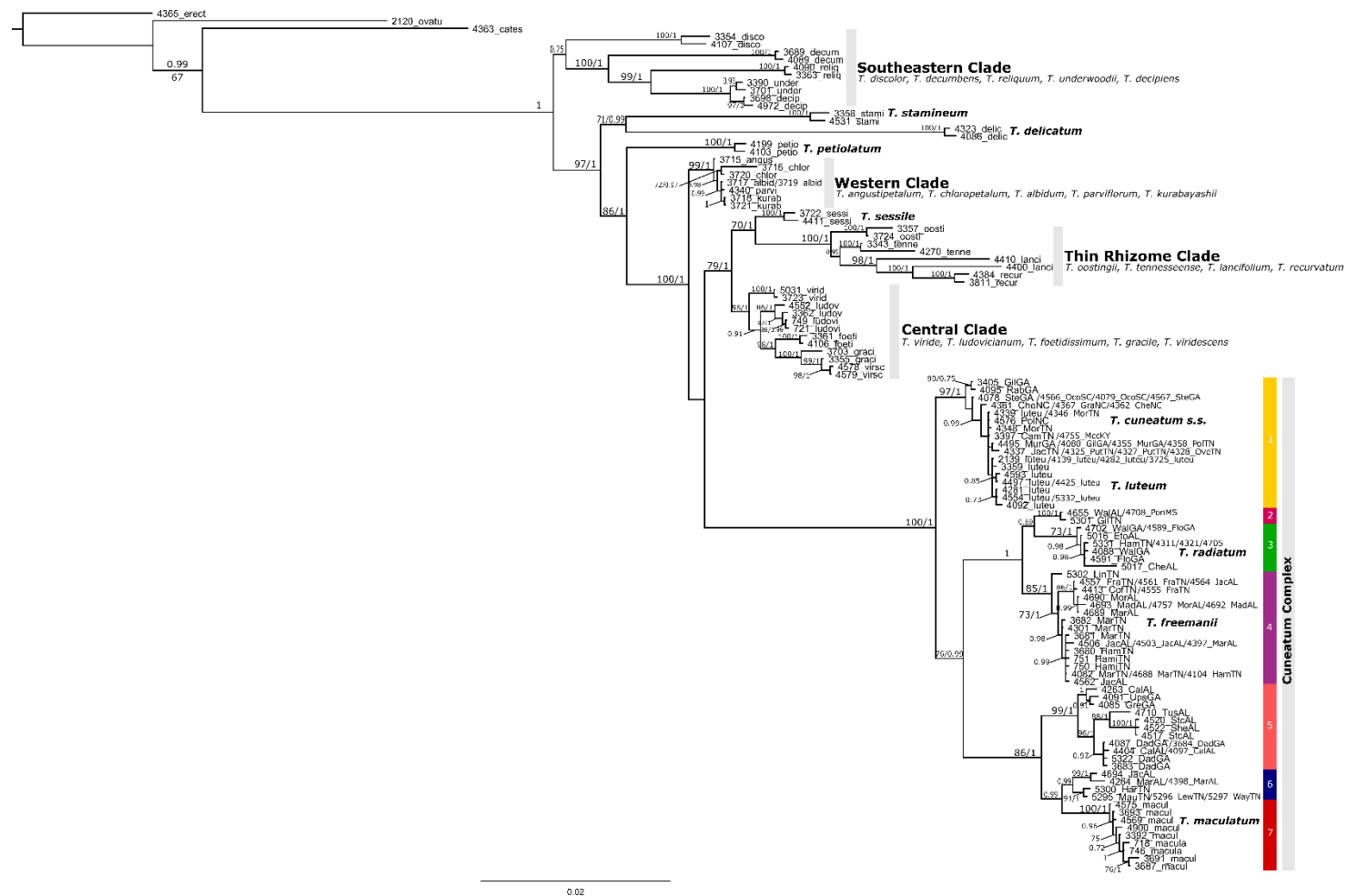


Figure 2.2. Bayesian inference phylogenetic tree. Phylogenetic tree using MrBayes showing relationships of *T. subgen. Sessilium* and the *T. cuneatum* species complex. Results from Maximum Likelihood majority rule consensus tree using RAxML are included in the tree. Maximum Likelihood bootstrap scores (> 60) for each branch are listed first and Bayesian posterior probabilities are listed second (> 0.6); either of the scores may be listed singly if the other is not available. Inferences based on plastid and nuclear ribosomal ITS data.

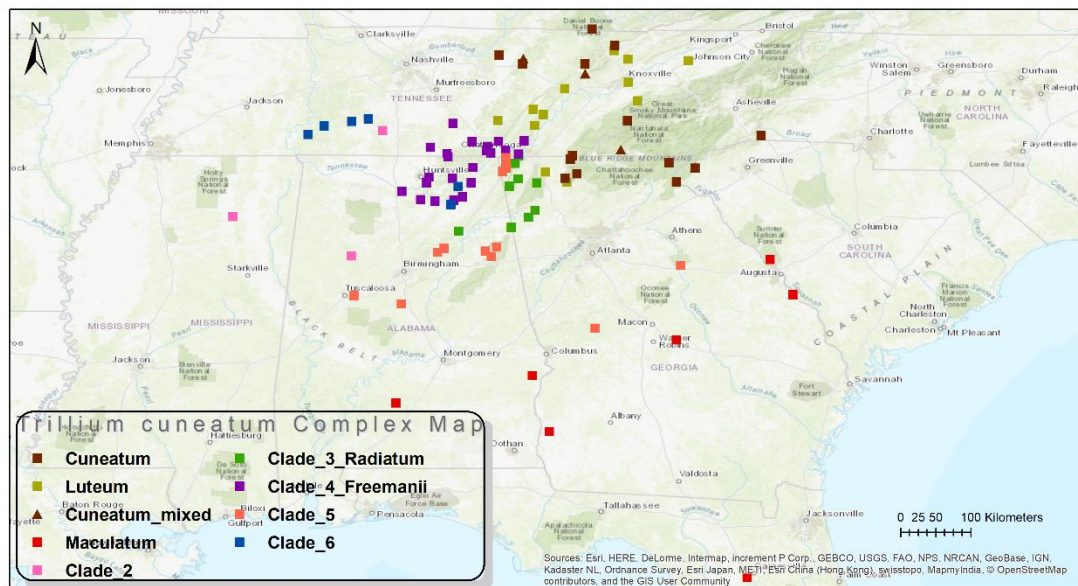


Figure 2.3. *Trillium cuneatum* complex map. A map of the southeastern US is shown depicting populations sampled in the study. Eight separate clades are color coded on the map. Mixed populations of *T. cuneatum* yellow and maroon or bronze color morphs are denoted with triangle icons.



Figure 2.4. Androecium of the species of the Cuneatum Complex. Androecium size and position are shown relative to ovary. A. *Trillium luteum*. B. *T. cuneatum* s.s. C. *T. maculatum*. D. *T. freemanii*. E. *T. radiatum*. Microscope images by J. Lampley.

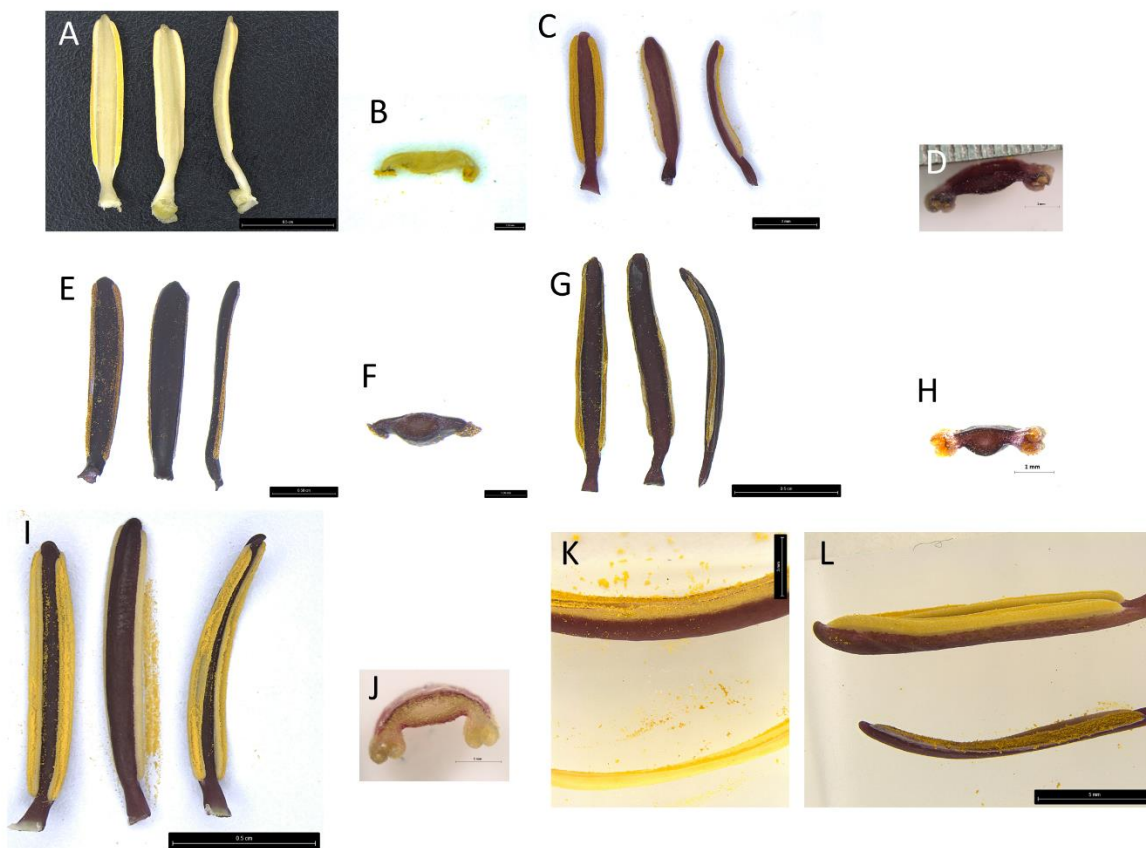


Figure 2.5. Stamens of the species of the Cuneatum Complex. A – J. Adaxial, abaxial, and lateral views of stamens and a cross-section of the anther are shown (L to R) for each species. Cross-section images are positioned with the abaxial side toward the top of the image. A – B. *Trillium luteum*. C – D. *T. cuneatum* s.s. E – F. *T. maculatum*. G – H. *T. freemanii*. I – J. *T. radiatum*. K. Stamen thickness comparison between *T. radiatum* (above) and *T. luteum* (below) is shown. L. Stamen thickness comparison between *T. radiatum* (above) and *T. cuneatum* s.s. (below) is shown. Microscope images by J. Lampley.

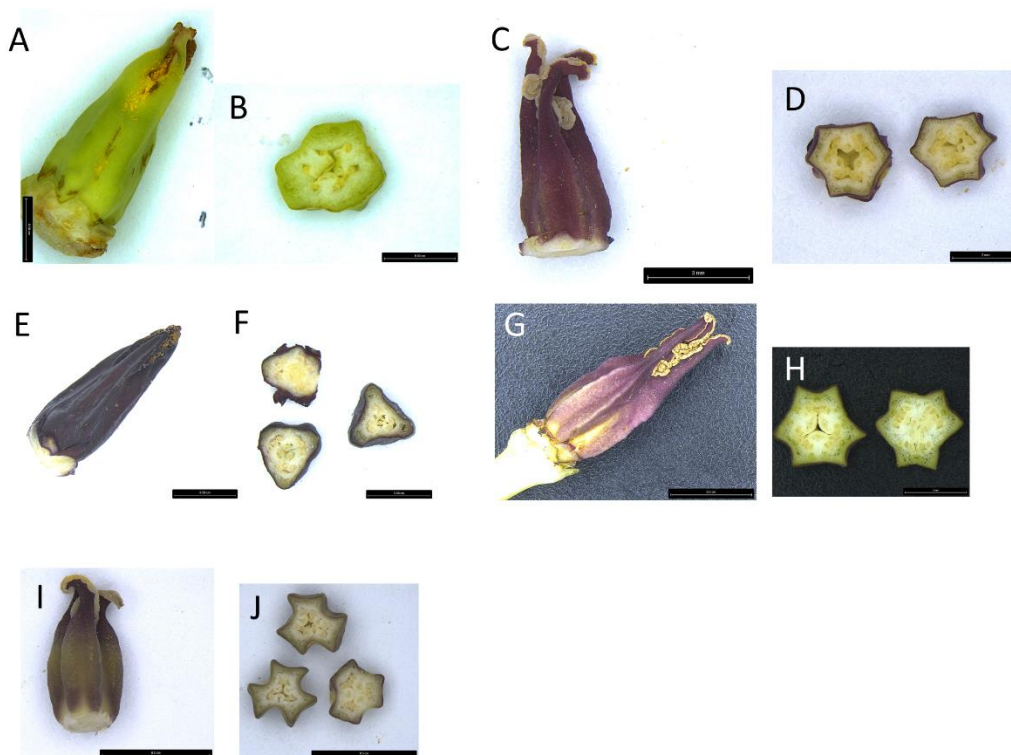


Figure 2.6. Images of the ovaries of the species of the Cuneatum Complex. Lateral view and cross-section of the ovary shown (L to R) for each species. A – B. *Trillium luteum*. C – D. *T. cuneatum* s.s. E – F. *T. maculatum*. G – H. *T. freemanii*. I – J. *T. radiatum*. Microscope images by J. Lampley.

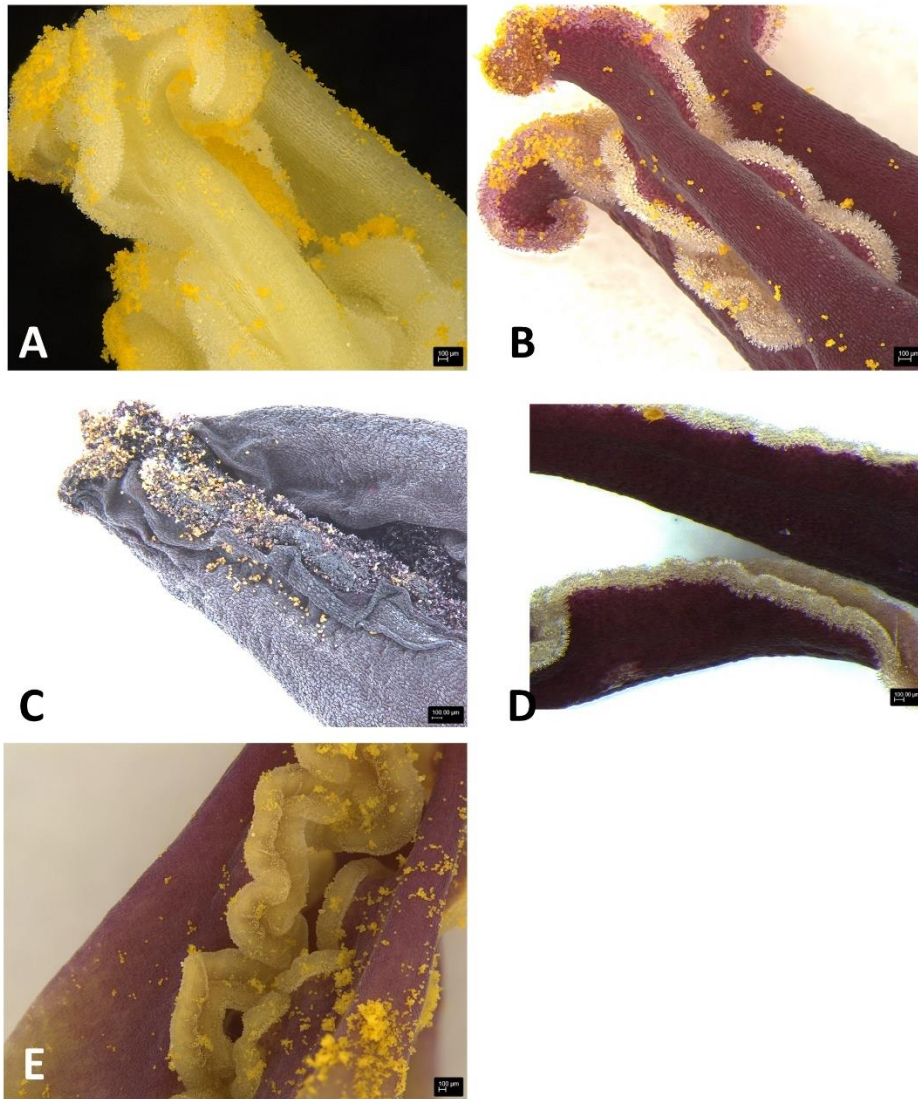


Figure 2.7. Stigmatic surface comparison of the species of the Cuneatum Complex. Stigmatic surfaces are shown for each species. A. *Trillium luteum*. B. *T. cuneatum* s.s. C. *T. maculatum*. D. *T. freemanii*. E. *T. radiatum*. Microscope images by J. Lampley.

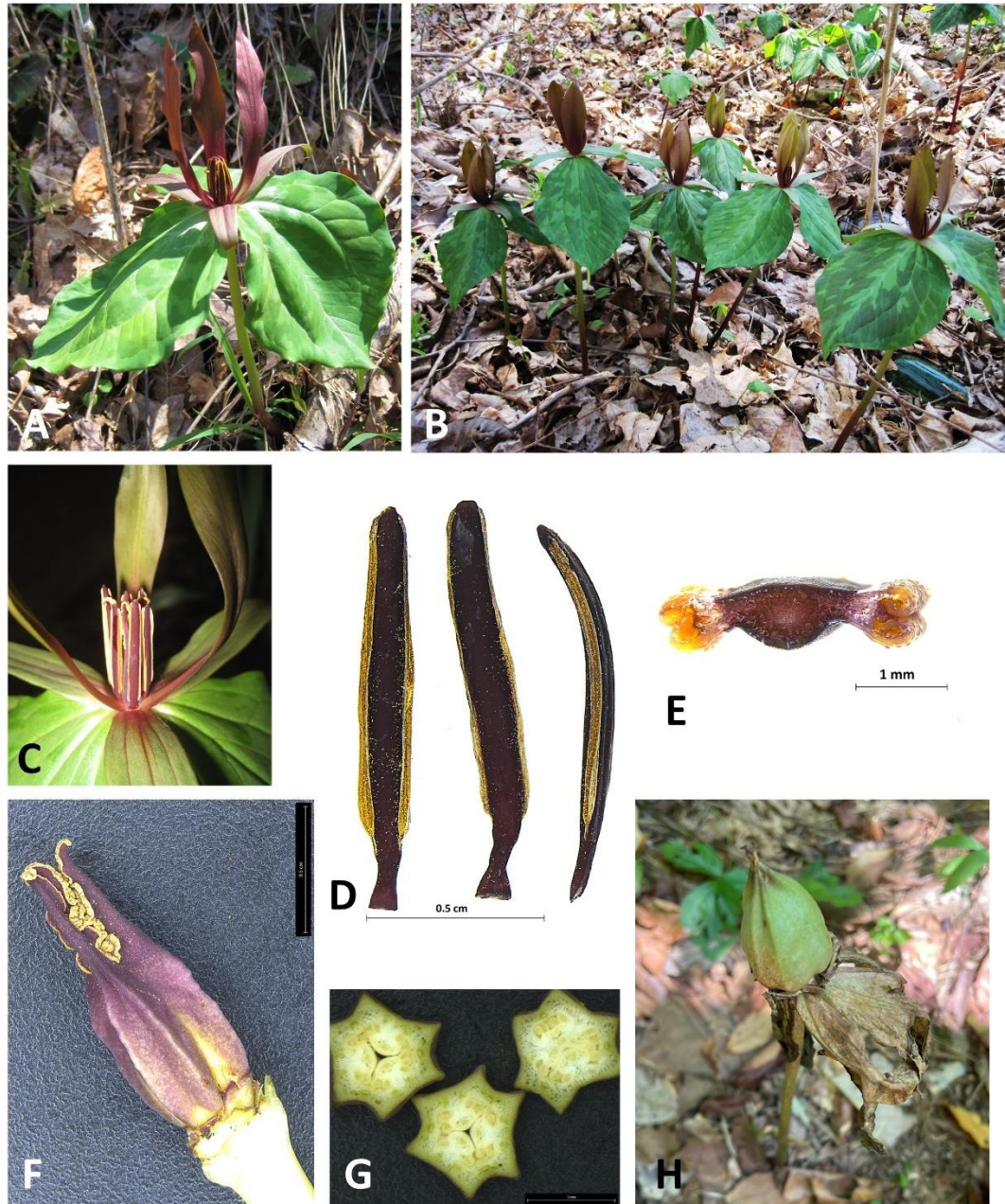


Figure 2.8. Images of *Trillium freemanii*. A. Flower of mature individual showing typical narrow petals held in a manner reminiscent of a candelabra, typical narrow arching sepals, and draping leaves. B. Scattered population growing in thick leaf litter of woodland floor. C. Androecium showing typical and relatively thin, narrow stamens; narrow petals display spreading and somewhat clawed bases which do not obscure the view of the center of flower. D. Stamen adaxial, abaxial, and lateral views showing latrorse to introrse dehiscence and relatively narrow pollen sacs with pollen. E. Anther cross-section showing lateral orientation of pollen sacs and relatively flat profile. F. Ovary during anthesis attached to scape with outer whorls removed showing sharp angles, purple coloration, and relatively long stigmatic branches. G. Sharply six-angled ovary in cross-section showing ovule placentation. H. Mature fruit on scape in early July. Photos by J. Lampley (A – G) and E. Schilling (H).

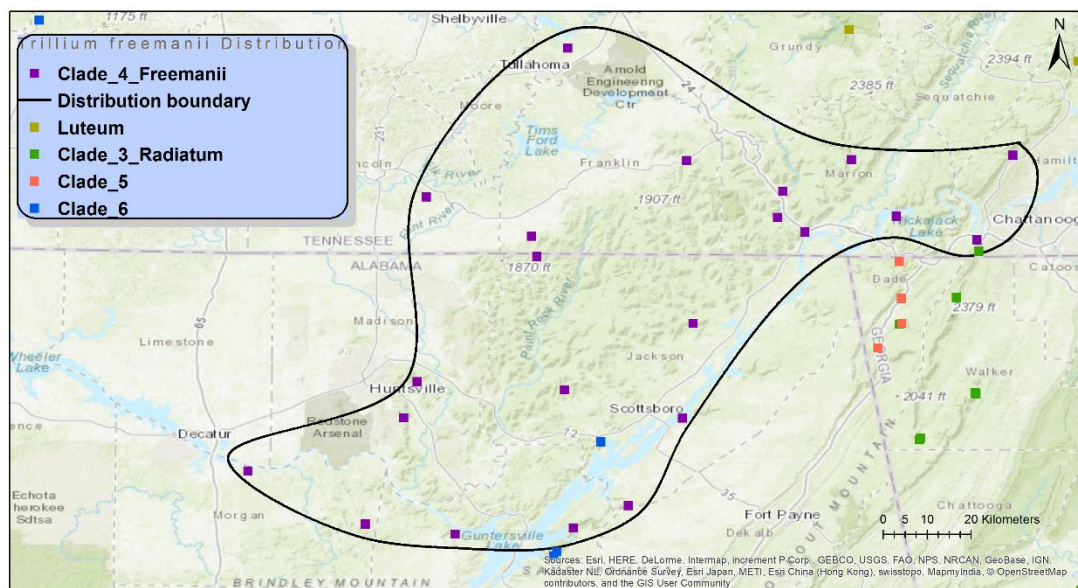


Figure 2.9. Map of *Trillium freemanii* distribution. A map of Tennessee, Alabama, and Georgia depicts the known populations of *T. freemanii* which are marked by purple squares. Other Cuneatum Complex lineages are marked by different colored icons on the map.

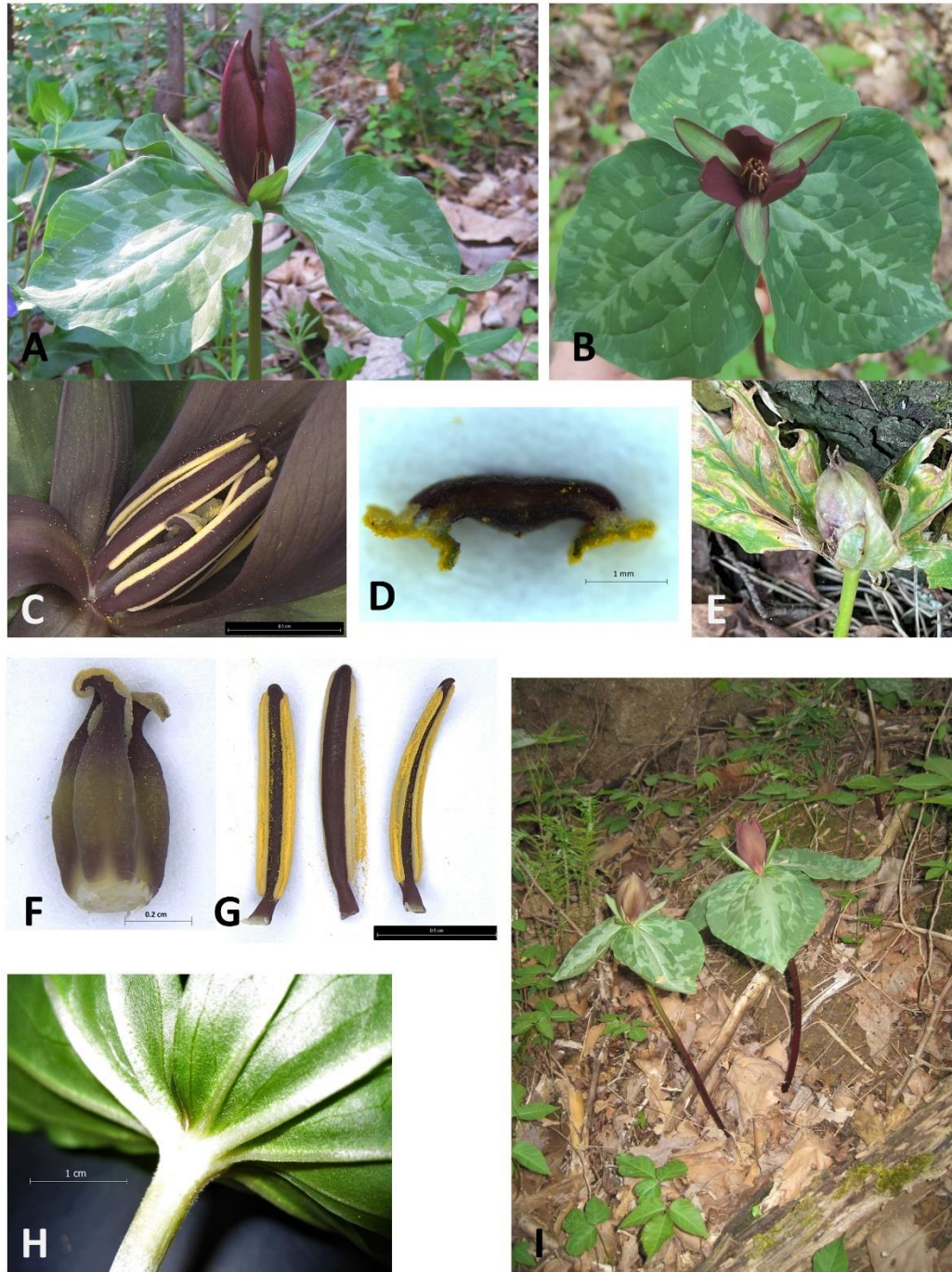


Figure 2.10. Images of *Trillium radiatum*. A – B. Lateral and superior views of *T. radiatum* showing radiate habit of the flowers. C. Androecium showing rounded abaxial surface of anther connectives and introrse dehiscence. D. Anther cross-section showing rounded connectives and prominent introrse pollen sacs. E. Mature fruit in early July. F. Ovary side view during anthesis showing deep angles, greenish-maroon coloration, and relatively short stigmatic branches compared to the length of the ovary proper. G. Stamen adaxial, abaxial, and lateral views (L to R) showing relatively short filaments, robust connectives with rounded abaxial surface and incurved anther apices, introrse dehiscence and prominent pollen sacs with pollen. H. Scape apex and abaxial leaf surfaces showing sparse pubescence on scape and scabrosity of primary veins. I. Scattered growing habit of *T. radiatum* population. Photos by J. Lampley (A – D, F – I) and E. Schilling (E).

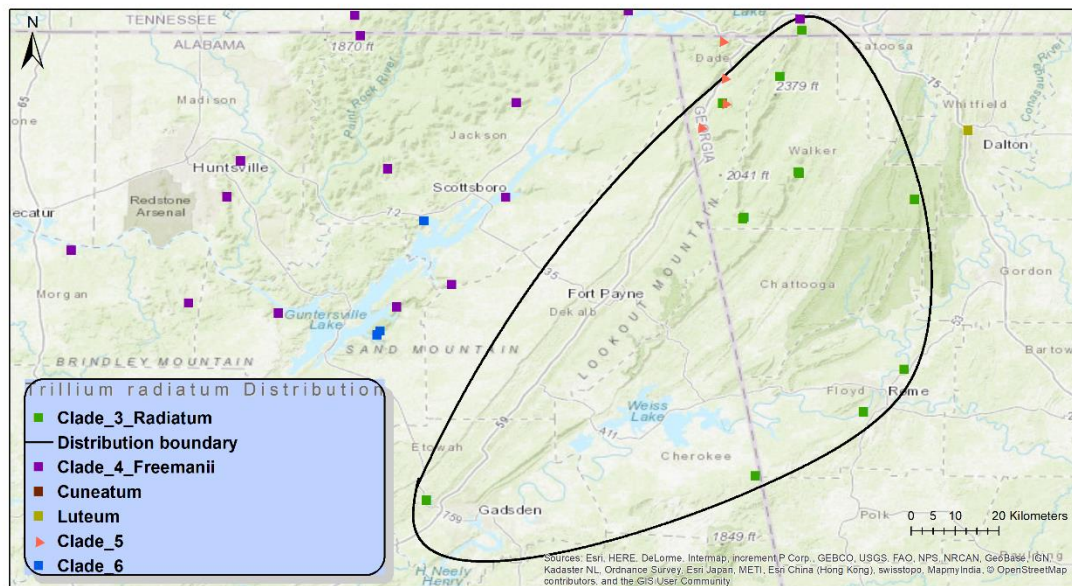


Figure 2.11. Map of *Trillium radiatum* distribution. A map of Tennessee, Alabama, and Georgia depicts the known populations of *T. radiatum* which are marked by green squares. Other Cuneatum Complex lineages are marked by different colored icons on the map.

Chapter 3

A phylogeographic analysis of *Trillium lancifolium* (Melanthiaceae)

INTRODUCTION

Trillium lancifolium and the Thin Rhizome Group

The regional endemic, *Trillium lancifolium* (Melanthiaceae), displays a widespread but discontinuous geographic distribution of isolated populations which is not well understood. Results of recent phylogenetic studies have indicated that populations of *T. lancifolium* make up a paraphyletic assemblage of lineages that also includes *T. recurvatum*, a species widely accepted as distinct (Schilling et al., 2013; Schilling et al., 2019; Lampley, unpub.). There is considerable variation in morphology and flowering phenology among *T. lancifolium* populations. Some of the lineages within *T. lancifolium* may represent cryptic taxa. This study will explore the phylogenetic relationships of *T. lancifolium* and close allies and the possible effects physiography and river drainages have had in its evolutionary and biogeographic history.

Several notable aspects of the geographic distribution of *Trillium lancifolium* make it unique among *Trillium* species. The species has the seventh largest range of sessile *Trillium* species (Extent of Occurrence = 150,000 km²) covering a large portion of the southeastern United States, including the states of Tennessee, Alabama, Florida, Georgia, and South Carolina (Fig. 3.1) (Meredith and *Trillium* Working Group 2019, 2020). This is an area comprising a wide variety of biogeographic regions. However, *T. lancifolium* only inhabits a small footprint scattered across this vast area (Area of Occupancy = 100 – 500 km²) with fewer than 90 populations (Meredith and *Trillium* Working Group 2019, 2020; NatureServe, 2021). This is in contrast to most other *Trillium* species which have either widespread and continuous, or very narrow, isolated distributions (Case and Case, 1997; Case, 2003; Kartesz, 2015). *Trillium lancifolium* is typically found in rich woods, rocky upland woodlands, thickets or canebrakes on floodplain alluvial soils beside creeks or rivers at lower elevations (20 – 200 m) (Small, 1897; Case, 2003; Weakley, 2020). Separate populations have been reported within the Savannah, Ocmulgee, Chattahoochee, Apalachicola, Tennessee, Coosa, Conasauga, Oostanaula, Tallapoosa, Alabama, Cahaba, Black Warrior, and Tombigbee River watersheds. *Trillium lancifolium* also spans the Eastern Continental Divide, the Fall Line (a geomorphologic break between upland physiographic provinces and the sedimentary Coastal Plain), and three major drainage basins (the Atlantic Seaboard, Gulf of Mexico Seaboard, and Ohio River drainage basins).

Trillium lancifolium together with *T. recurvatum* are part of a small clade that will be referred to here as the Thin Rhizome group. This group was first delimited based on morphology by Freeman (1975) as his informal Group 1. This group was subsequently expanded by addition of the recently described *T. oostingii* and *T. tennesseense* based on both morphological and molecular genetic evidence (Schilling et al., 2013). The four species are united in having reflexed sepals; slightly to strongly incurved anther connectives; and thin, brittle, elongate rhizomes, a combination of traits unique within *T.* subgen. *Sessilium* (Small, 1897; Case, 2003; Gaddy, 2008; Schilling et al., 2013). *Trillium lancifolium* and *T. recurvatum* differ in that *T. recurvatum* has shorter more lanceolate-ovate petals with an attenuate to weakly clawed base and a 2:1 length-width ratio, and petiolate leaves, whereas *T. lancifolium* has distinctly long-clawed, linear-spatulate, petals with at least a 4:1 length-width ratio and sessile leaves (Freeman, 1975; Weakley, 2020).

The Thin Rhizome group members are varied in geographic distributions, but all occur in floodplain habitats. *Trillium oostingii* is only known from south of the Fall Line, i.e., the Coastal Plain province, along the Wateree River, whereas *T. tennesseense* is found entirely in the Valley and Ridge province within the Holston River watershed. *Trillium recurvatum* differs from other

species of the Thin Rhizome group in that it is a widespread species inhabiting the watersheds and tributaries of the Mississippi, Tennessee, and Ohio Rivers stretching to Lake Michigan. Finally, *T. recurvatum* and *T. lancifolium* distributions are contiguous but apparently do not overlap, remotely co-occurring in only four places (Lawrence, Sumter, and Tuscaloosa Counties, AL and Hamilton Co., TN) leaving little opportunity for hybridization (Freeman, 1975; Kartesz, 2015; Keener et al., 2021).

Trillium lancifolium is relatively heterogeneous across its distribution (Fig. 3.2). Morphological differences have been noted by botanists among the populations of different regions (A. Floden, pers. comm., 2016). Examples of this variability include differences in petal color and shape, leaf shape, and overall size. Flowering phenology also varies in that there appears to be two different peak periods of blooming: February to early March or mid-April to early May (Case and Case, 1997).

One hypothesis which might explain some of the variability in *T. lancifolium* is that it is actually a pair of disjunct species that occur to the north and south of the Fall Line, respectively. The Fall Line is a geomorphological transition zone which separates the soft sedimentary Coastal Plain province from upland provinces and is distinctive for its waterfalls where rivers cross into the Coastal Plain. The Fall Line also forms the northern edge of the Coastal Plain Floristic Province, a distinct plant assemblage that has the sharpest boundary of any floristic province in North America (Takhtajan, 1986). The North American Coastal Plain has functioned as both a plant species refugium and a center of speciation due to its unique geology and warm climate (Sorrie and Weakley, 1999). At least 2 families, 47 genera and over 1300 species are endemic to this province owing in part to its availability for plant inhabitation since the Eocene (Sorrie and Weakley, 1999). In addition, there are numerous examples of species pairs separated by the Fall Line boundary with one species occurring in the Coastal Plain while the sister species occurs in the Piedmont or other upland province (A. Weakley, pers. comm., 2018). It has been proposed that *T. lancifolium* may be an example of one of these Fall Line species pairs based on its distribution north and south of the Fall Line (Fig. 3.1) (A. Floden, pers. comm., 2016). If this hypothesis is correct, morphological and genetic variability in *T. lancifolium* would correlate to either one group of populations occurring in the Coastal Plain province or to a second group of populations occurring in upland provinces north of the Fall Line.

Another hypothesis which may explain the patterns observed in *Trillium lancifolium* is that its populations have diversified and become isolated along river drainages. North American rivers and tributaries may have functioned as refugia by creating relatively warm, moist zones in which plants could escape the cold, arid climates induced by Pleistocene glaciation. The high specific heat capacity of water makes bodies of water slow to change temperature, creating microclimates with reduced temperature variability and oasis effects (Spronken-smith et al., 2000). Additionally, the floodplains beside rivers and creeks tend to be fertile from nutrient deposition. *Trillium* species tend to occur in rich, fertile and often calcareous soils (Case, 2003; Weakley, 2020), and rivers with their floodplain alluvial soils would make hospitable corridors in which species might make inroads into otherwise infertile new territory. The current distributions of several narrowly endemic *Trillium* species seem to be restricted to specific watersheds of various rivers of the Coastal Plain (Weakley, 2008). Rivers may also lead to diversification by acting as a barrier for some species: a recent study implicated the Tombigbee and Black Warrior Rivers as barriers to gene flow in *T. stamineum* populations (Wallace and Doffitt, 2013). If the river drainages have affected gene flow, morphological similarities and

genetic relatedness among *T. lancifolium* populations would be associated with specific watersheds.

One possibility that was considered in the current study is that hydrochory could be an important factor in the distribution of *Trillium lancifolium*. Hydrochory, or propagule dispersal by water conveyance, has not been observed in *Trillium*, but is a possible means of gene flow among populations which has been documented in riverine plants (Hyslop and Trowsdale, 2012). Documented seed dispersal in *Trillium* mediated by ants is slow, especially for narrow endemics (Kalisz et al., 1999; Miller and Kwit, 2018). However, plants often benefit from a secondary mode of dispersal including that of water dispersal (Beals, 1898; Vellend et al., 2003). *Trillium lancifolium* fruits from late spring through early summer, a time of heavy rains and incidental flooding in the southeastern US. In addition, the indehiscent fruits of sessile trilliums are capsules packed with spongy pith, rather than pulp, which surrounds the seeds (Berg, 1958; J. Lampley, pers. obs.). These characteristics create the ideal buoyancy required for hydrochory (Beals, 1898). If flood events were to allow for occasional hydrochorous dispersal in *T. lancifolium*, particular patterns of genetic structure along waterways would be evident. Upland areas within a particular watershed distant from sources of water may act as a barrier to gene flow in the floodplain-adapted species, whereas rivers and their tributaries may have the potential to be either a habitat corridor or a means of longer-distance water dispersal. If populations of a species upstream or downstream in a river system are more genetically similar to each other than to nearby populations from another watershed, it would suggest that river courses have directed gene flow in some way. If the opposite scenario is true, relatedness among populations would be a factor of proximity and more indicative of typical insect-mediated dispersal patterns (Loveless and Hamrick, 1984; Cain et al., 1998). The aspect that rivers might form barriers to dispersal as well as corridors might help to explain the puzzling distribution of *Trillium lancifolium*.

Objectives

The current study has the following objectives: 1) to obtain a resolved phylogeny to assess relationships within the Thin Rhizome group; 2) to examine the species-level taxonomy of *T. lancifolium* to determine if only one species or more are present; 3) to determine the relationship between phylogeny and distribution to evaluate the hypothesis that there are two taxa within the *T. lancifolium* circumscription separated by physiographic barriers, e.g. the Fall Line; 4) likewise, to evaluate the hypothesis that rivers and watersheds correlate with genetic structure and have contributed to diversification in *T. lancifolium* by affecting patterns of gene flow among populations; and 5) to make observations on the potential for *T. lancifolium* to survive water dispersal of fruits and seeds.

MATERIALS AND METHODS

Taxonomic Sampling

A total of 87 samples were used in the study (Table 3.1). Four species, *T. maculatum*, *T. petiolatum*, *T. underwoodii*, and *T. ovatum*, were represented by concatenations of sequences from more than one individual to complete the gene sampling for each species. To assess the relationships among the disjunct populations of *Trillium lancifolium*, plant material was collected in the field from 36 separate populations across the species distribution in AL, FL, GA, SC and TN. We considered that the non-sampled populations were nearby the ones that were sampled and not likely to differ significantly. Individuals were sampled from 10 different watersheds of the southeastern US from both north and south of the Fall Line, which included: Apalachicola

(Apalachicola-Chattahoochee-Flint (ACF)), Alabama, Black Warrior, Cahaba, Coosa, Ocmulgee, Savannah, Tallapoosa, Tennessee, and Tombigbee Rivers. Samples were also categorized by physiographic province, i.e. Coastal Plain, Piedmont, Valley and Ridge, or Cumberland Plateau. Other members of the Thin Rhizome group (*T. oostingii*, *T. tennesseense*, and *T. recurvatum*) were included in the study. *T. oostingii* and *T. tennesseense* have relatively narrow distributions and were considered to be sampled comprehensively. Samples of *T. recurvatum* were made to cover its range. Data from representatives of other major clades within *T.* subgen. *Sessilium*, as well as selected representatives of other clades of *Trillium* were included. *Trillidium govanianum* was included as an outgroup. Newly collected plant material was frozen at -70° C and kept until the time of extraction. Vouchers were deposited at the herbarium of the University of Tennessee, Knoxville (TENN).

Molecular Methods

Whole genomic DNA extractions were prepared with the DNeasy Plant Minikit (Qiagen, Inc., Hilden, Germany) using manufacturer recommended protocols. Field-collected leaf material (approximately 0.1 g/sample) was flash frozen in liquid nitrogen and ground up immediately before extraction. Eight of the DNA extraction samples had been kept in cold storage (-20°C) at the University of Tennessee Knoxville from a previous study (Farmer, 2007).

Four variable plastid gene regions were used in the study: the intergenic spacer *psbA-trnH*, the region between *ycf1* and *rpl23* that includes *trnI*^{CAU}, and partial regions of the *ycf1* and *ycf2* plastid coding genes. PCR amplification of these genes was performed in 20 µL reactions which included <0.01 µg/µL DNA template and master mix (10X PCR buffer, 1.5 mM MgCl₂, 0.2 mM each dNTP, 1.25 u GoTaq DNA polymerase (Promega, Madison, WI, USA), and 0.2 µM each of forward and reverse primers (Eurofins Scientific, Luxembourg)). The amplification procedure for *psbA-trnH* was the same as was performed by Shaw et al. (2005) and the procedure for *ycf1* and *ycf2* was the same as was performed in Lampley (Dissertation Ch. 2). Amplification protocol of gene *trnI*^(CAU) was done as for *trnH-psbA* but novel primers were developed based on comparisons of plastome sequences: (Forward primer Y1FA 5' to 3': AATGTGAGTGAAAGATCCCAC and Reverse primer R23R 5' to 3': TACTGCATATTTGATTCCATC).

PCR products were detected using gel electrophoresis on 1% agarose gels, purified using ExoSAP-IT PCR Product Cleanup Reagent (Affymetrix, Inc., USB, Cleveland, Ohio, USA), and measured for DNA concentration using a NanoDrop 2000 spectrophotometer (Thermo Scientific, Waltham, MA, USA). Sanger sequencing services were provided by the University of Tennessee Genomics Core (Knoxville, Tennessee, USA) using an Applied Biosystems 3730 Genetic Analyzer electrophoresis instrument and the BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Waltham, MA, USA). Sanger sequence data was manually edited using the Geneious v. 8.1.9 software platform (<https://www.geneious.com>). Next generation sequencing (NGS) via the Illumina HiSeq sequencing platform was used for 10 samples in the study (Table 3.1). NGS and Library preparation were performed by Global Biologics, LLC (Columbia, MO, USA). Assembly of NGS reads and extraction of gene regions for these 10 samples was performed as in the protocol in Lampley (Dissertation Ch. 1).

Phylogenetic Analyses

The MAFFT v7.017 (Katoh and Standley, 2013) utility within Geneious was used to align both Sanger and NGS sequence data. Final adjustment of alignments was done by eye in

Geneious. This was done separately for each gene. The partial gene region *ycf2* was treated as two separate partitions, as in Lampley (Dissertation Ch. 2). The program FastGap v.1.2 (Borchsenius, 2009) was then used to code indels as characters using the method of Simmons and Ochoterena (2000). Coded characters were included in the aligned matrix.

Phylogenetic inference was performed using Bayesian and Maximum Likelihood (ML) methods for the four gene region concatenated dataset. The ML analysis was implemented in RAxML v.7.2.8 (Stamatakis, 2006) and the Bayesian analysis was implemented in MrBayes v.3.2.6 (Ronquist and Huelsenbeck, 2003), both within Geneious. A General Time Reversible model with gamma-distributed rate variation and a proportion of invariant sites (GTR + I + G; Lampley, Dissertation Ch. 1) was used following results of Guindon and Gascuel (2003) and Darriba et al. (2012). In the Bayesian analysis, a total chain length of 1,100,000 generations was produced on four heated chains (temperature = 0.2) with a subsampling frequency of once every 250 generations. The effective sample size (ESS) value, calculated in Geneious, was used as a convergence diagnostic and the first 10% of trees (110,000) was discarded as “burn-in.” The ML analysis, using a bootstrapping algorithm, produced 1200 replicates starting with a complete random tree and from these a majority rule consensus tree was calculated.

Fruit Flotation Experiment

An experiment was conducted to determine the potential for reproductive success in various *Trillium* species during and after river flooding events. Ripe, undehisced *Trillium* fruits were harvested in the field in early summer (late May – June 2018 and 2019) and cleaned of extraneous debris. Fruits were placed in glass jars filled with distilled water and were kept undisturbed on a lab bench at RT and observed for changes. All fruits initially floated on the surface of the water. Once fruits sunk to the bottom of the jar, the number of flotation days was recorded. The fruits were then opened to remove seeds to be prepared for planting. Seeds were planted in ordinary seedling potting mix in plastic seed starter trays within a day of removal from the fruit to prevent seeds from desiccating. Seedlings were maintained in a greenhouse during summer and fall seasons and in a protected outdoor setting during winter and spring for cold treatment (Baskin and Baskin, 1998), and followed for four years. Cotyledon emergence was observed and recorded each spring. Rate of germination was determined by summing the cotyledon counts per fruit for that species in the most successful season for each and then dividing the result by the total number of seeds sown for a given species. Calculations of mean, standard deviation, and standard error were performed with Microsoft Excel (Microsoft 365 MSO).

RESULTS

Phylogenetic analysis

The aligned dataset used for the phylogenetic analysis, including intergenic spacer *psbA-trnH*, the gene region that includes *trnI*^{CAU}, and portions of *ycf1* and *ycf2*, had a total of 4,358 characters, including 121 indel coded characters. The matrix had 2,309 variable sites in the total matrix, not including coded indels. In the total matrix, *Trillium lancifolium* samples had an ungapped length difference of 279, not including samples with missing data. *Trillium recurvatum*, by comparison, had an ungapped length difference of 15 for the 12 samples included in the study. Individual gene length differences can be seen in Table 3.2. A notable gene difference in the study was found in samples of *T. lancifolium* from Clade A which each had two copies of *trnI*^{CAU}. All other samples of *T. lancifolium* and the Thin Rhizome group only had one

copy. A closely related genus of Asia and Europe, *Paris* s.s. (Melanthiaceae), is notable for having three copies of this gene (Huang et al., 2016).

The results of the phylogenetic analysis using Bayesian and Maximum Likelihood (ML) methods strongly supported the monophyly of the Thin Rhizome group and identified eight strongly supported clades (A – H) within a paraphyletic *Trillium lancifolium* complex (Fig. 3.3). The results of both analyses were congruent and therefore support scores for each are presented on the same tree. The trees were rooted on *Trillidium govanianum* in both analyses.

Early diverging splits in the tree were consistent with previous results (Schilling et al., 2019). *Trillium sessile* was placed as the sister group to the Thin Rhizome group clade with moderate support (71 BS, 1.0 PP). Within the Thin Rhizome clade, *T. tennesseense* samples formed a clade with strong support (98 BS, 1.0 PP) that was sister to a weakly supported clade of the remaining samples (68 BS, 0.94 PP). *Trillium oostingii* samples formed a clade with full support (100 BS, 1.0 PP) that was sister to a well-supported clade (95 BS, 1.0 PP) which included representatives of *T. lancifolium* and *T. recurvatum* which will collectively be referred to as the Lancifolium Complex.

Within the Lancifolium Complex clade, one weakly supported clade (64 BS, 0.98 PP) of *T. lancifolium* samples split from a second strongly supported clade (98 BS, 1.0 PP) of *T. recurvatum* and *T. lancifolium* samples. In the first clade, a fully supported subclade (100 BS, 1.0 PP) of *T. lancifolium* samples from northeastern Alabama, Clade A, was placed sister to another fully supported subclade (100 BS, 1.0 PP) of *T. lancifolium* samples from southwestern Alabama, Clade B. Samples from Clade A represented two populations from within the Tennessee river watershed and the Cumberland Plateau physiographic province, whereas samples from Clade B represented 11 populations from the Alabama, Black Warrior, and Tombigbee River watersheds, all within the Coastal Plain province. In the second clade, a fully supported Clade C (100 BS, 1.0 PP) which included all of the *T. recurvatum* samples in the study and one *T. lancifolium* representative was placed sister to a moderately supported clade (89 BS, 1.0 PP) of *T. lancifolium* samples. In this latter clade, two fully supported subclades (each 100 BS, 1.0 PP) emerged. The first subclade, Clade D, comprised a disparate collection of samples representing three populations and three different watersheds (Cahaba, Tallapoosa, and Alabama Rivers) all of the Coastal Plain province in southern central Alabama. The second subclade contained *T. lancifolium* samples from five watersheds, and showed further substructuring. In one weakly supported clade (0.53 PP), which was not resolved in the ML analysis, Clade E was sister to Clades F + G. Fully supported Clade E (100 BS, 1.0 PP) was formed by *T. lancifolium* samples from two populations all within the Savannah River watershed and falling either within the Coastal Plain or the Piedmont provinces. Clade F was formed by samples from two Florida populations, each occurring within the Apalachicola watershed and the Coastal Plain province. Clade G was composed of *T. lancifolium* samples from three populations within the Ocmulgee River watershed and which occurred either within the Coastal Plain or Piedmont provinces. Finally, Clade H was sister to clade E + F + G and was formed by samples of three watersheds (Tennessee, Tallapoosa, and Coosa Rivers). Clade H represented 16 populations of *T. lancifolium* which occurred mainly in the Valley and Ridge province, but also two populations occurred in the Piedmont.

Observations of fruit flotation and seedling germination

The fruit flotation study was performed on a total of 65 fruits from eight species of both sessile and pedicellate *Trillium* species (Table 3.3). *Trillium sulcatum* fruits floated for the

greatest length of time, with an average of 19.5 days. *Trillium pusillum* fruit floated the least amount of time, for less than a day. *Trillium lancifolium* fruits floated on average 5.7 days, whereas fruits of other Thin Rhizome group representatives, *T. recurvatum* and *T. tennesseense*, floated 11.5 days and 1.5 days, respectively. Cotyledon emergence was only observed for two species, *T. lancifolium* and *T. 'freemanii'*. *Trillium lancifolium* had the highest germination rate (7.5%), compared with *T. 'freemanii'* (1.3%).

DISCUSSION

The results of the current phylogenetic analysis added to the evidence that *Trillium lancifolium* as currently circumscribed is paraphyletic. *Trillium recurvatum* was supported as monophyletic, but it was nested within a clade of *T. lancifolium* samples. The analysis suggested that there are up to seven distinct lineages circumscribed as *T. lancifolium*, found in (1) northeastern Alabama, (2) southwestern Alabama, (3) south central Alabama, (4) eastern Georgia-western South Carolina, (5) northern Florida, (6) central Georgia, and (7) the eastern Alabama-northwestern Georgia-southern Tennessee region (see map, Fig. 3.4). The study also supported the monophyly of both of the recently described species, *T. tennesseense* and *T. oostingii*. Finally, rather than one explanation for the apparent diversification of *T. lancifolium*, the phylogenetic and spatial relationships suggest both rivershed and physiographic province boundaries have had a significant effect on the genetic structure of *T. lancifolium* populations.

Using more data and broader taxon sampling, the phylogenetic analysis provided better resolution regarding the relationships of the Thin Rhizome group and was in partial disagreement with previous studies. *Trillium oostingii* was placed as sister to *T. lancifolium* s.l. (i.e., *T. lancifolium* + *T. recurvatum*), whereas *T. tennesseense* was sister to this group. The analysis of Schilling et al. (2013) suggested that *T. tennesseense* was more closely related to *T. lancifolium* s.l. and that *T. oostingii* was sister to this group. Schilling et al. (2019) provided a well-supported phylogeny, however, the results placed *T. lancifolium* s.l. sister to a *T. oostingii* + *T. tennesseense* clade. All three studies were in agreement that *T. recurvatum* was embedded within a paraphyletic *T. lancifolium* s.l. clade. *Trillium oostingii*, a species of the Coastal Plain and the Wateree River watershed, is recognized by some authorities (Gaddy, 2008; Kartesz, 2015; Meredith and *Trillium* Working Group 2019, 2020; Weakley, 2020; NatureServe, 2021), but is instead considered a synonym of *T. lancifolium* by others (USDA, NRCS, 2021). *Trillium tennesseense* is variously accepted (Weakley, 2020; NatureServe, 2021) or is considered synonymous with *T. lancifolium* (USDA, NRCS, 2021). The strongly supported monophyly in the current analysis for both *T. oostingii* and *T. tennesseense* lends support to the recognition of each at the species level.

The current analysis revealed eight lineages within the Lancifolium Complex including *T. recurvatum*. Clade A included samples from northeastern Alabama. Each of these populations fell within the middle course of the Tennessee River and in the Sequatchie Valley of the Cumberland Plateau. Clade B was formed by samples of southwestern Alabama. These populations occurred within the Coastal Plain province and within two connected river watersheds: Black Warrior and Tombigbee, and a third neighboring watershed, Alabama. *Trillium recurvatum* was placed within the *T. lancifolium* s.l. clade rendering the species non-monophyletic. Clade C comprised all of the *T. recurvatum* samples in the study ranging from Illinois to Louisiana. However, one anomalous sample included in Clade C, 5012_etowAL from Big Wills Valley of the Valley and Ridge province, was identified in the field as *T. lancifolium*. It is unclear why this sample had a chloroplast more closely related to *T. recurvatum* with the

appearance of *T. lancifolium*, but it suggests hybridization, although *T. recurvatum* has not been collected in this area. Clade D samples occurred in south central Alabama in the Coastal Plain along three river watersheds: Cahaba, Tallapoosa, and Alabama. The Montgomery Co. sample of this clade occurred in the northern portion of the Alabama R. whereas the other samples on this river from Clade B occurred in the southern portion of the river. Clade E samples all occurred within the Savannah River watershed. The McCormick Co., SC population occurred within the Piedmont and the Burke Co., GA population was within the Coastal Plain. Clade F included samples from northern Florida. All fell within the Coastal Plain along the Apalachicola River watershed. Clade G populations were from the Ocmulgee River watershed in central Georgia. All but one of these samples occurred within the Coastal Plain. Finally, Clade H was formed by samples of eastern Alabama, northwestern Georgia, and southern Tennessee within three separate watersheds. Most samples were divided among the Coosa and the upper portion of the Tennessee River. One sample of this group was from a population in Haralson Co., Georgia and occurred relatively close to the headwaters of the Tallapoosa River. All but two of the samples of Clade H occurred within the Valley and Ridge province.

The hypothesis that there are two taxa within the *T. lancifolium* circumscription separated by one physiographic barrier such as the Fall Line was not supported. Thus, a simple solution is not available. However, relationships among populations seem to be partially correlated with physiographic province. Samples of Clades A, B, D, and F were all of one physiographic province. Clade H was the only clade almost entirely occurring in the Valley and Ridge province. The hypothesis of two taxa divided by the Fall Line did not seem to be supported because the two major clades, A + B and C – H, both had internal clades entirely of the Coastal Plain along with other clades of other provinces. By the branching order of the clades, it does seem possible that the Coastal Plain could have been a center of origin for this group, with multiple dispersals northward from there (Clade A, Clade H), eastward (Clade E, Clade G), and westward (*T. recurvatum*). *Trillium recurvatum* may have then moved northward up the Coastal Plain and the Mississippi River watershed after the Last Glacial Maximum of the Pleistocene.

In some instances, *T. lancifolium* populations reveal a genetic structure that is consistent with the hypothesis that rivers and watersheds have helped shape the patterns of gene flow among populations. For example, samples of Clade A, Clade E, Clade F, and Clade G all occur along only one river. In these cases, especially in Clades E and G which both occur across two physiographic regions, the Coastal Plain and the Piedmont, the hypothesis that the river watershed somehow effects gene flow seems plausible. However, in Clades D and H, the samples occur across neighboring watersheds, but are united by physiographic province, Coastal Plain and Valley and Ridge, respectively. As an alternative to the Coastal Plain origin scenario, it may be possible that there were multiple dispersal events out of a southern Appalachian refugium via ancient south flowing rivers. This could also account for *T. tennesseense* occurring north of the Appalachians and *T. oostingii* occurring south of the Appalachians. Either way, it seems likely that populations of *T. lancifolium* are relicts along these rivers and tributaries.

The results of the fruit flotation and seedling germination study provided evidence that some *Trillium* species can withstand at least some level of incidental flood events, if they should occur. It was shown that *T. lancifolium* fruits have the potential to survive inundation and dispersal by water for up to a week and maintain viable seeds. *Trillium lancifolium* was reported to have a 7.5% germination rate in the study. The species performed better than '*T. freemanii*' which had a germination rate of 1.3%, though it had a longer flotation time of nearly 17 days. *Trillium* '*freemanii*' is a putative taxon synonymous with *T. cuneatum* which inhabits locations

bounded by the Tennessee River. Flooding survival could be useful in the taxon, which is sometimes found in hollows with adjacent creeks. Pedicellate species of the Erectum group, *T. erectum* and *T. sulcatum*, had the longest fruit flotation times, both at approximately 19 days, though neither produced seedlings, and had more seeds per fruit (up to >100) compared with the sessile species. In comparison, *T. lancifolium* ranged from 3 to 27 seeds per fruit. The variability of seed count in a given species is likely due to the differences in age and condition of the rhizome and size of the plant (Knight, 2003). Larger fruit size was correlated with more seeds per fruit, though not necessarily correlated with length of flotation time (J. Lampley, pers. obs.). Potential limitations of this study were that there were only small sample sizes for each species and that, in a few instances, seeds were underdeveloped meaning that the fruit was harvested before fully ripening.

There is a puzzling pattern of variability in *Trillium lancifolium* that will require further study to resolve. However, based on the results of the phylogenetic analysis some options regarding the taxonomy of the Lancifolium Complex may be suggested. One option is that entities of the member clades should be elevated in rank as separate species. This could mean as few as one new species being described, of Clade A + B, while Clade D + E + F + G + H would retain the epithet *T. lancifolium*. Alternatively, as many as six new species could be split out for description. In this case, Clade F would likely retain the species epithet because the neotype is from Chattahoochee, FL within the Apalachicola watershed (Freeman, 1975). *Trillium lancifolium* is listed as State Endangered in Florida and Tennessee and has a global rank of G3 (vulnerable; Meredith and *Trillium* Working Group 2019, 2020). It is threatened by logging, invasive species, and feral hogs (NatureServe, 2021). If individual clades were split into multiple taxa, each of them would be in even greater need of protection. A second option is to expand the circumscription of *T. lancifolium* to include *T. recurvatum*. This would be controversial because *T. recurvatum* is easily identifiable having many morphological characteristics, besides its widespread biogeographic distribution, which distinguish it from *T. lancifolium*. Alternatively, *T. recurvatum* may be an example of a derivative, anacladogenetic species (Smith et al., 2018; Carnicero et al., 2019) that renders *T. lancifolium* paraphyletic. Thus, a third option would be to recognize the two species as currently circumscribed.

References

- Baskin, C.C. and J.M. Baskin. 1998. *Seeds: Ecology, Biogeography and Evolution of Dormancy and Germination*. New York: Academic Press.
- Beals, W.J. 1898. *Seed Dispersal*. Boston: Athenaeum Press.
- Berg, R.Y. 1958. *Seed Dispersal, Morphology, and Phylogeny of Trillium*. Oslo: H. Aschehoug and Co.
- Borchsenius, F. 2009. FastGap 1.2. Department of Biosciences, Aarhus University, Denmark. Available at http://www.aubot.dk/FastGap_home.htm.
- Cain, M., Damman, H., and A. Muir. 1998. Seed dispersal and the Holocene migration of woodland herbs. *Ecological Monographs*. 68(3): 325–347.
- Carnicero, P., Schönswetter, P., Garcia-Jacas, N. and M. Galbany-Casals. 2019. Is there a need for accepting paraphyletic taxa? A case study in the Sardinian endemic *Cymbalaria muelleri* (Plantaginaceae). *Botanical Journal of the Linnean Society*. 191: 325–338.
- Case, F. and R. Case. 1997. *Trilliums*. Portland, OR: Timber Press.
- Case, F. 2003. “*Trillium*.” In: Flora of North America Editorial Committee (Eds.). *Flora of North America North of Mexico*. Volume 26. Oxford: Oxford University Press.
- Darriba, D., Taboada, G.L., Doallo, R., and D. Posada. 2012. jModelTest 2: more models, new heuristics and parallel computing. *Nature Methods*. 9(8): 772.
- Farmer, S.B. 2007. A Systematic Study of *Trillium* subgenus *Delostylis*. PhD Diss., University of Tennessee. Available at https://trace.tennessee.edu/utk_graddiss/4235.
- Freeman, J. 1975. Revision of *Trillium* subgenus *Phyllantherum* (Liliaceae). *Brittonia*. 27: 1–62.
- Gaddy, L.L. 2008. A new sessile-flowered *Trillium* (Liliaceae: subgenus *Phyllantherum*) from South Carolina. *Phytologia*. 90(3): 382–390.
- Guindon, S. and O. Gascuel. 2003. A simple, fast and accurate method to estimate large phylogenies by maximum-likelihood. *Systematic Biology*. 52: 696–704.
- Huang, Y., Xiaojuan, L., Yang, Z., Yang, C., Yang, J., and Y. Ji. 2016. Analysis of complete chloroplast genome sequences improves phylogenetic resolution in *Paris* (Melanthiaceae). *Frontiers in Plant Science*. 7: 1797.
- Hyslop, J. and S. Trowsdale. 2012. A review of hydrochory (seed dispersal by water) with implications for riparian rehabilitation. *Journal of Hydrology*. 51(2): 137–152.
- Kalisz, S., Hanzawa, F.M., Tonsor, S.J., Thiede, D.A., and S. Voigt. 1999. Ant-mediated seed dispersal alters pattern of relatedness in a population of *Trillium grandiflorum*. *Ecology*. 80(8): 2620–2634.
- Kartesz, J.T. 2015. The Biota of North America Program (BONAP). North American Plant Atlas. Available at <http://bonap.net/napa>. Chapel Hill, N.C.
- Katoh, K. and D.M. Standley. 2013. MAFFT multiple sequence alignment software version 7: improvements in performance and usability. *Molecular Biology and Evolution*. 30: 772–780.
- Keener, B.R., Diamond, Jr., A.R., Davenport, L.J., Davison, P.G., Ginzburg, S.L., Hansen, C.J., Major, C.S., Spaulding, D.D., Triplett, J.K. and M. Woods. 2021. Alabama Plant Atlas. Available at <http://www.floraofalabama.org>. (Accessed 25 February 2021).
- Knight, T.M. 2003. Effects of herbivory and its timing across populations of *Trillium grandiflorum* (Liliaceae). *American Journal of Botany*. 90(8): 1207–1214.

- Loveless, M.D. and J.L. Hamrick. 1984. Ecological Determinants of Genetic Structure in Plant Populations. *Annual Review of Ecology and Systematics*. 15(1): 65–95.
- Meredith, C.R. and *Trillium* Working Group 2019. 2020. *Trillium lancifolium*. The IUCN Red List of Threatened Species 2020: e.T146086425A146089255. Available at <https://dx.doi.org/10.2305/IUCN.UK.2020-2.RLTS.T146086425A146089255.en>. (Accessed 25 February 2021).
- Miller, C.N. and C. Kwit. 2018. Overall seed dispersal effectiveness is lower in endemic *Trillium* species than in their widespread congeners. *American Journal of Botany*. 105(11): 1847–1857.
- NatureServe. 2021. NatureServe Explorer [web application]. NatureServe, Arlington, Virginia. Available at <https://explorer.natureserve.org/>. (Accessed July 10, 2021).
- Ronquist, F. and J.P. Huelsenbeck. 2003. MrBayes 3: Bayesian phylogenetic inference under mixed models. *Bioinformatics*. 19(12): 1572–1574.
- Schilling, E.E., Floden, A., and S.B. Farmer. 2013. A New Sessile-Flowered *Trillium* Species from Tennessee. *Castanea* 78(2): 140–147.
- Schilling, E.E., Floden, A., Lampley, J., Patrick, T.S. and S.B. Farmer. 2019. A New Species of *Trillium* (Melanthiaceae) from Central Georgia and its Phylogenetic Position in subgenus *Sessilium*. *Systematic Botany*. 44(1): 107–114.
- Shaw, J., E.B. Lickey, J.T. Beck, S.B. Farmer, W. Liu, J. Miller, K.C. Siripun, C.T. Winder, E.E. Schilling, and R.L. Small. 2005. The tortoise and the hare II: Relative utility of 21 noncoding chloroplast DNA sequences for phylogenetic analysis. *American Journal of Botany*. 92: 142–166.
- Simmons, M.P. and H. Ochoterena. 2000. Gaps as characters in sequence-based phylogenetic analyses. *Systematic Biology*. 49(2): 369–381.
- Small, J.K. 1897. Studies in the Botany of the Southeastern United States IX. The Sessile-Flowered Trillia of the Southern States. *Bulletin of the Torrey Botanical Club*. 24(4): 169–178.
- Smith, J.F., Ooi, M. T.-Y. and J.L. Clark. 2018. Incipient speciation in a neotropical Gesneriaceae: *Columnnea kucyniakii* is nested within *C. strigosa*. *Plant Systematics and Evolution*. 304: 511–519.
- Sorrie, B.A. and A.S. Weakley. 1999. Coastal Plain Vascular Plant Endemics: Phytogeographic Patterns. *Castanea*. 66: 50–82.
- Spronken-smith, R.A., Oke, T.R., and W.P. Lowry. 2000. Advection and the surface energy balance across an irrigated urban park. *International Journal of Climatology*. 20(9): 1033–1047.
- Stamatakis, A. 2006. RAxML-VI-HPC: Maximum Likelihood-based Phylogenetic Analyses with Thousands of Taxa and Mixed Models. *Bioinformatics*. 22(21): 2688–2690.
- Takhtajan, A. 1986. *Floristic regions of the world*. Berkeley, CA: University of California Press.
- USDA, NRCS. 2021. The PLANTS Database. National Plant Data Team, Greensboro, NC USA. Available at <http://plants.usda.gov>. (Accessed 10 July 2021).
- Vellend, M., Myers, J.A., Gardescu, S., And P. L. Marks. 2003. Dispersal of trillium seeds by deer: implications for long-distance migration of forest herbs. *Ecology*. 84(4): 1067–1072.
- Wallace, L.E. and C.H. Doffitt. 2013. Genetic Structure of the Mesic Forest-Adapted Herbs *Trillium cuneatum* and *Trillium stamineum* (Melanthiaceae) in the South-Central United States. *Castanea* 78(2): 154–162.

- Weakley, A.S. 2008. "Ecology and biogeography of *Trillium* in eastern North America: Where are the *Trilliums* and why are they there?" *Trillium* Symposium, Mt. Cuba Center, Hockessin, Delaware. Formerly available at <http://www.mtcubacenter.org/images/symposium-files/Weakley-Alan-Lecture-2.pdf>, (Accessed 09 December 2014).
- Weakley, A.S. 2020. *Flora of the Southeastern United States*, edition of 20 October 2020. Chapel Hill: University of North Carolina at Chapel Hill Herbarium (NCU), North Carolina Botanical Garden, University of North Carolina at Chapel Hill.

Appendix

Appendix C

Table 3.1. A list of samples used in the study. * denotes NGS samples. Physiographic provinces abbreviated as CL=Cumberland Plateau; CP=Coastal Plain; P=Piedmont; and VR=Valley and Ridge. ACF=Apalachicola-Chattahoochee-Flint rivers.

River	Phys	TaxonID	Species	ycf1 TY1F2	trnI Y1FA	psbA- trnH	ycf2 97F	ycf2 1164R	Voucher (TENN unless noted)	Location
ACF	CP	4533_gadsFL	<i>T. lancifolium</i>	X	X	X	X	X	Lampley and Schilling 310	Gadsden Co., FL
ACF	CP	3700_gadsFL	<i>T. lancifolium</i>	X	X	X	X	X	Floden s.n. April 2013	Gadsden Co., FL
ACF	CP	2113_MellFL	<i>T. lancifolium</i>	X	X	X	X	X	Mellard s.n. Mar 2001 - cult.	FL
Alabama	CP	4731_butlAL	<i>T. lancifolium</i>	X	X	X	X	X	Lampley and Schilling 440	Butler Co., AL
Alabama	CP	3696_wilcAL	<i>T. lancifolium</i>	X	X	X	X	X	Floden s.n. 2013	Wilcox Co., AL
Alabama	CP	3697_monrAL	<i>T. lancifolium</i>	X	X	X	X	X	Floden s.n. 2013	Monroe Co., AL
Alabama	CP	4406_dallAL	<i>T. lancifolium</i>	X	X	X	X	X	Barger & Holt OC-171 (AMAL)	Dallas Co., AL
Alabama	CP	*4410_dallAL	<i>T. lancifolium</i>	X	X	X	X	X	Barger & Holt OC-171 (AMAL)	Dallas Co., AL
Alabama	CP	4973_montAL	<i>T. lancifolium</i>	X	X	X	X	X	Lampley and Schilling 466	Montgomery Co., AL
Black W.	CP	3356_tuscAL	<i>T. lancifolium</i>	X	X	X	X	X	Floden 2204	Tuscaloosa Co., AL
Black W.	CP	4712_tuscAL	<i>T. lancifolium</i>	X	X	X	X	X	Lampley and Schilling 421	Tuscaloosa Co., AL
Tombigbee	CP	4525_mareAL	<i>T. lancifolium</i>	X	X	X	X	X	Lampley and Schilling 305	Marengo Co., AL
Tombigbee	CP	4516_sumtAL	<i>T. lancifolium</i>	X	X	X	X	X	Lampley and Schilling 306	Sumter Co., AL
Tombigbee	CP	4717_greeAL	<i>T. lancifolium</i>	X	X	X	X	X	Lampley and Schilling 439	Greene Co., AL
Tombigbee	CP	4722_clarAL	<i>T. lancifolium</i>	X	X	X	X	X	Lampley and Schilling 437	Clarke Co., AL
Tombigbee	CP	5426_washAL	<i>T. lancifolium</i>	X	X	X	X	X	Younge and Williams 065 (UWAL)	Washington Co., AL
Cahaba	CP	4523_perrAL	<i>T. lancifolium</i>	X	X	X	X	X	Lampley and Schilling s.n. 2016	Perry Co., AL
Coosa	P	4669_bartGA	<i>T. lancifolium</i>	X	X	X	X	X	Lampley and Schilling 352	Bartow Co., GA
Coosa	VR	4298_whitGA	<i>T. lancifolium</i>	X	X	X	X	X	Lampley et al. 251	Whitfield Co., GA
Coosa	VR	*4400_calhAL	<i>T. lancifolium</i>	X	X	X	X	X	Lampley and Lampley 282	Calhoun Co., AL
Coosa	VR	4401_calhAL	<i>T. lancifolium</i>	X	X	X	X	X	Lampley and Lampley 282	Calhoun Co., AL
Coosa	VR	4512_stclAL	<i>T. lancifolium</i>	X	X	X	X	X	Lampley and Schilling 299	St. Clair Co., AL

Table 3.1. Continued.

Coosa	VR	4526_stclAL	<i>T. lancifolium</i>	X	X	X	X	X	Lampley and Schilling 299	St. Clair Co., AL
Coosa	VR	4518_stclAL	<i>T. lancifolium</i>	X	X	X	X	X	Lampley and Schilling 301	St. Clair Co., AL
Coosa	VR	4590_floyGA	<i>T. lancifolium</i>	X	X	X	X	X	Lampley et al. 326	Floyd Co., GA
Coosa	VR	4592_floyGA	<i>T. lancifolium</i>	X	X	X	X	X	Lampley et al. 329	Floyd Co., GA
Coosa	VR	4675_gordGA	<i>T. lancifolium</i>	X	X	X	X	X	Lampley and Schilling 353	Gordon Co., GA
Coosa	VR	5012_etowAL	<i>T. lancifolium</i>	X	X	X	X	X	Lampley and Schilling 475	Etowah Co., AL
Ocmulgee	CP	4288_housGA	<i>T. lancifolium</i>	X	X	X			Lampley et al. 245	Houston Co., GA
Ocmulgee	CP	4289_housGA	<i>T. lancifolium</i>	X	X	X			Lampley et al. 245	Houston Co., GA
Ocmulgee	CP	4093_housGA	<i>T. lancifolium</i>	X		X	X	X	Floden and Schilling s.n. April 2014	Houston Co., GA
Ocmulgee	CP	4299_blecGA	<i>T. lancifolium</i>	X	X	X	X	X	Lampley et al. DNA4299 2015	Bleckley Co., GA
Ocmulgee	P	4966_jaspGA	<i>T. lancifolium</i>	X	X	X	X	X	Lampley and Schilling 472	Jasper Co., GA
Savannah	CP	3695_burkGA	<i>T. lancifolium</i>	X	X	X	X	X	Floden s.n. April 2013	Burke Co., GA
Savannah	CP	4570_burkGA	<i>T. lancifolium</i>	X	X	X	X	X	Lampley et al., 337	Burke Co., GA
Savannah	P	4574_mccoSC	<i>T. lancifolium</i>	X	X	X	X	X	Lampley et al., 341	McCormick Co., SC
Tallapoosa	CP	4538_macoAL	<i>T. lancifolium</i>	X	X	X	X	X	Lampley and Schilling 315	Macon Co., AL
Tallapoosa	CP	4539_macoAL	<i>T. lancifolium</i>	X	X	X	X	X	Lampley and Schilling 315	Macon Co., AL
Tallapoosa	P	4657_haraGA	<i>T. lancifolium</i>	X	X	X	X	X	Lampley and Schilling 347	Haralson Co., GA
Tennessee	CL	4505_jackAL	<i>T. lancifolium</i>	X	X	X	X	X	Lampley and Schilling 309	Jackson Co., AL
Tennessee	CL	4969_jackAL	<i>T. lancifolium</i>	X	X	X	X	X	Lampley and Schilling 458	Jackson Co., AL
Tennessee	CL	4683_marsAL	<i>T. lancifolium</i>	X	X	X	X	X	Lampley and Schilling 365	Marshall Co., AL
Tennessee	CL	4684_marsAL	<i>T. lancifolium</i>	X	X	X	X	X	Lampley and Schilling 365	Marshall Co., AL
Tennessee	VR	4968_hamiTN	<i>T. lancifolium</i>	X	X	X	X	X	Lampley et al., 457	Hamilton Co., TN
Tennessee	VR	4664_walkGA	<i>T. lancifolium</i>	X	X	X	X	X	Lampley and Schilling 346	Walker Co., GA
Tennessee	VR	3694_catoGA	<i>T. lancifolium</i>	X	X	X	X	X	Floden s.n. April 2013	Catoosa Co., GA
Tennessee	VR	4084_hamiTN	<i>T. lancifolium</i>	X	X	X	X	X	Floden et al. s.n. April 2014	Hamilton Co., TN
Tennessee	VR	4697_hamiTN	<i>T. lancifolium</i>	X	X	X	X	X	Lampley and Schilling 391	Hamilton Co., TN
		2109_Toost	<i>T. oostingii</i>	X	X	X	X	X	Farmer DNA2109 2005	Kershaw Co., SC

Table 3.1. Continued.

3357_Toost	<i>T. oostingii</i>	X	X	X	X	X	Floden 2012	Kershaw Co., SC
3724_Toost	<i>T. oostingii</i>	X	X	X	X	X	Floden 2013	Kershaw Co., SC
3343_Ttenn	<i>T. tennesseense</i>	X	X	X	X	X	Floden and Schilling 2012	Hamblen Co., TN
3344_Ttenn	<i>T. tennesseense</i>	X	X	X	X	X	Floden and Schilling 2012	Hamblen Co., TN
*4270_Ttenn	<i>T. tennesseense</i>	X	X	X	X	X	Floden 2015	Holston River
4732_Ttenn	<i>T. tennesseense</i>	X	X	X	X	X	Schilling 2017	Hamblen Co., TN
4354_Trecu	<i>T. recurvatum</i>	X	X	X	X	X	JT Lampley s.n. 2015	Wayne Co., IL
*4384_Trecu	<i>T. recurvatum</i>	X	X	X	X	X	Floden 2015	St. Louis Co., MO
4385_Trecu	<i>T. recurvatum</i>	X	X	X	X	X	Floden 2015	St. Clair Co., IL
4594_Trecu	<i>T. recurvatum</i>	X	X	X	X	X	Floden 2016	Tallahatchie Co., MS
4707_Trecu	<i>T. recurvatum</i>	X	X	X	X	X	Lampley and Schilling 394	Colbert Co., AL
4709_Trecu	<i>T. recurvatum</i>	X	X	X	X	X	Lampley and Schilling 406	Pontotoc Co., MS
3811_Trecu	<i>T. recurvatum</i>	X	X	X	X	X	Floden & Schilling 2467	Clay Co., TN
3394_Trecu	<i>T. recurvatum</i>	X	X	X	X	X	Farmer DNA3394 2012	Cumberland Co., TN
2111_Trecu	<i>T. recurvatum</i>	X	X	X	X	X	Farmer DNA2111 2005	Cumberland Co., TN
4120_Trecu	<i>T. recurvatum</i>	X	X	X	X	X	Floden 2014	A3LA 084
4121_Trecu	<i>T. recurvatum</i>	X	X	X	X	X	Floden 2014	A3LA 072
4696_Trecu	<i>T. recurvatum</i>	X	X	X	X	X	Floden 2017	Floden Garden
*4411_Tsess	<i>T. sessile</i>	X	X	X	X	X	Schilling 2015	Big Darby Creek, S. of Columbus OH
*4578_Tvscn	<i>T. viridescens</i>	X	X	X	X	X	Theo Whitsell	Arkansas
3715_Tangu	<i>T. angustipetalum</i>	X	X	X	X	X	Russ Graham	Dickey, Sierra Nevada, CA?
macula_chi	<i>T. maculatum</i>	3693	3693	4569	3693	3693	Floden et al. 2368; Lampley et al. 335	GA; Burke Co., GA;
petiol_chi	<i>T. petiolatum</i>	*4103	*4103	4199	*4103	*4103	Not listed 2014; Floden and Schilling 2014	Custer Co., ID?; Idaho
3358_Tstam	<i>T. stamineum</i>	X	X	X	X	X	Floden 2012	Madison Co., AL
4107_Tdisc	<i>T. discolor</i>	X	X	X	X	X	2014	Oconee Co., SC

Table 3.1. Continued.

underw_chi	<i>T. underwoodii</i>	3688	3701	3688	4729	4729	Floden 2013; Floden 2013; Lampley and Schilling 427	Jackson's Gap; Gadsden Co., FL; Clarke Co., AL
3360_Tdeci	<i>T. decipiens</i>	X	X	X	X	X	Floden 2125	Twiggs Co., GA
*4090_Treli	<i>T. reliquum</i>	X	X	X	X	X	Floden et al.	Upton Co., GA?
2132_Toazar	<i>T. pusillum</i> var. <i>ozarkanum</i>	X	X	X	X	X	Farmer & Estes s.n. May 2005	Russell Co., KY
2134_Talab	<i>T. pusillum</i> var. <i>alabamicum</i>	X	X	X	X	X	Farmer & Rhinehart s.n. April 2005	Cumberland Co., TN
ovatum_chi	<i>T. ovatum</i>	799	801	799	799	799	Karstad s.n. May 2002; Floden and Schilling 2014	Westbank, BC; Idaho
*4324_Tgran	<i>T.</i> <i>grandiflorum</i>	X	X	X	X	X	Lampley et al.,	Overton Co., TN
*4627_Tdgo	<i>Trillidium</i> <i>govanianum</i>	X	X	X	X	X	Susie Egan	Cottage Lake Gardens, Woodinville, WA

Table 3.2. Individual gene length differences of genes used in the study. Minimum and maximum lengths observed among samples are shown.

Marker	Sequence Length	
	<u>Min.</u>	<u>Max.</u>
<i>psbA-trnH</i>	657	861
<i>trnI</i> ^{CAU}	258	537
<i>ycf1</i>	507	717
<i>97F ycf2</i>	813	990
<i>1164R ycf2</i>	882	903

Table 3.3. Observations of fruit flotation and seedling germination in *Trillium*. (SE=standard error.)

Taxon	Mean flotation time (days) ± (SE)	# fruits tested	Min./Max. # seeds/fruit	% germination rate
<i>Trillium lancifolium</i>	5.7 ± 0.65	n = 23	3 to 27	7.50 %
<i>Trillium recurvatum</i>	11.5 ± 3.23	n = 6	6 to 45	0
<i>Trillium tennesseense</i>	1.5 ± 0.29	n = 4	3 to 31	0
<i>Trillium cuneatum</i>	4.3 ± 1.0	n = 15	0 to 92	0
<i>Trillium 'freemanii'</i>	16.9 ± 1.96	n = 8	4 to 68	1.30 %
<i>Trillium erectum</i>	19.0 ± 8.8	n = 4	18 to 123	0
<i>Trillium sulcatum</i>	19.5 ± 4.84	n = 4	41 to 103	0
<i>Trillium pusillum</i>	0.5	n = 1	10	n/a

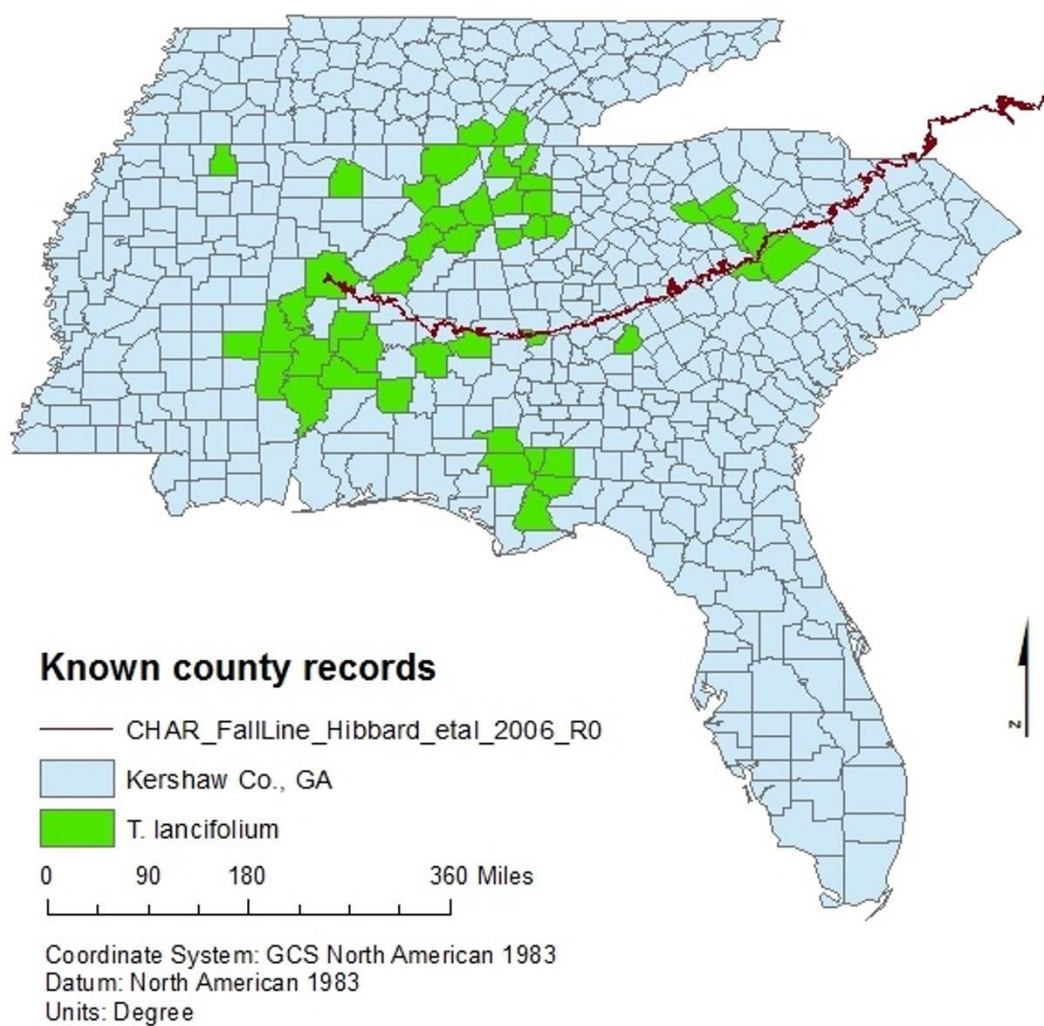


Figure 3.1. A map of the distribution of *T. lancifolium*. County-level data for *T. lancifolium* based on Biota of North America Program (BONAP), excluding *T. oostingii* sites (Kartesz, 2015). Map produced using ArcGIS software (Esri, Redlands, California, USA).

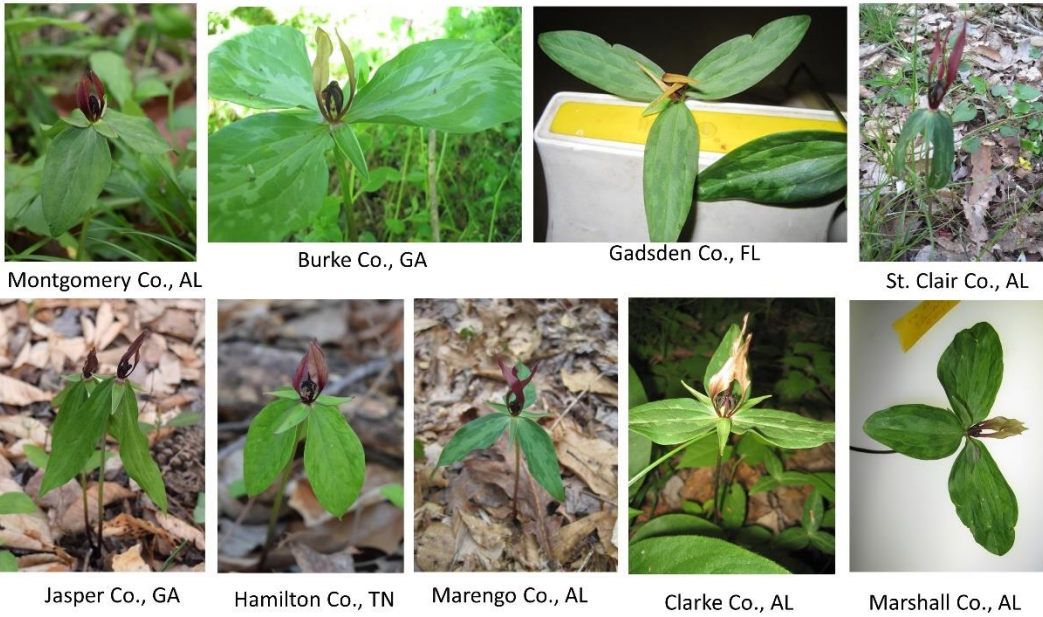


Figure 3.2. Images of *Trillium lancifolium* showing the morphological heterogeneity among various populations. Photographs by E. Schilling or J. Lampley.

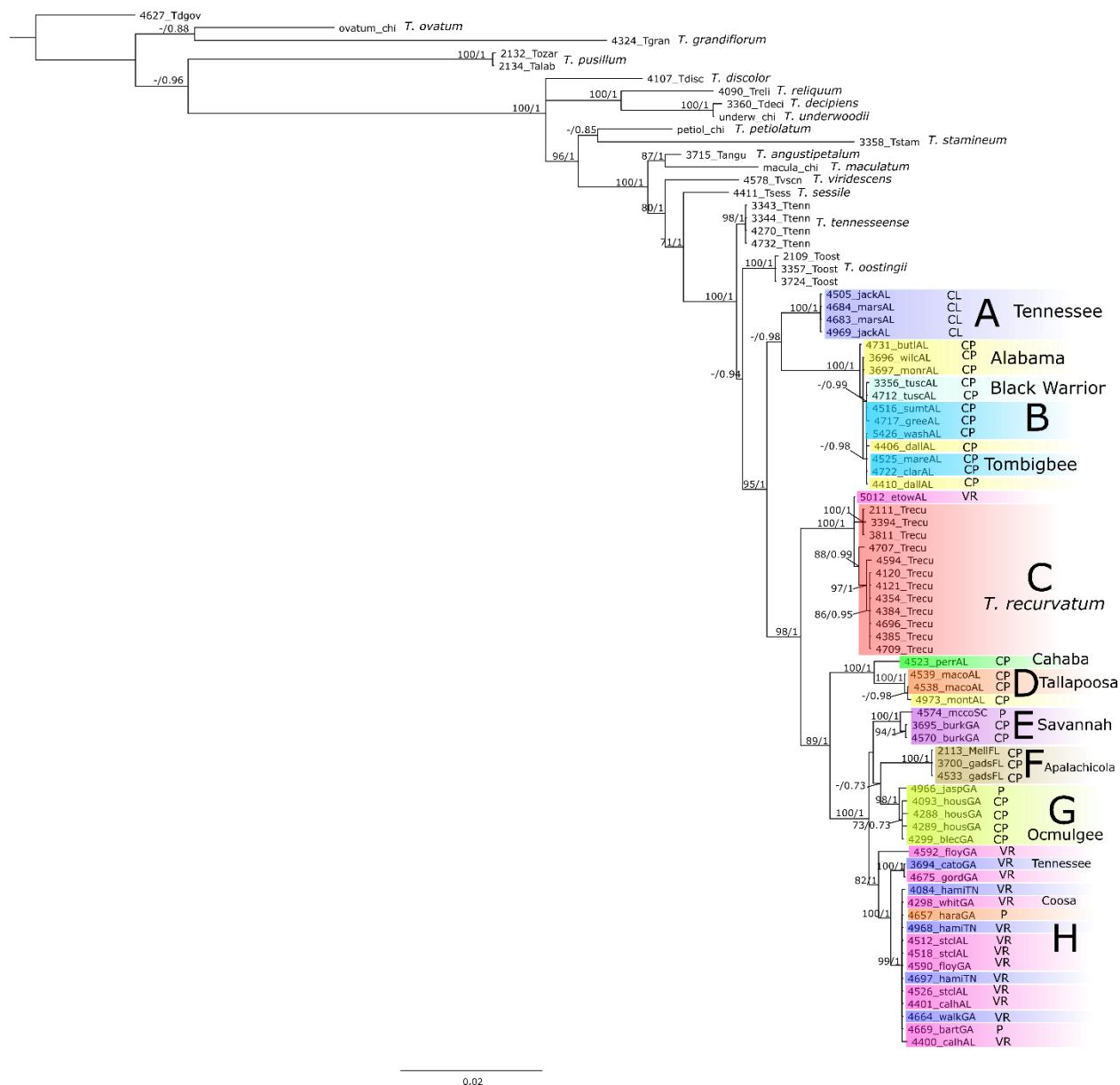


Figure 3.3. Bayesian inference phylogenetic tree using MrBayes showing relationships of *T. lancifolium* and the Thin Rhizome Group. Results from Maximum Likelihood majority rule consensus tree using RAxML are included in the tree. Maximum Likelihood bootstrap scores (> 60) for each branch are listed first and Bayesian posterior probabilities are listed second (> 0.6). Inferences based on plastid data. Clades are labeled A-H, by physiographic province (CL=Cumberland Plateau; CP=Coastal Plain; P=Piedmont; VR=Valley and Ridge), and by major watershed.

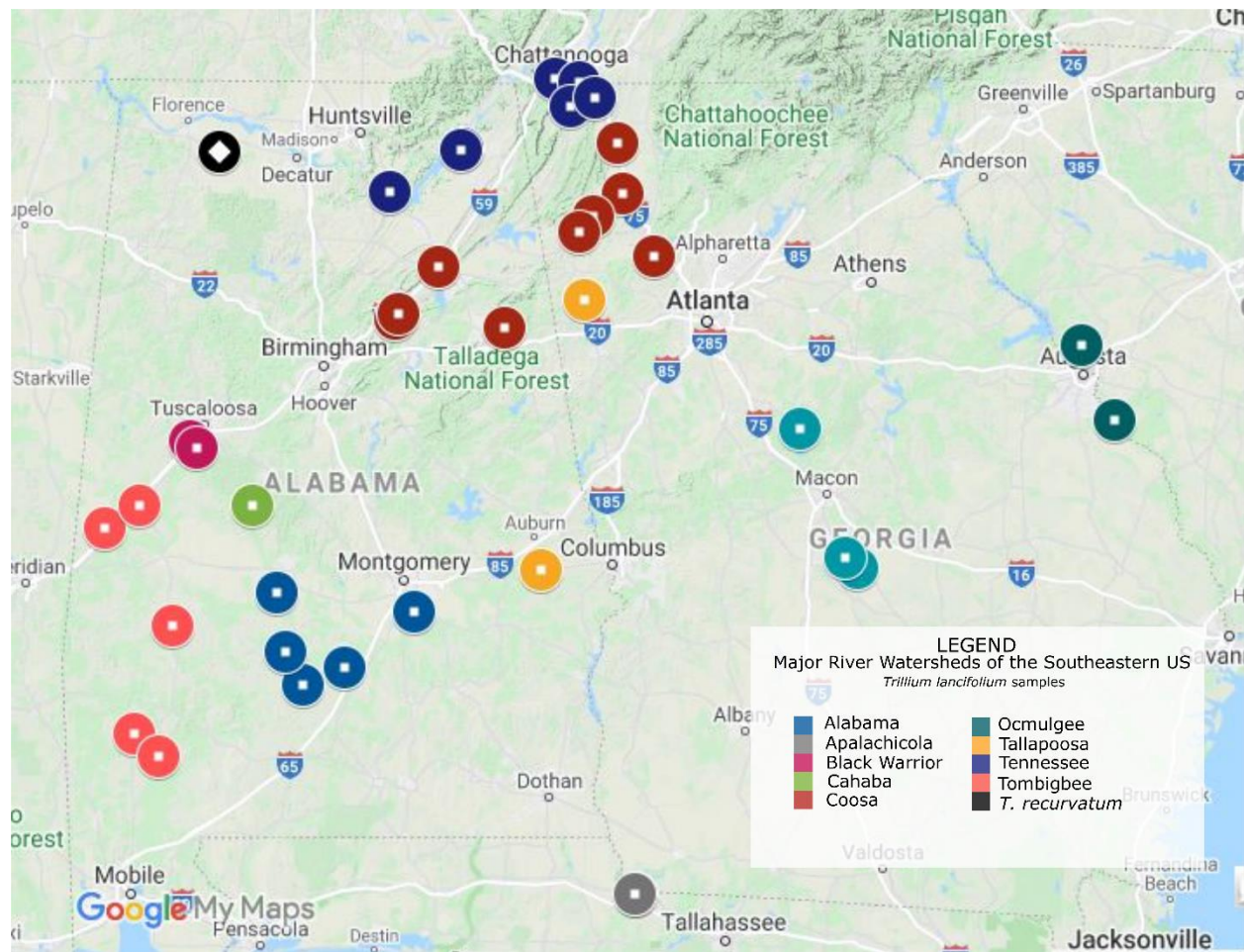


Figure 3.4. A map of the locations of *T. lancifolium* populations sampled in the study. Colors represent major watersheds, listed in Legend. Map produced using Google Maps (www.google.com/maps).

Conclusion

The current study helped resolve relationships within *Trillium* and Parideae at the generic, subgeneric, and species levels. Evidence was shown to help clarify our understanding of the timing of divergences within Parideae as well as the historical ranges where these divergences likely occurred. Relationships among *Trillium* subgenera were evaluated and a new classification was suggested. In addition, the *T. cuneatum* species complex was explored through broad sampling across its distribution. Multiple lineages were identified and two cryptic taxa were described as new species. Finally, the relationship between *T. lancifolium* and *T. recurvatum* was further resolved, however questions about diversification in *T. lancifolium*, with its disjunct populations scattered across numerous watersheds and physiographic provinces of the southeastern US had no straightforward solution.

In Chapter 1, new insights were offered regarding Parideae and *Trillium*. The results of the phylogenetic analysis placed *Pseudotrillium* sister to the remainder of Parideae. The results showed that *Paris* s.l. is monophyletic, and that *Paris* s.s. was sister to *Kinugasa* + *Daiswa*. The results showed the monophyly of *Trillidium* and supported the inclusion of *T. undulatum*. Four major lineages within *Trillium* emerged, partially corresponding with past morphological studies and subgeneric circumscriptions: the Erectum group, the Grandiflorum group, *T.* subgen. *Delostylium*, and *T.* subgen. *Sessilium*. The autonym *T.* subgen. *Trillium* was identified as comprising Erectum group species and *T.* subgen. *Callipetalon* was proposed for the Grandiflorum group representatives. *T.* subgen. *Delostylium* and *T.* subgen. *Sessilium* were kept as previously circumscribed. The results also provided evidence to support the recognition of *T. hibernsonii* as a species, and additionally, placed the species with the Erectum group instead of with *T. ovatum* in the Grandiflorum group. The divergence times estimated for Parideae diversification allow for Miocene disjunctions between eastern Asia and eastern North America and Bering Sea Land-bridge crossings. Diversification within *Trillium* and *Paris* lasted from the late Miocene up until the Pleistocene making a case for Pleistocene climate-driven diversification. Finally, the BioGeoBEARS range reconstruction analysis suggested that the origin for each the Parideae and *Trillium* s.l. lineages was in eastern North America.

Chapter 2 shed new light on relationships within the putative *Trillium cuneatum* complex and *T.* subgen. *Sessilium* as well as providing descriptions for two new taxa. The study employed a broad sampling of *T. cuneatum* populations and a dataset including both chloroplast and nuclear ribosomal sequence data. The results of the phylogenetic analyses provided more evidence that *T. cuneatum* is paraphyletic: its clade included widely recognized species *T. luteum* and *T. maculatum*. *Trillium maculatum* was shown as monophyletic in the results, however, *T. luteum* only formed a weakly supported clade. Five other clades were included within *T. cuneatum* besides the clade of *T. cuneatum* s.s. One clade included a sample of the alleged southern-most population of *T. luteum* from Alabama. This group formed its own clade, however, and did not show a close relationship to Valley and Ridge *T. luteum*. Another clade represented *T. cuneatum* samples from around Lookout Mountain in the southern Valley and Ridge province. Based on morphological observations this clade was described as *T. radiatum* sp. nov. Another clade represented *T. cuneatum* samples from the Tennessee River Gorge area and Jackson Mountains, stretching roughly from Chattanooga, TN to Decatur, AL. The clade was described as a species, *T. freemanii* sp. nov., owing to its distinct morphological characteristics. The remaining two clades included widespread or disjunct taxa and will require further study to determine if they might be named as species. Other results for *T.* subgen.

Sessilium showed that *T. viridescens* is sister to *T. gracile* rather than *T. viride*, *T. ludovicianum* is not sister to *T. gracile*, *T. sessile* is sister to the Thin Rhizome complex, and that *T. lancifolium* appeared to be paraphyletic. In addition, the results of the phylogenetic analysis informed the recommendation that the three section morphology-based classifications of *T.* subgen. *Sessilium* need to be expanded to five sections in all. Finally, microscopic morphological studies outlined differences between *T. cuneatum* s.s., *T. luteum*, *T. maculatum*, *T. freemanii* sp. nov., and *T. radiatum* sp. nov. in floral anatomy which distinguished each.

In Chapter 3, the results of the phylogenetic analysis shed light on the relationships within the putative *T. lancifolium* species complex and the Thin Rhizome group. The analysis, using both Bayesian and Maximum Likelihood methods, was based on plastid data including intergenic spacer *psbA-trnH*, the gene region that includes *trnI*^{CAU}, and portions of coding regions *ycf1* and *ycf2*. An examination of the data matrix revealed that samples of *T. lancifolium* from populations from northeastern Alabama had two copies of *trnI*^{CAU}. All other samples of *Trillium* in the study had only one copy of this gene. The phylogenetic results showed that the *T. lancifolium* clade included a monophyletic *T. recurvatum*, except for one anomalous sample of *T. lancifolium* from the Big Wills Creek valley in Alabama, and seven highly supported lineages of *T. lancifolium* samples. The results also provided further evidence of the monophyly of recently described species *T. tennesseense* and *T. oostingii*. In addition, *T. tennesseense* was placed as sister to the remaining Thin Rhizome group, while *T. oostingii* was placed as sister to the *T. lancifolium* + *T. recurvatum* clade. The hypothesis that there are two lineages within *T. lancifolium* which are separated by one physiographic barrier such as the Fall Line was not supported, however, four *T. lancifolium* clades were each of only one physiographic province (either the Cumberland Plateau or the Coastal Plain) suggesting that various physiographic barriers may be a factor in isolation and gene flow in *T. lancifolium*. In some cases, *T. lancifolium* populations seemed to correlate with specific watersheds. For example, four clades of *T. lancifolium* each consisted of only samples from populations occurring along a single river. Conversely, the Apalachicola, Black Warrior, Coosa, Ocmulgee, Savannah, and Tombigbee Rivers were observed to have only populations which belonged to a single clade in the *T. lancifolium* analysis. The results of the fruit flotation and seed germination study showed that *T. lancifolium* could survive flooding events during fruiting for roughly a week and still have viable seeds, suggesting that secondary seed dispersal by water could have occurred in the floodplain-adapted species *T. lancifolium*.

Vita

A native of southern Illinois, Jayne Lampley, was born in the town of Flora, Illinois. Eventually, her family settled in the town of Guntersville, Alabama where she attended middle and high schools. Jayne chose to attend Jacksonville State University in Jacksonville, Alabama where she majored in both Biology and Chemistry. There she graduated *summa cum laude* with a Bachelor of Science degree in 2010. She returned to Jacksonville State University to work toward a graduate degree having received a teaching assistantship in Chemistry, which was followed by an NSF sponsored research assistantship at the JSU herbarium. Her research involved both field and laboratory work, and was a phylogenetic and phytochemical study of *Polygonatum*. After completing her thesis, she graduated with a Master of Science degree in Biology in 2014. In the fall of 2014, she entered a doctoral program in Ecology and Evolutionary Biology and accepted a teaching assistantship at the University of Tennessee, Knoxville. There she joined the research lab of Professor Edward Schilling and began her systematic and biogeographic study of *Trillium*. This volume is the culmination of the work done for the fulfillment of her Doctor of Philosophy degree, which she received in 2021.