THESIS

CONSERVATION GENETICS AND PHYLOGEOGRAPHY OF A DISJUNCT PRAIRIE PLANT: *CLEMATIS FREMONTII* (RANUNCULACEAE)

Submitted by

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ABSTRACT

CONSERVATION GENETICS AND PHYLOGEOGRAPHY OF A DISJUNCT PRAIRIE PLANT: *CLEMATIS FREMONTII* (RANUNCULACEAE)

Premise: *Clematis fremontii* has a unique disjunct distribution with populations found in multiple distinct and widely separated mixed-grass and xeric limestone prairie habitats in the Great Plains, Ozarks, and Southeast. It is considered rare and endangered in much of its range. This study assesses genetic differentiation and diversity of *C. fremontii* in and among these disjunct areas in order to 1) inform conservation strategies, and 2) better understand the biogeographic processes that shaped its current range.

Methods: 116 samples collected from 17 populations across the species' range plus 10 outgroup samples of *C. ochroleuca* were sequenced using a double digest restriction-site associated DNA (ddRAD) approach. Genetic diversity and structure were analyzed using STRUCTURE, multivariate ordination, and other statistical approaches. hierarchical relationships were estimated with Tetrad, TNT, and a neighbor-net analysis.

Results: All populations showed moderate genetic diversity, and geographic regions showed moderate genetic differentiation from one another. Analyses generally demonstrated that initial divergence was between groups occurring east and west of the Mississippi River, but we found further structuring among disjunct regions. We also found evidence for secondary contact between eastern and western groups, particularly in the Georgia population.

Conclusions: Small, isolated populations of *Clematis fremontii* have higher levels of genetic diversity than we expected, but habitat loss still poses a major threat. Current levels of genetic diversity could indicate an extinction lag. Restoration and population augmentation efforts are needed for this species to persist long-term. Additionally, our data supports the hypothesis that separation of eastern and western *C. fremontii* populations dates to relatively recent Pleistocene events. This could be a vicariance event, such as meltwater mega-flooding of the Mississippi River, or an eastward expansion from a more widespread western group during an interglacial period. Our data did not support the hypotheses of ancient vicariance via the formation of the Mississippi embayment or very recent expansion dating to the Hypsithermal Interval. We also found that *C. fremontii* accumulated genetic variation upon isolation in xeric limestone prairie habitats, similar to a rapid radiation.

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DEDICATION

This research is dedicated to my dog, Banjo. He has no idea what I’ve been working on these past three years, but has been very supportive nonetheless.

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INTRODUCTION

Range disjunctions are major discontinuities in the geographic distribution of a species or group of related species. They have long captured the interest of scientists, as "their very existence would seem to demand the occurrence of some extraordinary historical event" (Hamilton and Eckert, 2007 pg. 1650). Such an event could be an instance of long-distance dispersal or a major vicariance episode. Vicariance of populations could be caused by the formation of a physical barrier, such as a river or mountain range, which separates a formerly continuous population into smaller metapopulations, or a climatic change that causes a species' range to contract, leaving disjunct populations stranded on isolated patches of suitable habitat (Baskin et al., 1997; Strong and Hills, 2003). The study of geographic patterns of genetic variation can be used to test the validity of competing hypotheses for the formation of a given range disjunction (Morris and Shaw, 2018; Prior et al, 2020; Mohn et al., 2021). It can also inform whether disjunct populations are valuable for conservation (Lesica and Allendorf, 1995; Allen et al., 2021), or if a broader, species-level conservation approach is appropriate (Hunter and Hutchinson, 1994).

Many types of grasslands in the eastern United States are hotspots for rare, endemic, and disjunct plant species (Noss, 2013; Cartwright and Wolfe, 2016; Cartwright, 2019). Notable examples include cedar glades (Baskin and Baskin, 2003), Appalachian shale barrens (Keener, 1983), high-elevation grassy balds (Wiser, 1994), and xeric limestone prairies (Allison and Stevens, 2001; Ware, 2002; Krakowiak and Shaw, 2019). Xeric limestone prairies (XLPs), as defined by Lawless et al. (2006), are open, non-forested areas occurring on shallow, rocky, soils derived from calcareous substrates with moderate to steep slopes. They are characterized by high abundance of C_4 perennial grasses such as *Schizachyrium scoparium* (Michx.) Nash, *Bouteloua curtipendula* (Michx.) Torr., *Andropogon gerardii* Vitman, and *Sorghastrum nutans* (L.) Nash, as well as high diversity of heliophytic forbs, many of which are rare at state, regional and/or global levels. Most XLPs represent an alternative stable state to deciduous forest (Gray, 1878; Noss, 2013) in that they remain open with an appropriate fire return interval or other equivalent disturbance but can succumb to woody encroachment without such disturbances (Beilmann and Brenner, 1951). They differ from glades, which are typically defined as flat, C_3 dominated climax communities (Baskin and Baskin, 2003; Cartwright and Wolfe, 2016), though the term "glade" has been misapplied to many XLPs (Baskin and Baskin, 2004; Krakowiak and Shaw, 2019), or defined more broadly by others (Ware, 2002). In the eastern United States, XLPs most commonly occur within the Ozark Plateau, Interior Low Plateau, and Ridge and Valley regions (Baskin and Baskin, 2000; Lawless et al., 2006).

The evolutionary and biogeographic origins of XLP communities are complex (Lawless et al., 2006). Some elements are ancient, evidenced by endemic distributions and primitive characteristics. Examples include *Liatris oligocephala* J. Allison, endemic to primary XLPs on Ketona dolomite outcrops in Bibb County, Alabama, and *Oenothera argillicola* Mackenzie, an endemic of shale barrens and XLPs in the mid-Appalachian region (Bartgis, 1993). The unique, simple cyme inflorescence of *L. oligocephala* is thought to be a primitive characteristic, possibly resembling the ancestral progenitor taxon of *Liatris* and *Carphephorus* (Allisons and Stevens, 2001; Lawless et al., 2006). In *O. argillicola*, distinctive cytological characteristics and its phylogenetic relation to extant species in the Southwestern United States (the center of diversity for *Oenothera*) suggest that this species could have entered the Mid-Appalachian region as early as the Tertiary (Keener, 1983; Dietrich et al., 1997). Others are thought to be recently derived from western prairie species, such as eastern members of *Echinacea* (Baskin and Baskin, 1986; Flagel et al., 2008) and *Dalea* (Diggs, 2013).

Clematis fremontii S. Watson occurs in a series of discrete and widely separated populations in mixed-grass prairie in the Smoky Hills region of Kansas and Nebraska (Locklear, 2017) and XLPs in the Ozarks and Southeast (NatureServe, 2022) (Figs. 1, 2). Historical taxonomic treatments of *C. fremontii* recognized an autonymic prairie variety and a "glade" variety, *C. fremontii* var. *reihlii*, to differentiate western and Ozark populations (Erickson, 1943). But, morphological variation within populations and even individual plants of this species is so extensive that these varieties are no longer recognized (Keener, 1967). Small, disjunct Southeastern populations in northern Arkansas (Graves, 2016), southeastern Tennessee (Horn and Shaw, 2007; Krakowiak et al., 2019), and northwestern Georgia (Ware, 2003) were all discovered much more recently.

Published hypotheses regarding the biogeographic processes that led to *Clematis fremontii*'s present distribution were all made prior to the discovery of populations east of the Mississippi River. Erickson (1945) suggested that western and Ozark populations of *C. fremontii* were connected by way of the Edward's Plateau in Texas (Fig. 1), similar to the present-day range of *Oenothera macrocarpa* Nutt. Interestingly, like *C. fremontii*, *O. macrocarpa* is also disjunct to the Southeast, where it occurs in the limestone glades of Middle Tennessee. Erickson (1945 pg. 426) suggested that separation between these two regions occurred during the "semi-arid period of the late Pleistocene, or, in view of the importance of competition from grasses, during the warmer, moister period which followed". Learn and Schaal (1987) suggested that the XLPs on which Missouri populations of *C. fremontii* occur are prairie remnants that have only been isolated since the end of the mid-Holocene Hypsithermal Interval, about 5,000 years ago (Sears, 1942).

Keener (1967, 1983) considered the biogeographic origins of *C. fremontii* from a broader taxonomic point of view in his studies of *Clematis* section *Viorna* subsection *Integrifoliae.* All members of this small group, aside from *C. fremontii*, strictly occur east of the Appalachian Mountains, with three mid-Appalachian shale barren endemics (*C. albicoma* Wherry, *C. coactilis* (Fernald) Keener, *C. viticaulis* Steele) and the relatively widespread *C. ochroleuca* Aiton (Fig. 1). Given their striking morphological similarities, he believed that the shale barren endemic *C. albicoma*, and *C. fremontii* possibly shared a widespread common ancestor that was split into eastern and western groups during the Pleistocene. Similarly, Erickson (1943) suggested that, given the narrow range of subsect. *Integrifoliae* species (excluding *C. ochroleuca*), their distribution could be relictual. Recent discoveries of eastern populations seem to support these hypotheses (Montgomery and Shaw, 2012).

A past study of phylogeographic relationships among populations of *C. fremontii* sampled cpDNA and nuclear ribosomal internal transcribed spacer (ITS) sequences (Montgomery, 2009), but it was found that these markers did not contain sufficient variability to distinguish populations of *C. fremontii* or even species within subsect. *Integrifoliae* from each other. Based on this low sequence variation, Montgomery and Shaw (2012) suggested that species in this group are recently diverged.

Similarly, Miikeda et al. (2006) reported tremendous morphological diversity yet low diversity in cpDNA and ITS sequences as evidence of a recent rapid radiation in *Clematis* (though no subsect. *Integrifoliae* species were included). To overcome these limitations, this study uses double digest restriction-site associated DNA sequencing (ddRAD) data to investigate the phylogeographic factors that gave rise to the present disjunct distribution of *C. fremontii*. This sequencing method has proved informative in other conservation genetic and phylogeographic studies as it is able to reveal a large number of polymorphic loci (Andrews et al. 2016; Prior et al., 2020). It has also proven useful in a recent phylogenetic study of North American *Clematis* (Harris, 2019).

We hypothesize that *C. fremontii* was formerly more widespread and that present disjunct metapopulations became isolated due to climatic change(s) and/or the formation of physical barriers. Ozark and eastern populations could be recent relics of a westward expansion (and subsequent contraction) of prairie habitat that occurred during and after the Hypsithermal Interval (Transeau, 1935; Hamilton and Eckert, 2007). Alternatively, *C. fremontii* could have expanded outward from the Ozarks during the Hypsithermal Interval to colonize populations in the western prairies and Southeast. If *C. fremontii* was widespread prior to the Hypsithermal Interval, vicariance of broad eastern and western groups could have occurred due to the formation of the Mississippi embayment during the late Eocene and Miocene (Cox and Van Arsdale, 2002) (Fig. 1). Vicariance between western and eastern groups could also be explained by more recent, Pleistocene flooding of the Mississippi River (Keener, 1983; Fildani et al., 2018; Prior et al., 2020).

Western and Ozark populations could have become isolated after the Hypsithermal Interval (Learn and Schaal, 1987), or their separation could also date to earlier Pleistocene vicariance events such as climatic change (Erickson, 1945), or flooding and/or temporary rerouting of the Missouri River and its tributaries (Bayne and Fent, 1963; Fildani et al., 2021). We hypothesize that separation between central and southern Ozark populations occurred in the last few hundred years due to recent reduction of open woodlands and savanna across this region (Beilmann and Brenner, 1951; Hanberry et al. 2014). Further, we hypothesize that populations in the Southeast and southern Ozarks have the lowest genetic diversity and show evidence of recent reduction in size, and thus be of higher conservation concern than those in Kansas and Nebraska. These western prairie populations primarily occur in areas with extremely low human population density (U.S. Census Bureau, 2020), sparse development aside from cattle ranches (which don't appear to strongly affect *C. fremontii* populations), and do not require disturbance to remain treeless (Albertson, 1937). Biogeographic hypotheses were tested with hierarchical analyses as well as analyses of population genetic diversity and structure. These data were also used to inform conservation strategies for this species.

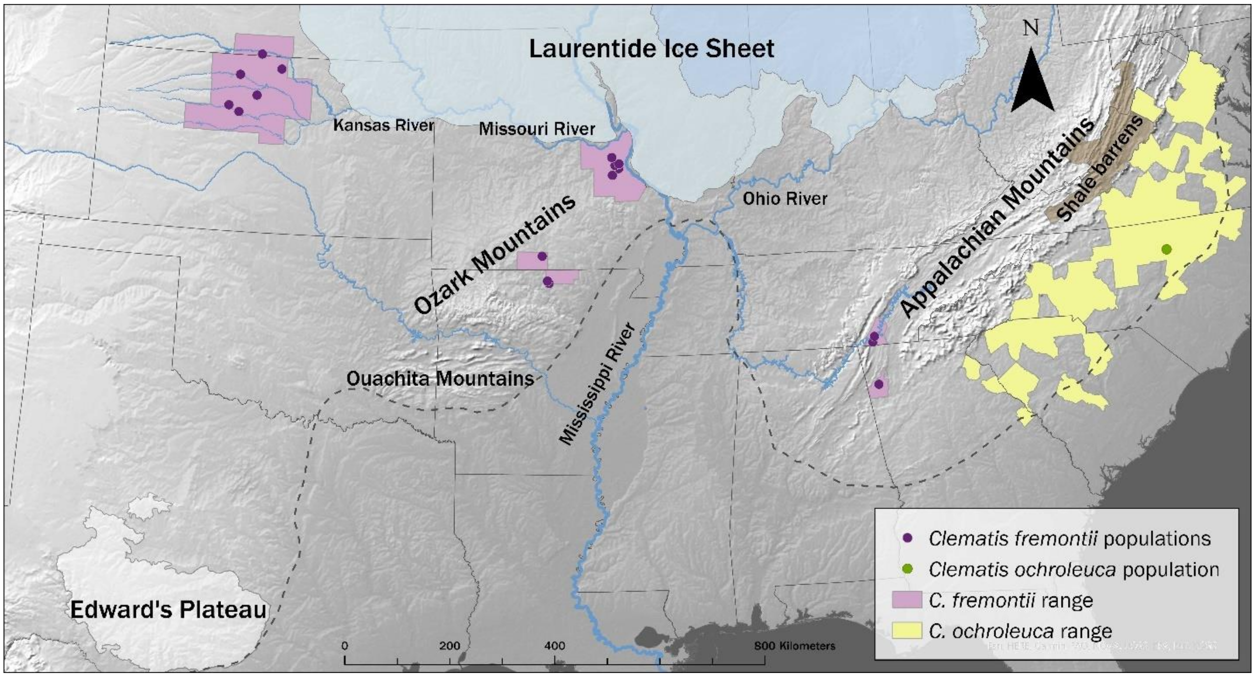

FIGURE 1. Map of the central and southeastern United States indicating distributions of *Clematis fremontii* and *C. ochroleuca* from county-level herbarium data (SERNEC, 2022), locations of sampled populations, and features that may have shaped the species' range. Features include the Ozark, Ouachita, and Appalachian Mountains, Edward's Plateau, mid-Appalachian shale barrens (depicted by the combined ranges of the three *Clematis* subsection *Integrifoliae* species endemic to them: *C. albicoma*, *C. coactilis, C. viticaulis*), and Mississippi River with various tributaries. The Mississippi River embayment and Coastal Plain (dashed line), which was inundated to varying degrees during the late Eocene and Miocene due to shifting sea levels (Cox and Van Arsdale, 2002), is indicated. The Mississippi-Missouri drainage basin was also subject to meltwater mega-floods during Pleistocene periods of deglaciation (Fildani et al., 2018). The extent of the Laurentide Ice Sheet during the last glacial maximum (dark blue), and its maximum extent from pre-Wisconsin glacial advances (light blue) is also shown (Korus et al., 2021).

MATERIALS AND METHODS

Study Species

Clematis fremontii is a non-vining species with large, coriaceous leaves and pink-purple urceolate flowers with thick, leather-like sepals. It is a long-lived perennial and grows in large clumps, with older individuals often having over 20 branches emerging from a woody, underground caudex with deep roots (Erickson, 1945; Keener, 1967). Leaves are amphistomatic with large stoma relative to other subsect. *Integrifoliae* species (Keener, 1967), a feature considered to be strongly associated with evolution in open habitats (Jordan et al., 2014). In Missouri populations, Erickson (1945) found bees (*Bombus* and *Apis* spp.) to be the most important pollinators, but also noted frequent visits by bumblebee-mimic sphinx moths (*Hemaris diffinis* (Boisduval)), various butterflies, and ants, though he doubted the involvement of these visitors in pollination. The breeding system is thought to be facultative or cyclic self-fertilization (Erickson, 1945; Keener, 1967). Like other members of subsection *Integrifoliae*, *C. fremontii* produces a dense head of achenes with long, persistent styles, but it is the only member in which styles are glabrous rather than plumose. Thus, seeds are mostly gravity-dispersed (Keener, 1967). However, seeds may travel short distances via the tumbleweed-like habit of larger senesced individuals (Erickson, 1945; Mehlman, 1993). Germination rates are apparently low, as evidenced by few observed seedlings in the field and extremely low greenhouse germination success (Erickson, 1945; Keener, 1967; J. Lee and K. Pittman, Missouri Botanical Garden, pers. comm.; M. Kintgen and B. Palmer, Denver Botanic Gardens, pers. comm.).

Clematis fremontii is considered rare and threatened across much of its range; it is listed as an S1 species (critically imperiled) in Georgia, Tennessee, Arkansas, and Nebraska, S3 (vulnerable) in Missouri, and S5 (secure) in Kansas (NatureServe, 2022). It is considered to be globally secure (G5), though its global status has not been recently assessed and is marked as ‘needs review’ (NatureServe, 2022). Threats include habitat loss, woody species encroachment due to altered (or non-existent) fire regimes, and potential loss of genetic diversity in small, isolated populations.

Site Selection and Permissions

Populations of *C. fremontii* were located through the use of herbarium specimens accessed with the SERNEC data portal, “Research Grade” iNaturalist observations, and advice from local Natural Heritage Program or botanical garden staff. In Kansas, five roadside populations roughly spanning the species’ distribution in the state were selected for sampling (e.g., Fig. 2C). The sampled Nebraska population represents the only recently verified population in the state and is also located on an unprotected roadside (Fig. 2B). The five sampled central Missouri populations all occur in state parks and other natural areas, including two that are specifically managed to conserve grassland habitat with prescribed burns and cedar (*Juniperus virginiana* L.) removal: Valley View Glades Natural Area (Fig. 2A) and Victoria Glades Conservation Area. The population sampled in southern Missouri and the two in Arkansas represent the only reported populations in the region; all three occur on unprotected roadsides (e.g., Fig. 2F). The two populations sampled in Tennessee and the one in Georgia also represent the only reported populations in their respective region. One Tennessee population occurs on a roadside property that was recently for sale, while the other occurs at a small National Historic Park located in a densely populated urban area (Fig. 2D) (Krakowiak and Shaw, 2019). The Georgia population is located alongside a walking trail on a private college campus (Fig. 2E). One population of *C. ochroleuca* near Durham, North Carolina was sampled as an outgroup. The primary author made an attempt to sample *C. ochroleuca* from the known populations in Georgia, but no individuals were located. Site specific permit information is presented in Appendix S1.

Sampling and DNA Extraction

Leaf samples were collected from *C. fremontii* individuals in 17 populations spanning its range in early May 2020 and mid-April 2021. For the outgroup species, *C. ochroleuca*, samples were collected from a population near the center of its range in early May 2021 (Fig. 1). In each population, 2-3 fresh, young leaves were collected from 10 individuals spaced at least 10 m apart and then dried in silica gel. However, the Georgia population was so small (<15 individuals observed) that more closely spaced plants

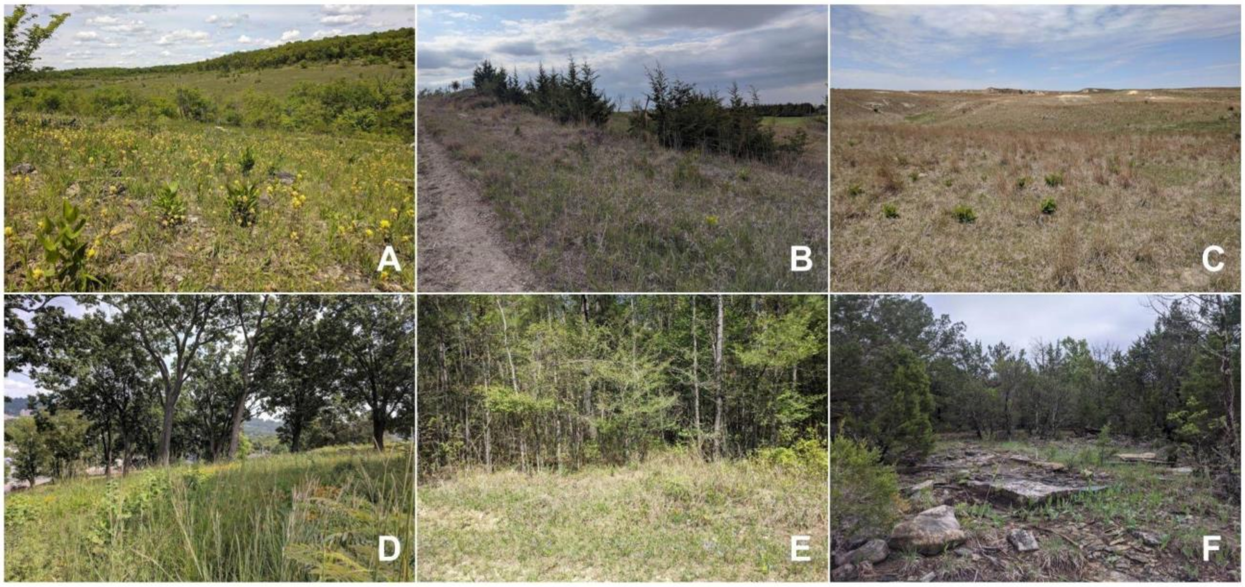

FIGURE 2. Habitat diversity observed by the primary author in places where *Clematis fremontii* occurs. A: Valley View Glades, Jefferson County, Missouri, B: roadside in Webster County, Nebraska, C: roadside in Smith County, Kansas, D: Orchard Knob Reservation, Hamilton County, Tennessee, E: trailside at Berry College, Floyd County, Georgia, F: roadside in Fulton County, Arkansas.

were sampled. One voucher specimen was collected per population and deposited at the University of Tennessee at Chattanooga Herbarium, except for those in Tennessee and Georgia which were already well documented (see Appendix S2 for voucher information). Genomic DNA was isolated from 10-20 mg tissue using silica-based columns following the protocol of Alexander et al. (2007), with modifications to the grinding process, increased incubation times, and lower elution volumes (Appendix S3). DNA concentrations were measured using a Qubit 2.0 or 3.0 fluorometer (Life Technologies, Waltham, Massachusetts, USA) and standardized to 40 ng/μL. Isolated DNA samples were stored at -30°C.

Library Preparation and Sequencing

One hundred and eighteen samples of *Clematis fremontii* were ultimately selected for sequencing. All ten *C. ochroleuca* samples were sequenced. One library (42 samples) was sequenced at the University of Colorado Boulder BioFrontiers Sequencing Core. Due to the closure of this facility, a second library containing the remaining 86 samples was sequenced at the Genomic Shared Resource at the University of Colorado Anschutz Medical Campus. DNA samples were processed according to a modified double digest RADseq protocol (Parchman et al., 2012; Tripp et al., 2017) using EcoR1 and Mse1 enzymes (New England Biolabs, Ipswich, Massachusetts, USA) to digest the DNA. Illumina adapters and barcodes (Tripp et al., 2017) were ligated to opposing ends of the fragments followed by PCR amplification. Samples for the first library were quality filtered and size selected using BluePippin (Sage Science, Beverly, Massachusetts, USA) at the BioFrontiers Sequencing Core. For the second library, AMPure XPbeads (Beckman Coulter, Jersey City, New Jersey, USA) were used to perform a two-sided size selection both before and after PCR. In both cases, 200-500 bp fragments were selected and libraries were checked for quality on a BioAnalyzer (Agilent Technologies, Wilmington, Delaware, USA). The first library was sequenced on an Illumina NextSeq with single-end 75 bp reads and the second on a NovaSeq 6000 SP flow cell with single-end 100 bp reads.

Sequence Data Processing

Raw Illumina reads were checked for quality with FastQC version 0.11.9 (Andrews, 2010). Trimmomatic version 0.39 (Bolger et al., 2014) was used to remove Illumina adapters, remove leading and trailing low-quality bases, scan reads with a 4-base sliding window and cut when the average quality per base drops below 15, drop reads which are <36 bp, and to crop reads from the NovaSeq run to match those of the NextSeq run. Files from the first run were demultiplexed with Fastq-Multx (Aronesty, 2013); files from the second run were demultiplexed by the sequencing facility. Single nucleotide polymorphism (SNP) discovery was performed de novo with iPyrad version 0.9.68 (Eaton and Overcast, 2020) using default parameters, except as noted here. The clustering threshold (14) was increased to 0.9, and the maximum number of indels allowed per locus (23) was reduced to zero. The first five and last ten base pairs of each assembled locus were trimmed (26) to eliminate barcode overhang due to demultiplexing errors from the second sequencing facility.

Three final datasets with different levels of missing data were produced. In the single dataset used for hierarchical analyses, loci present in at least 35% of samples were retained. Two datasets for population genetic analyses were produced, one with ingroups only, the other with the outgroup included. Both used a more conservative sampling threshold, allowing only the loci present in at least 70% of samples. In all cases, multiple runs allowing for varying levels of missing data were performed to ensure that inferences were robust to this parameter. Two ingroup samples were dropped due to low sample coverage. BayeScan version 2.0 (Foll and Gaggiotti, 2008) was used with default parameters to identify loci under selection, but no loci were identified for subsequent removal. The final hierarchical dataset included 126 individuals and 25419 loci with 180386 SNPs. The ingroup-only population genetic dataset included 116 individuals and 976 loci with 7503 SNPs. The outgroup-included population genetic dataset had 126 individuals and 882 loci with 7057 SNPs.

Hierarchical Analyses

To help test our phylogeographic hypotheses, we generated hierarchical trees with Tetrad, maximum parsimony analysis, and a neighbor-net analysis. Tetrad (Eaton and Overcast, 2020) uses the SVDQuartets algorithm of Chifman and Kubatko (2014) to infer quartet trees, and then uses the software wQMC to join all quartets into a supertree. All quartets were sampled, with 1,000 non-parametric bootstrap replicates. Additionally, the 60574 parsimony-informative nucleotide characters were analyzed in a hierarchical context using equally weighted parsimony in TNT version 1.5 (Goloboff and Catalono, 2016). The most parsimonious tree search consisted of 1000 tree-bisection-reconnection (TBR) search replicates and up to 50 trees held per search. Strict-consensus bootstrap support (Felsenstein, 1985; Davis et al., 1998) was determined using 1000 replicates, each consisting of 10 TBR searches, up to 50 trees held per search, and TBR collapsing. Bootstrap values were then mapped onto the most parsimonious tree. TreeGraph 2 version 2.15 (Stöver and Müller, 2010) was used to visualize trees. PAUP* version 4.0b10 (Swofford, 2001) was used to trim the 2,195,583-character matrix to the 96,595 variable characters. The variable characters were then uploaded into SplitsTree4 version 4.18.1 (Huson and Bryant, 2006), in which we conducted a neighbor-net analysis based on uncorrected p-distances.

Analyses of Genetic Diversity

We used analyses of genetic diversity to test our biogeographic hypotheses, and to compare levels of genetic diversity and inbreeding in all populations in order to inform conservation recommendations. VCFtools version 0.1.16 (Danecek et al., 2011) was used to further filter the population genetic datasets for use in generating statistics. The max-missing parameter was set to 0.7, thereby excluding sites with greater than 30% missing data. Recoded VCF files were converted into Arlequin format using PGDSpider version 2.1.1.5 (Lischer and Excoffier, 2012). For the ingroup-only dataset, Arlequin version 3.5.2.2 (Excoffier et al., 2005) was used to calculate observed and expected heterozygosity, average number of alleles, number of polymorphic loci for each population, and pairwise genetic distance between populations (F_{ST}). Inbreeding coefficients (F_{IS}) and proportions of polymorphic loci were calculated from these statistics in an Excel spreadsheet. Stacks Populations version 2.2 (Catchen et al., 2013) was used to determine the number of private alleles for each population. Genetic diversity values for five regions defined by geographic separation (Kansas/Nebraska, central Missouri, southern Missouri/Arkansas, Tennessee, Georgia) were also calculated by taking the averages of values for populations. Average F_{ST} values for regional comparisons were also calculated. Isolation by distance was tested by plotting average geographical distance between regions against Slatkin's linearized F_{ST} for regional comparisons, calculated by $F_{ST}/(1-F_{ST})$ (Slatkin, 1993). Significance of results was calculated using a Python implementation of the Mantel test with 100000 permutations and the Pearson correlation coefficient (Mantel, 1967). The population genetic dataset that included the outgroup was only used for calculating F_{ST} of *C. fremontii* populations relative to *C. ochroleuca.*

Analyses of Genetic Structure

Population genetic structure was assessed with STRUCTURE version 2.3.4 (Pritchard et al., 2000), principal components analysis (PCA), and two analyses of molecular variance (AMOVA). For STRUCTURE analyses, groups (K) were tested for values of 1 to 6. For each value of K, ten repetitions were run with a 5000 generation burn-in period and 10000 generations after burn-in. Structure Harvester web version 0.6.94 (Earl and vonHoldt, 2012) was used to generate ΔK plots in order to determine the best supported K value (Evanno et al., 2005). A principal components analysis (PCA) was run with the ipyrad-analysis toolkit PCA tool (Eaton and Overcast, 2020) using default values for "mincov" and "minmap" parameters to filter SNPs shared across less than 50% of all samples and require 50% of samples to have data in each of the five geographic regions defined above. Missing values were imputed with the “sample” method. To reduce the effects of linkage on results and visualize confidence levels, 100 replicate analyses were run using a random seed, each subsampling a different random set of unlinked SNPs (one per locus). Arlequin version 3.5.2.2 (Excoffier et al., 2005) was used to run a standard analysis of molecular variance (AMOVA) with groupings of populations into five regions (as defined in Table 2) and two regions (based on the best supported K value from STRUCTURE analysis). Relative support for these groupings was considered by comparing levels of ‘among regions’ variance.

RESULTS

Observations From Field Sites

During sample collection, the primary author noted extensive variation in the size, color, and shape of sepals, as well as variation in pedicel length, leaf shape and pubescence, plant height, and internode length. Variation was considerable among and within regions, within populations, and in some cases in individual plants. This finding is consistent with others' observations of this species (Erickson, 1943; Keener, 1967; Graves, 2016). Floral color variation was most notable in Kansas populations, with numerous individuals showing a variety of colors on the underside of sepals (Fig 3B, F) which wasn't commonly observed elsewhere. It was also noted that the color of individual flowers shifts as they age. Young flowers cupped between fresh leaves of Tennessee plants, for example, generally start out pale and shift to a darker magenta (Fig. 3H, A). Conversely, some young flowers in Kansas were observed to start out extremely dark purple (Fig 3G).

FIGURE 3. Floral color and morphological variation in *Clematis fremontii* observed by the author. A: Tennessee, B: Kansas, C: Georgia, D: southern Missouri, E: central Missouri, F: Kansas, G: Kansas, H: Tennessee.

Populations of *C. fremontii* in Kansas/Nebraska were the most extensive and least spatially discrete. It was also observed that, unlike many other native mixed grass prairie plants (Eddy, 1990), this species is avoided by cattle, likely due to the presence of cyanogenic compounds and bitter-tasting saponins (Greshoff, 1909; Seigler, 1976). In some central Missouri populations, such as in Victoria Glades Conservation Area and Valley View Glades Natural Area (Fig. 2A), individual plants likely numbered in the tens of thousands on open hillsides. The smallest central Missouri population sampled was in Don Robinson State Park, where <20 individuals were found in a semi-open area with high canopy cover. Populations in southern Missouri and Arkansas were all relatively small (50-100 individuals), and more heavily dominated by *Juniperus virginiana*. The Tennessee population at Orchard Knob Reservation (~300 individuals) is protected from development, but encroachment of woody and invasive species is a major threat (Fig. 2D). The other Tennessee population is larger, but increasingly dominated by *J. virginiana*, and is apparently sometimes used for dumping trash and large brush piles (Krakowiak et al., 2019). The Georgia population was by far the smallest sampled. Less than 15 plants could be located on the sunny edge of a dense pine woodland (Fig. 2E), with only one individual in flower. Compared to previous reports (Montgomery, 2009), it seems that this population has decreased in size in recent years.

Hierarchical Analyses

Trees produced by Tetrad (Fig. 4A, Appendix S4) and TNT (Appendix S5) showed populations of *C. fremontii* to form a well-supported species that is distinct from the outgroup, *C. ochroleuca*. The tree produced with Tetrad showed Southeastern populations of *C. fremontii* to be sister to all others, though this branch had low bootstrap support (63%). This branching pattern shows initial divergence between groups occurring east and west of the Mississippi River. Every other major split had bootstrap support ≥ 95%. The clade of Kansas and Nebraska populations was sister to all Ozark (central/southern Missouri and Arkansas) populations. Within the Southeastern clade, all three populations formed exclusive lineages with 100% bootstrap support, with Georgia sister to the two Tennessee populations. Within the Ozark clade, southern Missouri and Arkansas populations formed a clade with 99% bootstrap support, and the three populations within each formed clades with 100% bootstrap support. Individual populations within the larger central Missouri group generally did not form well-supported clades; MO5 had the highest bootstrap support (72%). Within the Kansas/Nebraska clade, KS1 was resolved as a clade with 87% bootstrap support, and NE was resolved as a clade with 99% bootstrap support. These findings show further structuring within eastern and western groups.

The most parsimonious tree (Appendix S5) showed the clade of Kansas/Nebraska as sister to all other *C. fremontii* populations, though bootstrap support for this relationship was low (59%). Support for the sister relationship of Ozark and Southeastern populations was also low (45%). Relationships within regions were very similar to those found in the Tetrad analysis.

The neighbor-net analysis based on uncorrected p-values revealed short, wide branches separating major regions, with substantially longer, primarily terminal branches within individual regions, showing lower diversity between regions, and higher diversity within. Branches between regions were generally similar in length and did not show a strong east-west split (Fig. 4B).

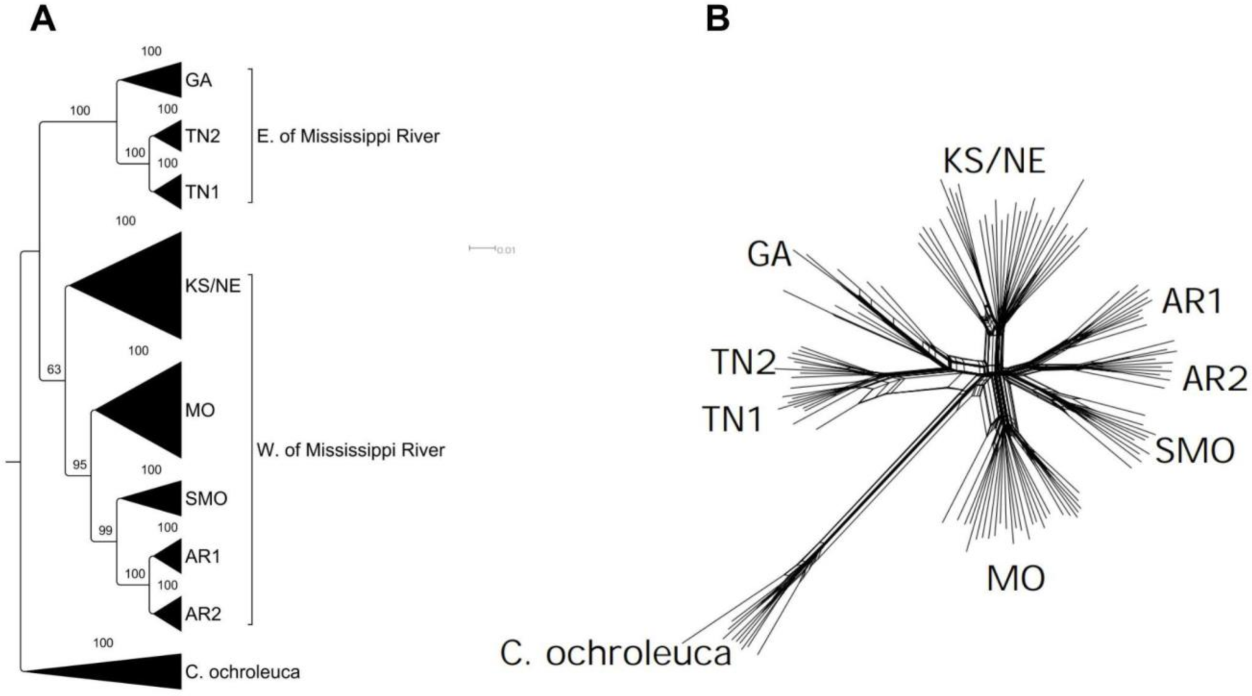

Figure 4. Results of hierarchical analyses of 116 *Clematis fremontii* and 10 *C. ochroleuca* individuals. (A) Summarized hierarchical tree of 116 *C. fremontii* individuals and 10 *C. ochroleuca* individuals produced with Tetrad. Bootstrap values are shown above branches and clades. The full tree is shown in Appendix S4. (B) Neighbor-net analysis based on uncorrected p-values. See Table 1 for population abbreviations.

Patterns of Genetic Diversity

Observed heterozygosity (H_O) values ranged from 0.22 (SMO) to 0.37 (MO4), showing moderate genetic diversity in all populations. Expected heterozygosity (H_E) ranged from 0.21 (SMO) to 0.36 (KS2) (Table 1). Of the five regional groups, Kansas/Nebraska and central Missouri had the highest levels of genetic diversity as measured by heterozygosity (H_O=0.34, H_E=0.33 for both regions) (Table 2). Tennessee had intermediate values (H_O=0.27, H_E=0.25) while southern Missouri/Arkansas and Georgia had the lowest (H_O=0.23, H_E=0.22 for both regions) (Table 2). Because H_O exceeded H_E in all populations, all F_{IS} values were negative. F_{IS} values for individual populations ranged from -0.12 (TN1) to -0.01 (AR2) (Table 1). This finding could be indicative of heterozygote excess, but because these values were only slightly negative, is likely better interpreted as a lack of inbreeding. Average number of alleles per population ranged from 2.000 (MO4) to 2.012 (AR2). All populations had private alleles, ranging from 87 in MO3 to 264 in SMO. Proportions of polymorphic loci in individual populations ranged from 12.11% (MO3) to 21.83% (AR2) (Table 1). The three lower diversity regions had more unique diversity as measured by number of private alleles per population and percent polymorphic loci (Table 2).

Population pairwise F_{ST} estimates from the ingroup-only dataset ranged from -0.01 (KS3 vs. KS4) to 0.20 (TN1 vs. MO3, SMO, and AR1), showing generally moderate differentiation between populations (Table 3). Among regions, the comparison of Tennessee and southern Missouri/Arkansas had the highest F_{ST} (0.18), while Kansas/Nebraska versus Central Missouri had the lowest (0.07) (Table 4). Mean F_{ST} values for comparisons between populations within regions were all much lower, aside from the southern Missouri/Arkansas group (0.10). There was no evidence for a pattern of isolation by distance based on linearized F_{ST} values for intra-regional population comparisons (p=0.453, r=0.114) (Fig. 5). Population pairwise F_{ST} estimates between *C. fremontii* populations and *C. ochroleuca* ranged from 0.16 (MO4) to 0.26 (TN2) (Appendix S6), with an average of 0.21. Most F_{ST} estimates between populations of *C. fremontii* were lower than comparisons with *C. ochroleuca*, but not all.

TABLE 1. Population abbreviation, locality information (some coordinates obscured for protection), sample size (n), and genetic diversity statistics for all populations including observed heterozygosity (H_O), expected heterozygosity (H_E), inbreeding coefficient (F_{IS}), mean number of alleles (A), number of private alleles (PA), and proportion of polymorphic loci (%P). Standard deviations are given in parentheses.

Abbr.	Site, County, State	Lat, Long	n	H_O	H_E	F_{IS}	A	PA	%P
NE	Red Cloud, Webster, NE	40.0, -98.5	5	0.36 (0.21)	0.34 (0.15)	-0.05	2.004 (0.07)	126	14.16
KS1	Delta Cemetery, Jewell, KS	39.797, -98.055	5	0.34 (0.20)	0.32 (0.13)	-0.06	2.006 (0.08)	114	13.74
KS2	F. Rd., Smith, KS	39.664, -98.973	5	0.36 (0.20)	0.36 (0.15)	-0.02	2.006 (0.08)	118	13.99
KS3	Tipton, Osborne, KS	39.329, -98.578	5	0.33 (0.17)	0.32 (0.12)	-0.01	2.003 (0.05)	149	15.19
KS4	Saline Rd., Russell, KS	39.031, -98.958	5	0.34 (0.20)	0.33 (0.15)	-0.03	2.006 (0.01)	167	16.45
KS5	King Hill, Rooks, KS	39.133, -99.186	5	0.33 (0.19)	0.32 (0.13)	-0.03	2.005 (0.07)	118	14.29
MO1	Washington State Park, Washington MO	38.1, -90.7	5	0.34 (0.21)	0.33 (0.16)	-0.03	2.002 (0.04)	93	12.56
MO2	Victoria Glades, Jefferson, MO	38.203, -90.544	5	0.32 (0.20)	0.32 (0.13)	-0.02	2.007 (0.09)	90	13.30
MO3	Valley View Glades, Jefferson, MO	38.257, -90.625	5	0.34 (0.19)	0.32 (0.13)	-0.06	2.002 (0.04)	87	12.11
MO4	Sandy Creek Covered Bridge, Jefferson, MO	38.3, -90.5	5	0.37 (0.20)	0.36 (0.15)	-0.03	2.000 (0.00)	122	14.27
MO5	Don Robinson State Park, Jefferson, MO	38.4, -90.7	7	0.31 (0.19)	0.30 (0.16)	-0.03	2.005 (0.07)	143	15.41
SMO	Hwy 181, Ozark, MO	36.7, -92.2	10	0.22 (0.16)	0.21 (0.12)	-0.05	2.007 (0.08)	264	20.32
AR1	Wild Cherry, Fulton, AR	36.3, -92.1	10	0.25 (0.19)	0.22 (0.14)	-0.10	2.010 (0.10)	200	19.38
AR2	Elizabeth, Fulton, AR	36.3, -92.1	10	0.23 (0.15)	0.23 (0.13)	-0.01	2.012 (0.02)	262	21.83
TN1	Orchard Knob Reservation, Hamilton, TN	35.0, -85.3	10	0.27 (0.21)	0.25 (0.14)	-0.12	2.004 (0.06)	136	15.80
TN2	Adams Rd., Hamilton, TN	35.1, -85.2	9	0.27 (0.20)	0.25 (0.13)	-0.10	2.005 (0.07)	122	16.67
GA	Berry College, Floyd, GA	34.3, -85.2	10	0.23 (0.17)	0.22 (0.12)	-0.03	2.004 (0.06)	223	19.09

TABLE 2. Regional genetic diversity statistics including number of populations sampled (Pop.), total sample size (n), averages values for observed heterozygosity (H_O), expected heterozygosity (H_E), inbreeding coefficient (F_{IS}), and number of alleles, sum of private alleles for all populations in the region (PA), average number of private alleles per population (Avg PA) and average proportion of polymorphic loci (%P).

Region	Pop.	n	H_O	H_E	F_{IS}	A	Sum PA	Avg PA	%P
Kansas/Nebraska	6	30	0.34	0.33	-0.03	2.005	792	132	14.64
Central Missouri	5	27	0.34	0.33	-0.04	2.003	535	107	13.53
Southern Missouri/Arkansas	3	30	0.23	0.22	-0.05	2.010	726	242	20.51
Tennessee	2	19	0.27	0.25	-0.11	2.005	258	129	16.23
Georgia	1	10	0.23	0.22	-0.03	2.004	223	223	19.09

TABLE 3. Population pairwise F_{ST} estimates; p-values symbolized above the diagonal (p-value <0.01= **, p-value <0.05= *, p-value >0.05= ns). F_{ST} values >0.15 are shown in bold.

	NE	KS1	KS2	KS3	KS4	KS5	MO1	MO2	MO3	MO4	MO5	SMO	AR1	AR2	TN1	TN2	GA
NE		*	**	**	**	**	**	*	**	**	**	**	**	**	**	**	**
KS1	0.01		**	**	**	**	*	**	**	**	**	**	**	**	**	**	**
KS2	-0.01	0.00		ns	ns	**	**	**	**	*	**	**	**	**	**	**	**
KS3	-0.02	0.02	-0.03		*	**	*	**	**	**	**	**	**	**	**	**	**
KS4	0.00	0.02	-0.04	-0.01		*	**	*	*	*	**	**	**	**	**	**	**
KS5	0.00	0.03	-0.03	0.00	-0.02		**	**	**	**	**	**	**	**	**	**	**
MO1	0.07	0.10	0.07	0.07	0.06	0.07		ns	*	ns	ns	**	**	**	**	**	**
MO2	0.11	0.10	0.06	0.09	0.08	0.07	-0.03		ns	ns	*	**	**	**	**	**	**
MO3	0.12	0.11	0.08	0.10	0.07	0.08	-0.00	-0.02		*	*	**	**	**	**	**	**
MO4	0.07	0.06	0.04	0.05	0.06	0.05	-0.04	-0.03	-0.02		**	**	**	**	**	**	**
MO5	0.09	0.07	0.05	0.05	0.06	0.07	-0.03	-0.01	-0.02	-0.02		**	**	**	**	**	**
SMO	0.12	0.12	0.07	0.12	0.09	0.09	0.10	0.12	0.11	0.09	0.06		**	**	**	**	**
AR1	0.11	0.11	0.08	0.12	0.10	0.13	0.10	0.11	0.12	0.10	0.07	0.12		**	**	**	**
AR2	0.11	0.12	0.08	0.09	0.08	0.09	0.08	0.10	0.11	0.07	0.08	0.10	0.08		**	**	**
TN1	0.18	0.17	0.15	0.17	0.15	0.16	0.19	0.18	0.20	0.16	0.13	0.20	0.20	0.18		**	**
TN2	0.15	0.14	0.11	0.14	0.13	0.14	0.14	0.15	0.16	0.12	0.09	0.17	0.18	0.16	0.04		**
GA	0.09	0.09	0.07	0.08	0.06	0.07	0.08	0.08	0.09	0.05	0.05	0.10	0.11	0.10	0.14	0.10	

TABLE 4. Average significant population pairwise F_{ST} estimates for each region.

	Kansas/ Nebraska	Central Missouri	Southern Missouri/ Arkansas	Tennessee	Georgia
Kansas/Nebraska	0.00				
Central Missouri	0.07	-0.01			
Southern Missouri/Arkansas	0.10	0.10	0.10		
Tennessee	0.15	0.15	0.18	0.04	
Georgia	0.08	0.07	0.11	0.12	~

Genetic Structure

Structure Harvester indicated K=2 as the best supported number of groups, showing broad separation between populations occurring east and west of the Mississippi River (Fig. 6A). The eastern group comprised populations in Tennessee and Georgia, while the western group comprised populations in Kansas/Nebraska, central Missouri, and southern Missouri/Arkansas. Of the eastern populations, Georgia showed the strongest signature of admixture with the western group. The second-best supported number of groups was K=4, though support was much lower (see Appendix S7 for graph of ΔK vs. K). This value of K corresponded to four major groups: Kansas/Nebraska, central Missouri, southern Missouri/Arkansas, and Tennessee/Georgia (Fig. 6B).

Principal component analysis explained 7.3% and 5.5% of variance on PC axes zero and one, respectively (Fig. 7). This analysis generally showed an east-west split, but each of the five geographic regions formed a distinct cluster. Similar to STRUCTURE results, the Georgia population occurred in an intermediate position between populations west of the Mississippi and Tennessee populations on PC axis zero, indicating possible admixture. The relatively broad span of replicates demonstrates variability in results, though major patterns appear consistent. AMOVA showed greater among regions variance with the five-region grouping (8.40%) than with two (5.49%), indicating that a five-region grouping was a better fit for this analysis. Further, when five regions were considered, 2.83% of variance was explained by variation among populations within regions, whereas 7.43% was explained with the two-region grouping. These results indicate further genetic structuring within the eastern and western groups identified by STRUCTURE K=2. For both groupings, variation within populations was by far the highest value (five regions: 88.87%, two regions: 87.08%) (Table 5).

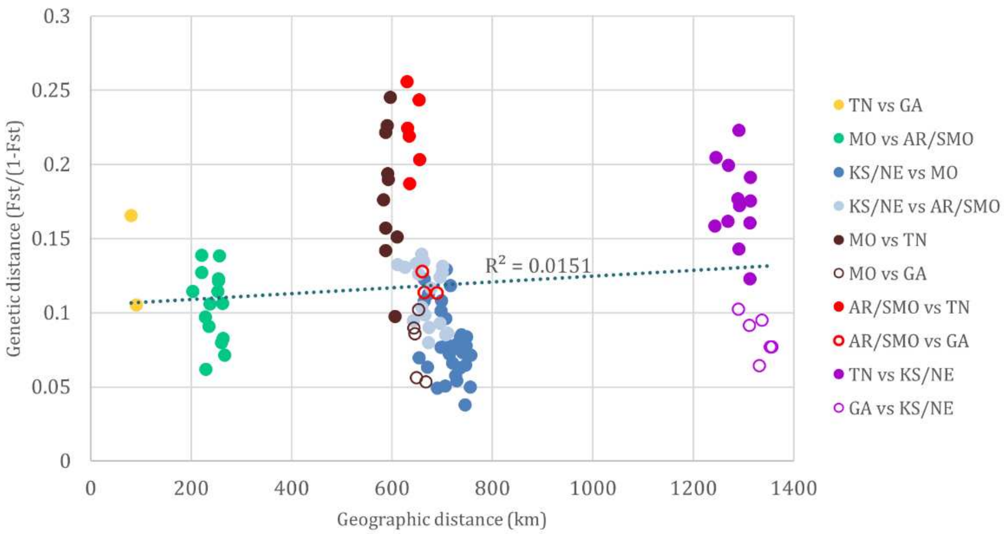

FIGURE 5. Isolation by distance analysis comparing Slatkin's linearized F_{ST} to straight-line distances between populations among regions. There was no evidence for correlation of genetic and geographic distances (p=0.453).

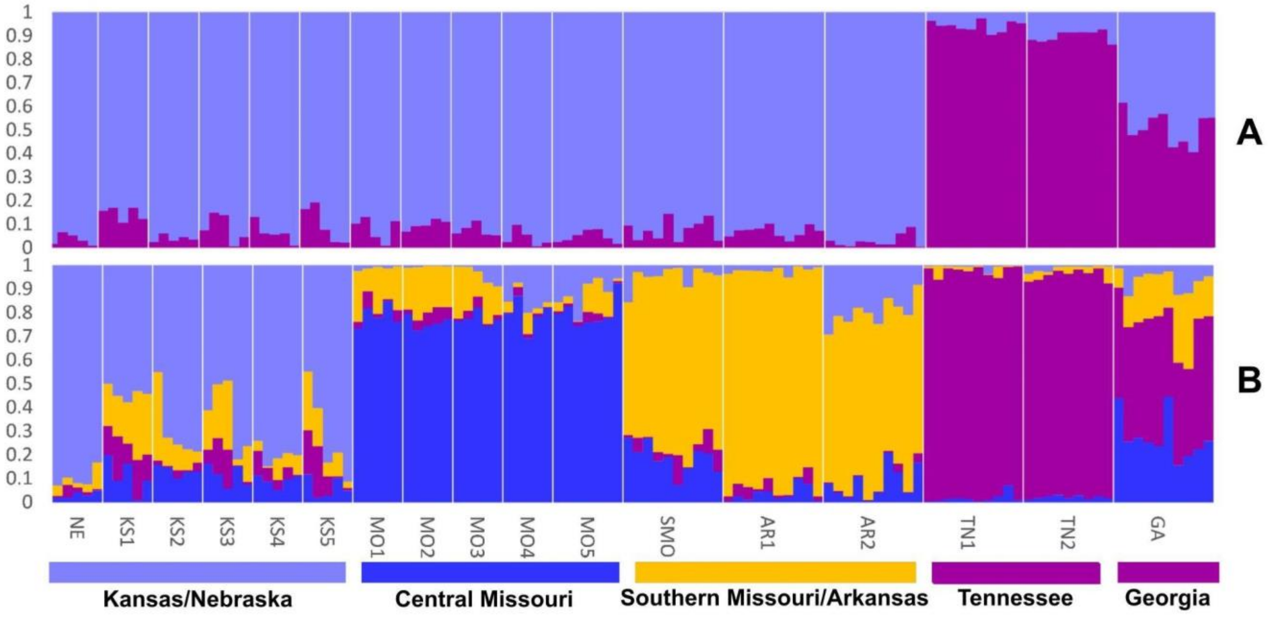

FIGURE 6. Results of Structure analysis of 116 *Clematis fremontii* individuals. Colored bars represent individuals; thin white bars separate populations (see Table 1 for population abbreviations). Colored bars at the bottom represent the five regions considered in other analyses. (A) The best supported number of genetic clusters (K=2) indicates structure between groups occurring east and west of the Mississippi River. (B) The second-best supported number of genetic clusters (K=4) shows four regional groups: western prairies (Kansas and Nebraska), northern Ozarks (central Missouri), southern Ozarks (southern Missouri and northern Arkansas), and Southeast (Tennessee and Georgia).

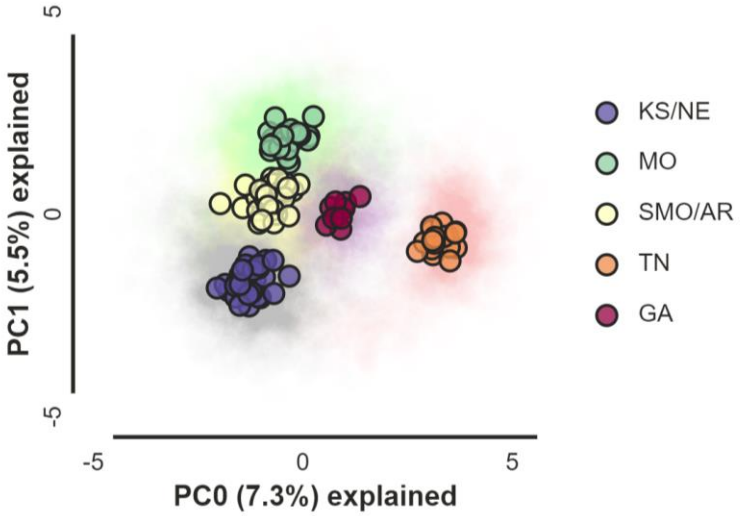

FIGURE 7. Principal component analysis plot of PC0 (7.3%) and PC1 (5.5%). Replicate results are shown in low opacity, and the centroid of all replicated points for each sample is shown with a black border. The broad span of replicates shows variability in results but stability of major groupings.

TABLE 5. Analysis of molecular variance (AMOVA) with five and two defined regions.

Source of variation	d.f.	Sum of squares	Var. components	% of var.
Five regions: 1) KS/NE, 2) central MO, 3) southern MO/AR, 4) TN, 5) GA				
Among regions	4	1508.56	6.17	8.40
Among populations within regions	12	1097.81	2.08	2.83
Within populations	215	14015.41	65.19	88.87
Two regions (STRUCTURE K=2): 1) West of Mississippi River, 2) East of Mississippi River				
Among regions	1	522.66	4.11	5.49
Among populations within regions	15	2083.71	5.56	7.43
Within populations	215	14015.41	65.19	87.08

DISCUSSION

Population Genetic Diversity in *C. fremontii*

Genetic diversity provides a basis for adaptation and resilience to environmental stress and change (Lande, 1988). In species with disjunct distributions, it is generally expected that peripheral populations will have less genetic diversity than central populations due to isolation, small population size, increased inbreeding, and potential founder effects, which all promote genetic drift (Nei, 1975; Allendorf, 1986; Lesica and Allendorf, 1995). We therefore expected to find the most population genetic diversity in the western prairie populations (Kansas and Nebraska), as *C. fremontii* is most common in this region, somewhat less in central Missouri where *C. fremontii* has a more restricted range, and low diversity in regions with very few known populations, including southern Missouri/Arkansas (three known populations), Tennessee (two), and Georgia (one). We also expected to find increased levels of inbreeding in smaller, disjunct populations. Our estimates of genetic diversity met some of these expectations, but not all.

Kansas/Nebraska and central Missouri populations had equally high values for observed and expected heterozygosity, and genetic diversity was highest in these regions. Smaller, disjunct populations in southern Missouri, Arkansas, Tennessee and Georgia had lower values for observed and expected heterozygosity on average, but still showed moderate levels of diversity, counter to our expectations (Table 2). We also found that the number of private alleles per population and proportion of polymorphic loci were consistently higher in populations with lower diversity (those in southern Missouri, Arkansas, Tennessee, and Georgia) than in Kansas/Nebraska or central Missouri populations (Table 1). We suggest that these populations are undergoing genetic bottlenecks due to habitat loss from woody encroachment. Fortunately, life history traits such as perenniality with long lifespans (up to 70 years) (Keener, 1983) and a mating system that allows outcrossing have likely buffered these populations from more severe genetic diversity losses (Raduski et al., 2021).

The finding of slightly negative, near-zero inbreeding coefficients (F_{IS}) shows that populations of *C. fremontii* are not suffering genetic losses from inbreeding (Table 1). This was an unexpected finding, especially for populations with few individuals, because *C. fremontii* is not considered to be an obligate outcrosser. In this species, the outermost stamens mature first, initially facilitating cross-pollination. The innermost stamens mature later, allowing them to come into contact with the stigmatic surface, facilitating self-pollination if outcrossing did not already occur (Erickson, 1945; Keener, 1967). This apparent lack of inbreeding and potential slight heterozygote excess could be indicative of small reproductive population size, and/or low regeneration rates due to low seed viability and infrequent germination (discussed in following section) (Rasmussen, 1979; Stoeckel et al., 2006; Wahlsteen, 2021).

We found moderate levels of genetic diversity even in small populations of *C. fremontii*, including those at Sandy Creek Covered Bridge Historic Site (MO4), Don Robinson State Park (MO5), and in southern Missouri, Arkansas, Tennessee, and Georgia. Despite containing only a few hundred individuals in a small, isolated patch of open habitat, MO4 had the highest values for H_O and H_E of any population sampled in this study—higher than nearby central Missouri populations with thousands of individuals and abundant XLP habitat. Similarly, the Georgia population still showed moderate levels of genetic diversity despite containing <15 individuals (Table 1). But, the retention of neutral genetic diversity in small populations of *C. fremontii* does not mean they are invulnerable (Teixeira and Huber, 2021), and could be indicative of a delayed extinction, or extinction lag, rather than stability (Kuussaari et al., 2009).

Implications for conservation of *C. fremontii*

Based on our findings of moderate genetic diversity even in small populations of *C. fremontii*, we suggest that these populations represent relictual fragments of once larger, diverse populations that today experience low regeneration, as evidenced by a lack of inbreeding (Wahlsteen, 2021). These findings also suggest that current levels of genetic diversity could be more attributable to historical landscape patterns than current (Tilman et al., 1994). Multiple lines of evidence have indicated that grassland and savanna habitat was historically more widespread in the Southeast and Ozarks (Noss, 2013), including studies of historical climate (Transeau, 1935; Sears, 1942), modern disjunct distributions of grassland adapted insects (Olson et al., 2017) as well as reptiles and amphibians (Schmidt, 1938), fossil evidence of prairie vertebrates in the Ozarks and Southeast (Guilday et al., 1969; Guilday, 1971; Parmalee and Klippel, 1981), historical accounts, including land surveys of tree densities (Beilmann and Brenner, 1951; Hanberry et al., 2014), palynological studies (Delcourt et al., 1986), microscopic charcoal records (Ballard et al., 2017), and soil profile studies (Driese et al., 2008). If current levels of genetic diversity are indeed more attributable to a historical landscape with greater availability of grassland habitat, this could indicate an extinction lag (Tilman et al., 1994).

Extinction lags occur when there is a time delay between impacts on a species, such as habitat loss, and the species' ultimate disappearance. This pattern has been detected in other long-lived species such as trees (Vellend et al., 2006), alpine plants (Alexander et al., 2018), and Eurasian grassland plants (Deák et al., 2021), where habitat loss and climate change have swiftly decreased/are swiftly decreasing available habitat for these species, but their long-life spans cause slower genetic losses. The implication of an extinction lag is that small, disjunct *C. fremontii* populations could face extinction even without further habitat loss or fragmentation.

Clematis fremontii, as well as other subsection *Integrifoliae* species, exhibits extremely low germination rates both in field and greenhouse settings (Erickson, 1945; Platt, 1951; Uttal, 1961; Keener, 1967; Justin Lee and Katherine Pittman, Missouri Botanical Garden, pers. comm.; Mike Kintgen and Brooke Palmer, Denver Botanic Gardens, pers. comm.). Barnes (2004) reported *C. fremontii* to have deep simple epicotyl morphophysiological dormancy. That is, seeds of this species require both a warm and cold stratification period in order to germinate; warm stratification is required to break the dormancy of the radicle, while subsequent cold stratification is required to break dormancy of the shoot (epicotyl) (Baskin and Baskin, 2014). It is possible that *C. fremontii* seeds are not readily germinating in the field because seeds are unable to break one or both types of dormancy. Anthropogenic climate change, which has caused general warming trends as well as lower consistency in seasonal climatic patterns, has led to changes in dormancy and germination in other plant species (Klupczyńska and Pawłowski, 2021). No matter the cause, low germination rates in *C. fremontii* could hinder its ability to adapt to change, further increasing the potential for eventual extinction (Aiken et al., 2008; Alexander et al., 2018).

Populations of *C. fremontii* in the Ozarks and Southeast are small, largely unmanaged, extremely susceptible to stochastic loss, and actively declining due to encroachment from woody species. Immediate conservation efforts are needed to protect this species in the long term. Because many grasslands in this region represent an alternative stable state to forest, the reintroduction of fire or other equivalent disturbance mechanisms, such as periodic mowing and brush removal, to these areas is a vital first step for their restoration and long-term maintenance. Due to small population sizes and low germination rates, population augmentation would also be warranted. Disjunct populations of *C. fremontii* do not currently experience gene flow, so in the absence of a nuanced understanding of local adaptation in these populations, we do not advise augmenting populations with individuals from other regions.

One course of action would be to collect seeds, germinate them ex situ, and transplant young plants back into the source population. Because attempts at germination have largely been unsuccessful, further research will be needed to elucidate the specific conditions needed to fully break dormancy in *C. fremontii* seeds. For the federally endangered Alabama leather flower, *Clematis socialis*, researchers at the Center for Plant Conservation have focused on in vitro propagation of stem cuttings and tissue due to low seed viability and germination rates (Trusty et al., 2009). Using these methods, they have had success in maintaining a diverse ex situ collection of *C. socialis*. Ex situ collections are generally kept as a safeguard against extinction, especially for species in which in situ conservation is difficult or impossible. This is a relevant consideration for *C. fremontii* populations, many of which occur on privately owned land (Keyser et al., 2019). A combined strategy of in situ and ex situ conservation efforts could be an effective approach for the conservation of *C. fremontii* (Volis, 2017).

Phylogeographic Patterns

We analyzed population genetic diversity, structure, and hierarchical relationships among populations of *C. fremontii* to test various hypotheses regarding the formation of its present disjunct distribution. Isolation by distance analysis did not show evidence for a correlation between genetic and geographic distances (Fig. 5). This result refutes the hypothesis that Ozark (Missouri and Southern Missouri/Arkansas) and Southeastern (Tennessee/Georgia) populations of *C. fremontii* are relics of a recent westward expansion of prairie habitat during the Hypisthermal Interval. Additionally, populations in Tennessee/Georgia were more differentiated from Ozarks populations than were populations in Kansas/Nebraska, despite the fact that these two regions are equidistant from the Ozarks (Fig 5). This result refutes the hypothesis of simultaneous Hypsithermal expansion outward from the Ozarks. The findings of moderate F_{ST} values across all comparisons (Table 3) and moderate levels of genetic diversity range wide (Table 1) also refute the hypothesis that *C. fremontii* represents an ancient lineage that underwent vicariance millions of years ago.

Instead, these findings, along with the results of STRUCTURE (K=2) (Fig. 6), PCA (Fig. 7), and the hierarchical tree produced by Tetrad (Fig. 4A) supports our hypotheses of more recent, Pleistocene vicariance of eastern and western populations. During Pleistocene interglacial periods, the Mississippi River frequently experienced meltwater mega-floods (Fildani et al., 2018). These events could have caused vicariance of western and eastern populations, with subsequent isolation maintained by intervening unsuitable habitat along the Mississippi River (Mohn et al., 2021). An additional scenario that we did not initially hypothesize but cannot be ruled out is a Pleistocene interglacial eastward expansion from a joint western population. Our finding of nearly identical levels of genetic diversity in Kansas/Nebraska and central Missouri regions (Table 2), and relatively low differentiation between them ($F_{ST} = 0.07$) despite substantial geographical distance supports the hypothesis that these two populations were formerly continuous (Erickson, 1945; Learn and Schaal, 1987), and suggests that their separation was more recent than that of eastern and western groups. The finding of genetically well-differentiated clades occurring east and west of the Mississippi River is known as a Mississippi River Discontinuity (Soltis et al., 2006). If no significant sub-structuring is found within these clades, two glacial refugia occurring east and west of the Mississippi River can be inferred (Soltis et al., 2006). Our finding of multiple strongly supported clades within both western and eastern regions (Fig. 4A), separation between regions in PCA (Fig. 7), and more among regions variance detected through AMOVA with a five-region grouping than with two (eastern and western groups) (Table 5) refutes the hypothesis of dual refugia.

We hypothesized that central Missouri populations would show evidence of very recent separation from southern Missouri/Arkansas populations due to reduction of open woodlands and savanna in the Ozarks over the last few hundred years (Beilmann and Brenner, 1951; Hanberry, 2014). However, we found moderate differentiation between these regions ($F_{ST} = 0.10$), and a strongly supported southern Missouri/Arkansas clade (Fig. 4A), which refutes our hypothesis. As expected, Tennessee populations showed low genetic differentiation from one another (F_{ST}=0.04), supporting our hypothesis that these populations were recently isolated from eachother. However, the Georgia population was more differentiated from Tennessee populations (F_{ST}=0.12) than it was from Kansas populations (F_{ST}=0.08), which was unexpected. STRUCTURE results showed the Georgia population to have a stronger signature of admixture with populations west of the Mississippi River than either Tennessee population did (Fig. 6). Principal components analysis also showed Georgia in an intermediate position between western and eastern groups (Fig. 7). Xeric adapted plant communities in the Southeast and Ozarks are thought to have greatly expanded their ranges and achieved their maximum extent during the Hypsithermal Interval (Delcourt et al., 1986). This could have led to subsequent admixture of previously isolated groups, which could help explain these patterns, as well as the low support for the sister group relationship of eastern and western lineages. Similarly, Flagel et al. (2008) attributed low phylogenetic resolution in the western prairie and XLP genus *Echinacea* in part to secondary, postglacial contact between species with incomplete reproductive barriers.

Both AMOVA results showed that variance within populations explained the highest percentage of variation in *C. fremontii* (87.08% and 88.87%), while variance among regions and among populations within regions was much lower (Table 5). Similarly, hierarchical analyses showed strong support for disjunct regions (as defined in Table 2), and even for some populations within these regions (Appendix S4 and S5). This pattern can be visualized in the neighbor-net network (Fig. 4B), which shows short, wide internal branches among regions compared to long terminal branches within regions. We propose that *C.*

fremontii has retained a similar ecological niche over time, in that it has always occurred on open, grassland habitat. That *C. fremontii* has amphistomatic leaves with large stoma, which are known adaptations to open, xeric habitats (Jordan, 2014; Richardson, 2017), supports this idea.

We suggest that, following the reduction of open habitat during a cooler, wetter, climatic period, formerly more extensive *C. fremontii* populations either migrated into, or became stranded in, edaphically controlled XLPs, in which they accumulated genetic variation in response to geographic isolation and the unique qualities of this habitat type. A similar scenario was proposed to have led to rapid radiation of prairie clovers (*Dalea* spp.) in the Southeast and Ozarks (Diggs, 2013). The fact that *C. fremontii* has tracked this niche over time rather than adapting to the development and expansion of deciduous forest has likely been a key factor in the isolation of populations (Wiens, 2004; Kozak et al., 2006). Additionally, the isolation of XLPs has likely been accentuated by the loss of regular fire return intervals and other natural disturbance mechanisms (Noss, 2013). This phenomenon could also be relevant to the divergence of other subsection *Integrifoliae* species, most of which are found in mid-Appalachian shale barrens, which are floristically and ecologically similar to XLPs (Platt, 1951; Keener, 1983; Lawless et al., 2006). Presently isolated *C. fremontii* populations may eventually diverge to the extent of allopatric speciation.

CONCLUSIONS

Our data demonstrate that ddRAD-seq is a useful tool for studying population genetic diversity and structure in *C. fremontii*. We found that small disjunct populations of *C. fremontii* have moderate genetic diversity and are not suffering genetic losses from inbreeding. However, the XLP habitat in which populations outside of Kansas/Nebraska occur is diminishing. We recommend conservation action focused on habitat restoration by reinstating a disturbance regime, ideally through controlled burning, and possible augmentation of populations through ex situ germination efforts. Conservation of this species would greatly benefit from further research to determine the mechanisms that break dormancy and allow for seedling establishment. Because many populations occur on privately owned land, we suggest that creation of a permanent ex situ collection would also be prudent, with a focus on protecting genetic diversity from small, unprotected and/or unmanaged populations in southern Missouri, Arkansas, Tennessee, and Georgia. Xeric limestone prairie communities contain numerous rare and endangered plant species (Lawless et al., 2006), so restoration of habitat for *C. fremontii* would be beneficial for these as well. Additionally, as anthropogenic climate change increases mean temperatures and aridity, grassland restoration can be a sustainable choice (Török et al., 2021).

Our results demonstrate that *C. fremontii* populations are not recent relics of a Hypsithermal expansion, nor did they undergo ancient vicariance due to the formation of the Mississippi embayment. Instead, we found support for Pleistocene vicariance of a widespread population, possibly due to flooding of the Mississippi River (Fildani et al., 2018), or eastward expansion of a joint western population during a Pleistocene interglacial period. In either case, we believe that initial separation occurred between groups east and west of the Mississippi River, and that present isolation of western and eastern metapopulations is more recent. This present isolation is likely attributable to the onset of a cooler, wetter, climatic period that ultimately reduced the extent of grasslands in these regions. Hypsithermal climatic shifts that caused the expansion of pre-existing grassland habitat in the Ozarks and Southeast (Delcourt et al., 1986) could have led to secondary contact between formerly separate groups. This secondary contact could have weakened current signals of genetic structure between eastern and western groups (Flagel et al., 2008), and could explain the unexpected signal of western admixture with the Georgia population. Our results also demonstrate that populations of *C. fremontii* have accumulated genetic variation upon isolation in XLPs.

Future Directions

Ecological niche modeling could provide further insights on phylogeography of *C. fremontii*, though the broad geographic distribution of this species across regions with major differences in climate and its presence in small, isolated patches of appropriate habitat would make this challenging. Though observed morphological variation did not appear to conform to any consistent geographic patterns, future studies of *C. fremontii* could involve a common garden experiment and morphometric analysis to study morphological variation more closely. This could be used to determine if any of the regional groups, which we found to be genetically well-supported, could merit subspecific recognition. Recent common garden and morphometric studies of two *Clematis* sect. *Viorna* species complexes, *C. reticulata* (Murphy, 2020) and *C. viorna* (Irick and Estes, 2019; Irick and Estes, in prep.), have led to the recognition of several new species.

It would also be informative to include the other subsection *Integrifoliae* species in future genetic analyses. This would enable a test of Keener's (1967; 1983) hypothesis regarding the shared origin of *C. fremontii* and *C. albicoma*. Population-level sampling of the three narrowly distributed shale barren endemics (*C. albicoma*, *C. coactilis*, and *C. viticaulis*), along with range-wide sampling of the more widely distributed *C. ochroleuca*, could be used to compare levels of genetic diversity among narrowly endemic and widespread species in this group, and could help determine the order and timing of divergences among species. The inclusion of all subsection *Integrifoliae* species could also be used to test the hypothesis of recent radiation and divergence in this group (Montgomery 2009; Montgomery and Shaw, 2012).

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APPENDICES

Appendix S1. Site specific collection permit information.

Site name, county, state, population abbr.	Permit Information or Number
Washington State Park, Washington, Missouri, MO1	Formally approved by Ken McCarty, Director of Missouri State Parks Natural Resource Management Program
Victoria Glades Conservation Area, Jefferson, Missouri, MO2	Formally approved by Malissa Briggler, Missouri state botanist
Valley View Glades Natural Area, Jefferson, Missouri, MO3	Formally approved by Malissa Briggler, Missouri state botanist
Sandy Creek Covered Bridge, Jefferson, Missouri. MO4	Formally approved by Ken McCarty, Director of Missouri State Parks Natural Resource Management Program
Don Robinson State Park, Jefferson, Missouri, MO5	Formally approved by Ken McCarty, Director of Missouri State Parks Natural Resource Management Program
Orchard Knob Reservation, Hamilton, Tennessee, TN1	CHCH-2021-SCI-0004
Berry College, Floyd, Georgia, GA	ELM-2021-001

Appendix S2. Herbarium accession information for all voucher specimens, including population abbreviation (Pop. abbr.), site name, county, and state, herbarium code (UCHT=University of Tennessee at Chattanooga Herbarium, TENN=University of Tennessee Herbarium, GA=University of Georgia Herbarium), and collector name(s).

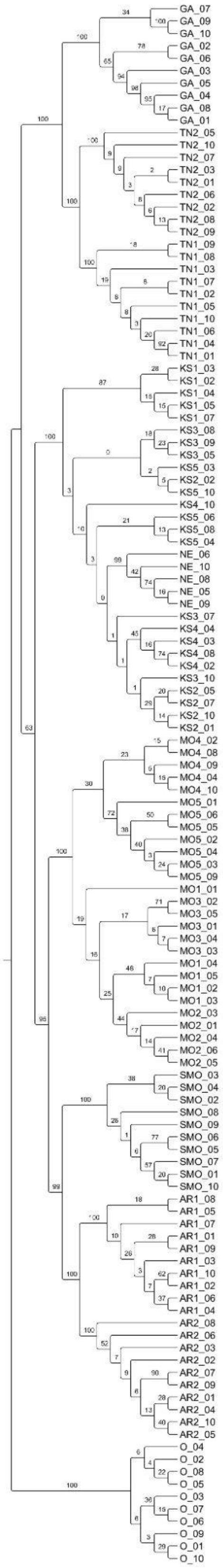
Pop. abbr.	Site, county, state	Herbarium code	Collector name
NE	Red Cloud, Webster, NE	UCHT	Alaina Krakowiak
KS1	Delta Cemetery, Jewell, KS	UCHT	Alaina Krakowiak
KS2	F. Rd., Smith, KS	UCHT	Alaina Krakowiak
KS3	Tipton, Osborne, KS	UCHT	Alaina Krakowiak
KS4	Saline Rd., Russell, KS	UCHT	Alaina Krakowiak
KS5	King Hill, Rooks, KS	UCHT	Alaina Krakowiak
MO1	Washington State Park, Washington, MO	UCHT	Alaina Krakowiak
MO2	Victoria Glades, Jefferson, MO	UCHT	Alaina Krakowiak
MO3	Valley View Glades, Jefferson, MO	UCHT	Alaina Krakowiak

MO4	Sandy Creek Covered Bridge, Jefferson, MO	UCHT	Alaina Krakowiak
MO5	Don Robinson State Park, Jefferson, MO	UCHT	Alaina Krakowiak
SMO	Hwy 181, Ozark, MO	UCHT	Alaina Krakowiak
AR1	Wild Cherry, Fulton, AR	UCHT	Alaina Krakowiak
AR2	Elizabeth, Fulton, AR	UCHT	Alaina Krakowiak
TN1	Orchard Knob Reservation, Hamilton, TN	UCHT	
TN2	Adams Rd., Hamilton, TN	TENN	Joey Shaw with Dennis D. Horn and Jim Cash
GA	Berry College, Floyd, GA	GA	Richard and Teresa Ware

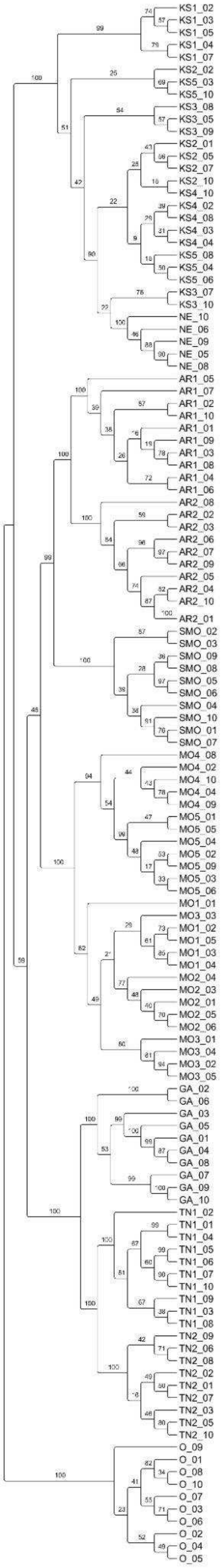
Appendix S3. Alterations to Alexander et al. (2007) genomic DNA extraction protocol.
Modifications listed by step number:

- Steps 2 & 3: Tissue was ground using three, 2.3 mm zirconia/silica beads and a Qiagen TissueLyser.
- Step 5: The buffered grindate was incubated at 65 °C for 1 hour, gently inverting the tubes every 10 minutes.
- Steps 6 & 7: After adding potassium acetate, tubes were placed in a -30 °C freezer overnight, then centrifuged from frozen for 15 minutes at maximum force the next day.
- Step 10: After adding binding buffer, the tubes were left to incubate at room temperature for 20 minutes.
- Step 17: DNA was eluted using 30-50 µL of TE buffer depending on desired concentration, with some samples receiving a 2nd elution of the same volume into a separate tube.

Appendix S4. Full results of Tetrad analysis.



Appendix S5. Full results of maximum parsimony analysis.



Appendix S6. F_{ST} estimates for *C. fremontii* populations against the outgroup, *C. ochroleuca*. All values significant (p=0.00).

	NE	KS1	KS2	KS3	KS4	KS5	MO1	MO2	MO3	MO4	MO5	SMO	AR1	AR2	TN1	TN2	GA
Clematis ochroleuca	0.20	0.20	0.18	0.20	0.18	0.20	0.22	0.23	0.25	0.16	0.17	0.24	0.24	0.21	0.30	0.26	0.21

Appendix S7. Graph of ΔK vs. K from Structure analysis.