Distribution and Genetic Diversity of the Rare Plant Veratrum woodii (Liliales: Melanthiaceae) in Georgia: A Preliminary Study with AFLP Fingerprint Data

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Abstract—*Veratrum woodii*, a long-lived herbaceous perennial species, has a fragmented distribution with populations scattered in the southeastern and lower midwestern USA. In Georgia, the species has a protection status of rare. This preliminary study focused on verifying historic and/or unvouchered populations in Georgia and characterizing variation and genetic structure within and among all populations in the state. We analyzed AFLP (amplified fragment length polymorphism) data as dominantly inherited markers for 16 populations sampled from Georgia, Florida, and Missouri. Our results suggest that this species overall has relatively low levels of genetic diversity and that differentiation among populations is comparable to species with similar life history traits. Measures of genetic diversity, such as mean H_e, indicate that variation of populations has some partitioning between disjunct northern and southern Georgia (and Florida) populations. However, our analyses imply that watershed assignment, rather than geographic distance, provides a better explanation for variation and population structure. We hypothesize that southern relict populations in Georgia may have served as refugia during Pleistocene glaciations. We conclude that life-history characteristics, low levels of genetic variation, and suppression of ecological disturbance collectively jeopardize populations of *Veratrum woodii* in Georgia.

Keywords—Disjunct populations, founder effect, refugia, Southeast USA, watersheds.

Veratrum woodii Robbins ex Alph.Wood [*Melanthium woodii* (Robbins ex Alph.Wood) Bodkin, *V. intermedium* Chapm.; Wood's false-hellebore, Ozark bunchflower] has a disjunct distribution comprising isolated localities in 14 states, ranging from the Interior Highlands eastward through areas of the Midwest and the Southeast (Fig. 1A). These perennial herbs generally grow in basic soils of moist to mesic deciduous forests on hillsides, terraces, river bluffs, and ravines at elevations up to 900 m (Zimmerman 1958; Bodkin and Utech 2002). Populations often are restricted to relatively steep, north- and east-facing lower slopes in these habitats (Zomlefer 1997; Yatskievych 1999).

The general geographic range of *V. woodii*, southeastern and lower midwestern USA but absent north of the Last Glacial Maximum (ca. 21,500 BP), has been considered the result of climate-driven range contractions during a series of Pleistocene glacial maxima (Davis 1983; Delcourt 2002; Donoghue and Smith 2004; Graham 2011). Disjunct populations of certain plant species within this broad area, often delimited geographically by major river systems, watershed basins, and/or mountain ranges, have been regarded as the locations of Pleistocene-era refugia, now recognized as "hotspots" of endemism and biodiversity (summary in Soltis et al. 2006). A biogeographic study by Bellemare and Moeller (2014), which included *V. woodii*, showed that this distribution pattern is shared by 188 other small-ranged herbs associated with eastern North American temperate deciduous forest habitats. As with *V. woodii*, many of these species persist in microhabitat types (such as mesic sites, valleys, and north-facing slopes) that may moderate unfavorable climatic conditions.

Many of the small-ranged herbs in the Bellemare and Moeller (2014) study have rarity rankings by federal and state agencies. *Veratrum woodii* has a global conservation rank of G5, defined as "secure: common, widespread, and abundant" (NatureServe 2016). However, the species is listed as a conservation concern in 13 (out of 14) states (see Table 1 for summary), with state conservation rankings as S1 (critically imperiled; five states), S2 (imperiled; five states including Georgia), and S3 (vulnerable; three states). These states also

have designated protection status for *V. woodii* ranging from endangered (two states), threatened (five states), rare (three states including Georgia) to watch-listed/possibly rare (three states). The species is designated as "apparently secure" (S4) only in Missouri, where it is fairly widespread (but scattered) mainly in the eastern half of the state (Yatskievych 1999).

The rarity of *Veratrum woodii* throughout most of its range has been attributed to the restricted range and habitat type coupled with several aspects of its life history, outlined below, that limit reproduction and dispersal. The plants arise from a stout rhizome with a fibrous-sheathed bulb (Fig. 1C), which early in the spring produces several relatively large (to ca. 60 cm long), broad, plaited leaves initially clustered into a loose rosette (Fig. 1D). In the summer, a flowering individual produces a long stem, up to ca. 2 m tall (*Zomlefer 1853*, GA), which terminates in a paniculate inflorescence bearing star-like maroon flowers of ca. 1.5 cm diameter. Distinguishing floral features for the species include the two glistening perigonal glands at the base of each tepal, epitepalous and strongly excurved stamens (Fig. 1B), and a densely tomentose pubescence of the immature ovary (Zomlefer 1997). All flowers appear morphologically perfect but those of lower inflorescence branches are usually functionally staminate (Zimmerman 1958). Mature three-lobed capsules release several winged seeds ca. 1 cm long.

Little is known about the pollination biology of *V. woodii*. The characteristic shiny perigonal glands are purportedly nonnectariferous (Bodkin and Utech 2002). Deam (1940) reported various species of flies as floral visitors for a garden plant of *V. woodii* in Indiana, and flower beetles are also likely pollinators as in other species of *Veratrum* (Robertson 1896; Bodkin 1978; Kato et al. 2009). The flowers are protandrous, which promotes outcrossing. Self-pollination may possibly occur, as in *V. virginicum* (L.) W.T.Aiton and *V. latifolium* (Desr.) Zomlefer (Bodkin 1978), when the incurved staminal filaments curl in age toward the diverging mature styles.

However, sexual reproduction in *Veratrum woodii* is limited. Zimmerman (1958), Bodkin (1978), and Ebinger (1996) summarize anecdotal reports corroborating the sporadic (and
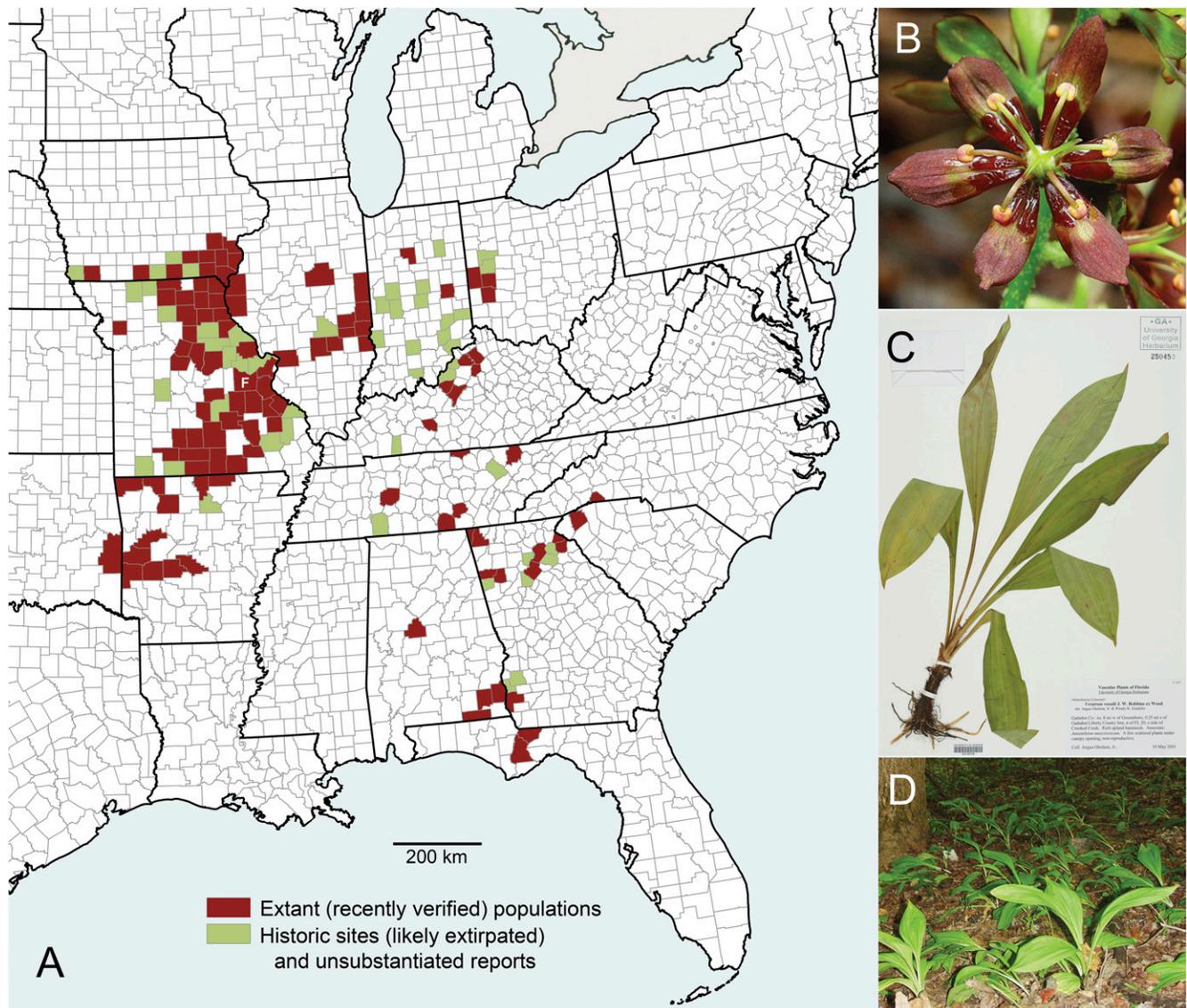
FIG. 1. Distribution map and photographs of *Veratrum woodii*. A. Map of the midwestern and southeastern USA showing the general range of *V. woodii*. F = Franklin County, Missouri, the location of population 16 (*Yatskievych 11–34*, GA, MO). State distributions from NatureServe (2016) and Georgia distribution from the current study, supplemented by Deam (1940), Steyermark (1963), Chester et al. (1993), Ebinger (1993), Yatskievych (1999), Alabama Natural Heritage Program (2012), Kentucky State Nature Preserves Commission (2013), Arkansas Natural Heritage Commission (2015), Indiana Department of Natural Resources (2016), Illinois Natural History Survey (2017), Ohio Department of Natural Resources (2017), Robinson and Finnegan (2017), Keener et al. (2018), Krakow (2018), South Carolina Heritage Trust (2018), and Tennessee Department of Environment and Conservation (2018). Base map generated by Steven C. Hughes from Census Bureau data (https://www.census.gov/). B. Close-up of flower (from Missouri) showing glistening perigonal glands and excurved stamen filaments, × 3. C. Juvenile specimen showing fibrous-sheathed bulb, from Gadsden County, Florida, 10 May 2001 (*Gholson s. n.*, GA), × 0.17. D. Population of non-reproductive plants in the "rosette" phase, Dade County, Georgia, 24 July 2010 (*Zomlefer 2414*, FLAS, GA, VSC), ×0.05. Photo credits: B. © Dan Tenaglia (Missouriplants.com), used with permission from Karen (Tenaglia) Hoksbergen; C. Steven C. Hughes; D. Wendy B. Zomlefer.

sometimes synchronized) flowering of *V. woodii* populations observed by botanists throughout the range of the species for over 130 yr. An individual plant flowers irregularly, perhaps once every four to seven years—if at all (Deam 1940; Steyermark 1963; Mathew 1989). Consequently, a population typically has few flowering shoots in a given year, and completely non-flowering populations, comprising only plants in the vegetative ("rosette") phase, are commonly encountered.

A formal study of the phenology and fruit set of *V. woodii* plants was conducted by Ebinger (1993, 1996) from 1985 to 1993 for 31 populations (1300 plants) in east-central Illinois. These populations ranged in size from a few individuals to ca.

400 plants (one population). During the initial seven-year study period, only four plants produced inflorescences, and two of those set fruit. In 1993, the total was 96 flowering individuals (in 16 populations), and 70 of these plants produced capsules, with 0–8 seeds/capsule. Seed production for that year was low overall, averaging, for a particular plant, ca. 1.5 seeds per capsule, and 13% of the 218 capsules examined lacked seeds.

Understory light conditions may explain the suppression of flowering (and flower pollination) in *V. woodii*. Formal field studies on other infrequently flowering forest geophytes of eastern North America have demonstrated that reproductive success (mass flowering and fruiting) may be stimulated by the

TABLE 1. State-level rarity classification for *Veratrum woodii* in the 14 states of its range (see Fig. 1A). State conservation rank: S1 = critically imperiled (five or fewer occurrences or 1000 or fewer individuals), S2 = imperiled (6 to 20 occurrences or 1,001 to 3,000 individuals), S3 = vulnerable (rare; typically 21 to 100 occurrences or 3,001 to 10,000 individuals), S4 = apparently secure (uncommon but not rare and with some cause for long-term concern; 101 or more occurrences or 10,001 or more individuals). State protection status: E = endangered (in danger of extinction throughout all or part of range), INV = inventory element (possibly rare, under study), R = rare (may not be endangered or threatened but should be protected because of scarcity), T = threatened (likely to become endangered in the foreseeable future throughout all or parts of range), WL = watch list (known or suspected conservation concern, further documentation needed). State conservation ranks from NatureServe (2016); state status from Alabama Natural Heritage Program (2012), Kentucky State Nature Preserves Commission (2013), Arkansas Natural Heritage Commission (2015), Boyle (2015), Indiana Department of Natural Resources (2016), Florida Natural Areas Inventory (2017), Illinois Natural History Survey (2017), Ohio Department of Natural Resources (2017), Oklahoma Natural Heritage Inventory (2017), Robinson and Finnegan (2017), Iowa Department of Natural Resources (2018), Krakow (2018), and Tennessee Department of Environment and Conservation (2018); state conservation ranks and protection status for South Carolina provided by H. Brown (South Carolina Heritage Trust, pers. comm.).

State	State conservation rank	State protection status
Alabama	S1	R
Arkansas	S3	INV
Florida	S2	E
Georgia	S2	R
Illinois	S3	T
Indiana	S3	WL
Iowa	S2	T
Kentucky	S2	T
Missouri	S4	—
North Carolina	S1	T
Ohio	S2	T
Oklahoma	S1	R
South Carolina	S1	INV
Tennessee	S1	E

creation of more open habitats via fire [e.g. *Xerophyllum asphodeloides* (L.) Nutt.; Bourg et al. 2015] and/or canopy gaps (e.g. *Cypripedium acaule* Aiton; Gill 1996). The increased sunlight in these disturbed areas reduces competing ground layer species and also attracts significantly more pollinators that typically avoid dense shade. Ebinger (1993, 1996) noted that canopy openings (created by wind-throws) or fire appeared to promote flowering in *V. woodii* in Illinois. Flowering of *V. woodii* has been suppressed and plants persist in the rosette stage in many sites in Missouri characterized by a closed canopy and dense ground layer of shade-tolerant species; the best floral displays have occurred in years following prescribed burns (G. Yatskievych pers. obs.).

Despite the infrequency of flowering and low seed set, the number of individuals increased in all populations observed by Ebinger (1996) over the eight-year study period, possibly due to vegetative reproduction via forking/splitting of the rhizomes, a process documented in this species by Deam (1940). Rhizomes and seeds of *Veratrum* species generally require winter dormancy (Youngken 1953; Taylor 1956). The flattened, ellipsoid, and winged seeds of *V. woodii* are likely adapted for short-distance wind dispersal: similar seeds of *V. album* L. can be carried by wind for at least several meters (Hesse et al. 2008). Dispersal of *V. woodii* rhizomes or bulbs by animals is unlikely due to toxic bioactive steroidal alkaloids that have been well-documented for most species in the genus (Pammel 1910; summary in Chandler and McDougal 2014) and are often most concentrated in the rootstock. Plants of *V.*

woodii are likely long-lived based on the reports for other species in the genus, as for example, at least 100 yr for a wild plant of *V. viride* Aiton and at least 30 yr for a cultivated specimen (Taylor 1956; Mathew 1989).

Veratrum woodii has not been the subject of genetic analyses previously at the population level. For this preliminary study, we used AFLP (amplified fragment-length polymorphism) loci technique, a reliable and reproducible dominant–recessive DNA marker system, for assessing genetic variation in natural populations (summary in Reisch and Bernhardt-Römermann 2014). In particular, AFLPs have advantages for population studies of rare and endangered species: a priori sequence data are not required, and the process involves the simultaneous amplification of a large number of polymorphic DNA fragments (Blears et al. 1998; Bian et al. 2015). Large numbers of loci may reduce sampling error due to averaging estimates of population differentiation across multiple sites (Falk 1991; Travis et al. 1996).

The distribution of *V. woodii* populations in Georgia also was not clear at the time of this study: previously vouchered localities for this rare plant had not been confirmed for at least three decades, and more recent sightings had not been vouchered. Consequently, the goals of this study were to: 1) verify previously vouchered localities of *Veratrum woodii* in Georgia, dated from 1947–1988; 2) voucher "sight records" in Georgia compiled in the Georgia Department of Natural Resources portal, *Georgia Natural Rare Elements Database* (Krakow 2018); 3) characterize genetic variation and its structure within and among populations in Georgia using analyses of AFLP data; and 4) infer the population history of *V. woodii* in Georgia based on the results of the genetic analyses correlated with geographic distribution.

Materials and Methods

Plant Material—Potential locations for *V. woodii* populations in Georgia were compiled from specimen labels at GA Herbarium (11 locations in five counties, dated 1947–1988) and data provided by the Georgia Department of Natural Resources, Wildlife Resources Division (13 legacy and recent unvouchered "sight records"; Krakow 2018). Two sites in the Chattahoochee River National Recreation Area, recently (2007) vouchered by Zomlefer for a floristic survey (Zomlefer et al. 2012), were revisited to obtain fresh leaf material, and three well-documented populations in Gadsden County in the panhandle of Florida also were sampled. An additional voucher specimen (and leaf material) from a population in Franklin County (east-central) Missouri was provided by George Yatskievych for comparison.

The first author conducted nine intensive field trips (2 April 2010–1 October 2011) in Georgia and Florida with the assistance of coauthors J. R. Comer, J. R. Allison, and/or colleagues listed in acknowledgments. Sampling was undertaken in accordance with the following special permits: Chicopee Woods Nature Preserve, plant collection permit; Florida Department of Agriculture and Consumer Services, Division of Plant Industry, regulated plant harvesting permit # 922; Florida Department of Environmental Protection, Division of Recreation and Parks, research/collecting permit 10050314; Georgia Department of Natural Resources, protected plant permit 10006; and National Park Service, Chattahoochee National Recreation Area, permit # CHAT-2010-SCI-0004. Vouchers were prepared with standard field and herbarium protocols for rare perennials, leaving the rootstock intact and removing only a few leaves (and sometimes some of the upper portion of the plant). Leaves were sampled from individuals at least two meters apart (to avoid ramets of clonal individuals) and from a maximum of 30 individuals per population. Leaf tissue was preserved in silica gel for DNA extraction (Chase and Hills 1991). Voucher specimens were deposited at GA Herbarium, with duplicates at FLAS and VSC.

Sample Preparation—Leaf material of 15 individuals per population (or the entire population if fewer than 15 individuals) were randomly selected for DNA extraction and AFLP analysis (216 individuals from 16 populations: Florida, Georgia, and Missouri; Table 2). A modified CTAB

TABLE 2. A list of the 16 populations of *Veratrum woodii* sampled for this study (see Figs. 1A, 2), including population number, location (state, county, locality, elevation) and estimated population size, physiographic region and watershed, and voucher specimen information (collector, collector number, [herbarium acronym]). * = population vouchered for the first time via this study; † = population on public (protected) land. Physiographic regions = Environmental Protection Agency level III ecoregions (US EPA 2017): P = Piedmont, RV = Ridge and Valley, SP = Southeastern Plains, SW = Southwestern Appalachians. Watershed boundaries (USDA, NRCS 2018): C–A = Chattahoochee–Apalachicola, C–T = Coosa–Tallapoosa, MT–C = Middle Tennessee–Chickamauga, O–A = Oconee–Altamaha, S = Savannah.

Population number	Location (state, county: locality, elevation) and estimated population size	Physiographic region; watershed (Georgia and Florida)	Voucher specimen(s)
1*	Georgia, Dade: Rising Fawn, Crawfish Creek/Allison Creek, 235 m; ca. 150 individuals	RV; MT–C	*Zomlefer 2414* (FLAS, GA, VSC)
2*	Georgia, Dade: Rising Fawn, Johnson Crook, 565 m; 5 individuals	SA; MT–C	*Zomlefer 2416* (GA)
3*	Georgia, Walker: Lookout Mountain, Allen Spring Gap, 590 m; ca. 50 individuals	SA; MT–C	*Zomlefer 2412* (GA, VSC)
4*	Georgia, Polk: Cedartown, tributary of Cedar Creek, 330 m; ca. 60 individuals	P; C–T	*Zomlefer 2430* (GA)
5*†	Georgia, Paulding: Dallas, Paulding Forest Wildlife Management Area, Pegamore Creek, 298 m; 17 individuals	P; C–T	*Zomlefer 2432* (GA)
6†	Georgia, Gwinnett: Cumming, Chattahoochee River National Recreation Area, Bowmans Island, Richland Creek, 314 m; ca. 80 individuals	P; C–A	*Zomlefer 1832* (GA)
7†	Georgia, Gwinnett: Sugar Hill, Chattahoochee River National Recreation Area, Orrs Ferry, sewer easement of Chattahoochee River, 300 m; 100+ individuals	P; C–A	*Zomlefer 1853* (GA)
8*†	Georgia, Hall: Gainesville, Elachee Nature Preserve, Chicopee Woods Aquatic Center, Walnut Creek, 306 m; 70 individuals	P; O–A	*Zomlefer 2405* (FLAS, GA, VSC)
9*	Georgia, Franklin: Jewelville, Nails Creek, 247 m; 6 individuals	P; S	*Zomlefer 2407* (GA)
10*†	Georgia, Stephens: Toccoa, Stephens County Recreation Area, Tugaloo River, 228 m; ca. 40 individuals	P; S	*Zomlefer 2406* (FLAS, GA, VSC)
11	Georgia, Early: Blakely, Grimsley Mill Branch, 68 m; 7 individuals	SP; C–A	*Allison 3328* (GA)
12†	Georgia, Decatur: Bainbridge, Faceville Landing, Lake Seminole, 31 m; ca. 80 individuals	SP; C–A	*Zomlefer 2404* (GA)
13†	Florida, Gadsden: Torreya State Park, north side of Crooked Creek, 39 m; ca. 40 individuals	SP; C–A	*Zomlefer 2408* (FLAS, GA)
14†	Florida, Gadsden: Torreya State Park, Crooked Creek at Aspalaga Rd., 31 m; ca. 50 individuals	SP; C–A	*Zomlefer 2409* (FLAS, GA)
15	Florida, Gadsden: Chattahoochee, Flat Creek, 33 m; 600+ individuals	SP; C–A	*Zomlefer 2411* (GA)
16†	Missouri, Franklin: Villa Ridge, Missouri Botanical Garden Shaw Nature Reserve, 150 m; ca. 75 individuals	—	*Yatskievych 11–34* (GA, MO)

method (Doyle and Doyle 1987) was used for DNA extraction. The analyses of AFLP followed the general procedure described by Vos et al. (1995) with minor modifications. Invitrogen's (Carlsbad, CA) AFLP® Core Reagent kit was used for restriction digestion (enzymes *EcoRI* and *MseI*) and adapter ligation following manufacturer protocols, scaling reactions to one-half and using 9 μl of DNA. Pre-selective amplifications were prepared using Invitrogen's Pre-Amp primer mix I (Carlsbad, California) and Promega's PCR master mix (Madison, Wisconsin), as follows: 1 μl Pre-Amp primer mix, 10 μl PCR master mix, 5 μl H$_2$O, 4 μl of the restriction/ligation; thermocycler protocol 94°C for 2 min; then 94°C for 20 s; 56°C for 30 s; 72°C for 2 min × 20; 60°C for 30 min, and then held at 4°C.

Three μl of the pre-selective PCR products were diluted in 72 μl TE buffer for selective amplification. Based on preliminary runs of 10 primer combinations, the following three primer pairs were chosen for selective amplification (*EcoRI/MseI*; *EcoRI* dye labeled): AGG/CAC, AGG/CTA, and ACG/CAG (Invitrogen, Carlsbad, California). The following protocols were used for the selective amplifications: 0.5 μl of each primer (10 μM), 5 μl PCR master mix, 3 μl H$_2$O, 1 μl diluted pre-selective product; 94°C for 30 s then 65°C for 30 s with step down 0.7°C–72°C for 1 min × 12; 94°C for 30 s, 56°C for 30 s, 72°C for 1 min × 32; and then held at 4°C. Capillary electrophoresis was carried out by the Georgia Genomics Facility (University of Georgia, Athens, Georgia) with an Applied Biosystems (Carlsbad, California) 3730*xl* DNA Analyzer. The Thermo Fisher Scientific (Waltham, Massachusetts) GeneScan™ 500 ROX™ dye size standard was used as the reference for assigning fragment size. Peaks were automatically scored with default settings of Applied Biosystems Peak Scanner™ software (v. 1.0), followed by additional manual scoring to ensure inclusion of only unambiguous peaks with heights > 200 (in relative fluorescent units). The concatenated data matrices have been deposited in the Dryad repository (Zomlefer et al. 2018).

Analyses of Genetic Diversity and Population Structure—Analyses of AFLP patterns were based on the premise that 1) AFLPs are diploid dominant markers with alleles either present (amplified) or absent (non-amplified), and 2) populations were in Hardy-Weinberg equilibrium (Treier and Müller-Schärer 2011). These assumptions seem valid for

Veratrum woodii, a diploid (2n = 16; Löve 1985) and likely cross-pollinated (Deam 1940) species. Significant departures from Hardy-Weinberg equilibrium, although not tested directly, probably were unlikely.

Separate estimates of genetic diversity were generated with the software GenAlEx (v. 6.502, Peakall and Smouse 2006, 2012; Smouse et al. 2015) for all 16 populations (N = 216 samples, including the Missouri population), and for the 15 populations from Georgia and Florida (N = 200, i.e. excluding Missouri). The program add-on processed allele frequency data to estimate expected heterozygosity (with standard error) from binary presence-absence data matrices and to calculate percent polymorphic loci, observed number of loci, and private loci within each population.

The software Arlequin (v. 3.5.2.2; Excoffier and Lischer 2010) was used to infer F$_{ST}$ (9999 permutations) and to generate a pairwise F$_{ST}$ matrix (distance method, threshold set at $p < 0.05$; Weir and Cockerham 1984; Excoffier et al. 1992; Weir 1996) with (16 × 15)/2 = 120 pairs of populations. Estimates of Nei's (1972) genetic distance were used to construct an unrooted neighbor joining tree with PHYLIP v. 3.695 (Felsenstein 1989, 2005). The *Clones* function within the R-source package AFLPDat (Ehrich 2006) was used to calculate genotypic diversity (Nei 1987) and to estimate the effective number of genotypes within populations (Parker 1979): since this species can clonally reproduce, such analysis could influence inferences from the dataset.

An analysis of molecular variation (AMOVA) was employed to partition the total genetic diversity into within and among population components ($\Phi_{ST} \approx F_{ST} = G_{ST}$; Nei 1973, 1977; Ma et al. 2015), as well as variation of populations grouped geographically (all populations) and according to watershed (Georgia and Florida populations; USDA, NRCS 2018). The pairwise F$_{ST}$ matrix was used to determine whether populations within watersheds were more genetically similar than pairs of populations located in different watersheds. For each of the four Georgia–Florida watersheds with two or more populations (see Fig. 2), mean pairwise F$_{ST}$ values among populations within a watershed were compared with mean values between 1) populations in that watershed and 2) populations within the other five watersheds. Mantel tests were used to evaluate the relationship between geographic distance and genetic differentiation [represented by pairwise F$_{ST}$/(1 - pairwise F$_{ST}$); Rousset 1997]. Geographic distance was linearized
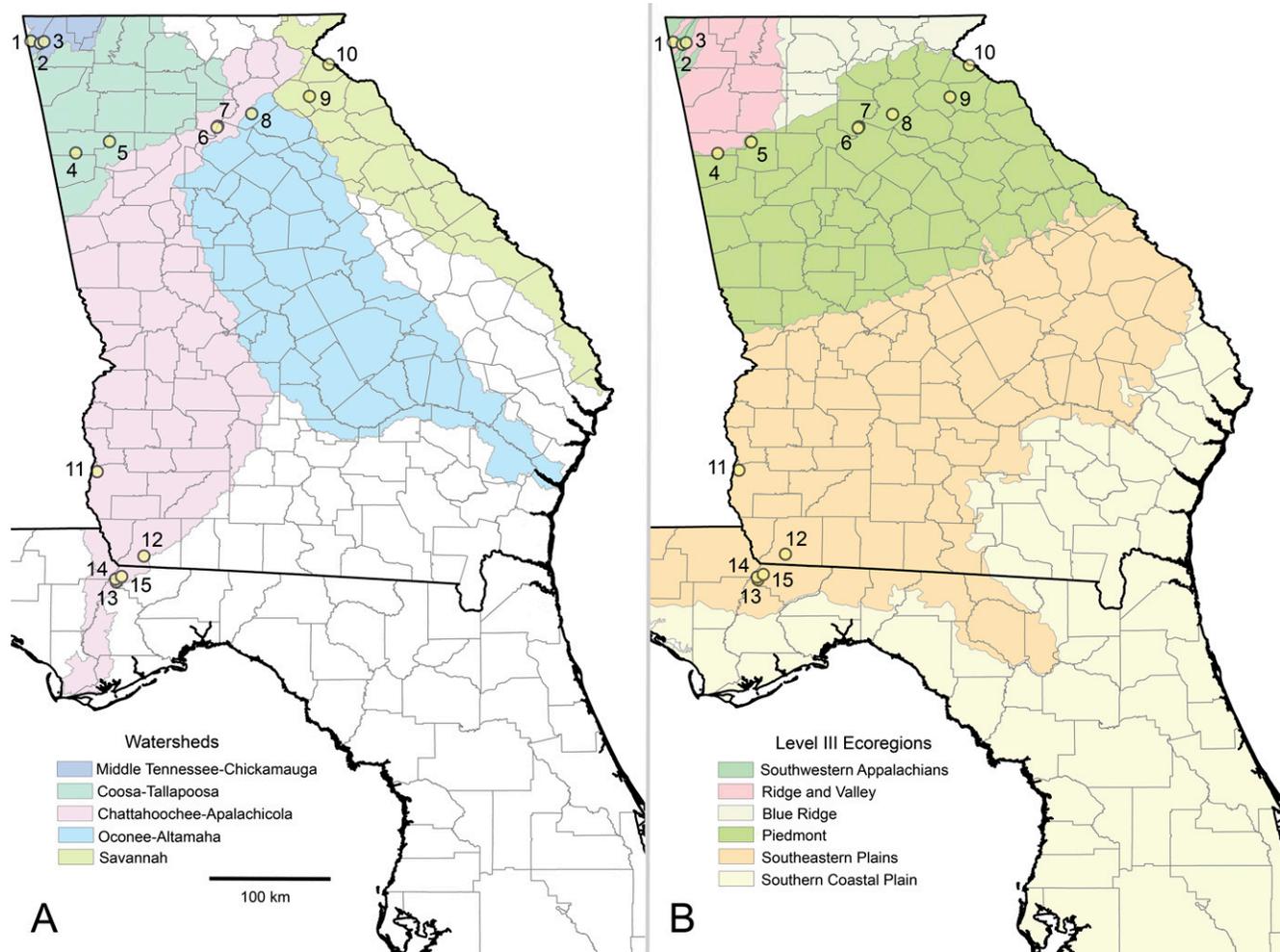
Fig. 2. Maps of Georgia and northern Florida showing location of populations 1–15 of *Veratrum woodii* sampled for this study (Table 2), overlain with associated watersheds (A) and physiographic regions (B). (See Fig. 1A for general location of population 16, Franklin County, Missouri). Watershed boundary data from USDA, NRCS (2018); level III ecoregion data from US EPA (2017). Maps generated by Steven C. Hughes.

(log [1 + geographic distance]) from voucher GPS coordinates standardized to decimal degrees. Conversions were executed in AFLPDat using the *Arlequin.M* function in R (Ehrich 2006) for Arlequin to create the input for the Mantel tests. Mantel tests were conducted with parameters set at 9999 permutations for 1) all 16 populations and 2) all populations minus the population from Missouri (population 16).

The program STRUCTURE (v. 2.3.4, Pritchard et al. 2000; Falush et al. 2003; Hubisz et al. 2009) was utilized to analyze the presence–absence data matrix from the AFLP analysis, an appropriate approach when no a priori relationships among individuals and populations were assumed (Kalinowski 2011). Parameters for simulations included 20,000 burn-in and 50,000 samples using Markov chain Monte Carlo (MCMC) methods for K-values 1 through 8, with the admixture model applied to infer alpha (α), and sampling locations were not used (no LOCPRIOR). These parameters and simulations were repeated for both the correlated allele frequencies model and the independent allele frequencies model. STRUCTURE HARVESTER (Earl and Vonholdt 2012) harvested STRUCTURE simulation files, for both the correlated and allele frequencies models, utilizing the Evanno et al. (2005) method to seek the optimal ΔK-value of each model type. Output files from STRUCTURE then were processed in the main pipeline of CLUMPAK (http://clumpak.tau.ac.il, Kopelman et al. 2015) for all K values, which processes simulation data in both CLUMPP (Jakobsson and Rosenberg 2007) and DISTRUCT (Rosenberg 2004).

RESULTS

Extant Georgia Populations—The results of the field survey, including voucher information, are summarized in Table 2 and Fig. 2. The 12 populations (in 10 counties) located in Georgia (Fig. 2B) have a disjunct distribution between the northern and southwestern parts of the state, corresponding to several physiographic regions: Ridge and Valley (population 1), Southwestern Appalachian (2 and 3), and Piedmont (4–10) in the north, and Southeastern Plains (Coastal Plains) in the south (populations 11–15, including Florida). These sites are restricted to a few river systems in five watersheds (Fig. 2A). The habitat for *V. woodii* in the Ridge and Valley and Southeastern Appalachian ecoregions is limestone and ravine forest (mesic hardwood forest) at 235–600 m elevation, whereas the Piedmont region populations inhabit oak–hickory–pine forests in moist sites along steep ravines and north-facing hillsides at 250–330 m (Zomlefer specimen label data). In the disjunct Southeastern Plains region, *V. woodii* occurs at much lower elevations (30–70 m) on slopes under beech–magnolia forest (mesic slope forest or southern mixed hardwood forest).

Eight populations were vouchered for the first time (indicated by an asterisk [*] in Table 2), and the survey also validated the presence of two populations (11 and 12) last located (and collected) in 1988. Population sizes in Georgia ranged from five to ca. 150 individuals (populations 2 and 1, respectively). Six populations in Georgia occur on conservation lands († in Table 2). Other reported (and unvouchered) sites could not be traced due to vague locality information, and

some legacy populations have been extirpated. For example, a population vouchered from Hall County in 1955 (*Duncan 19360*, GA) is now likely under Lake Lanier, a reservoir created by the Buford Dam in 1956. The construction of a housing development (Dockside at Water's Edge) in 1991 destroyed the habitat of an unvouchered population in DeKalb County (J. A. Allison pers. obs.), although some plants were rescued and planted at the Atlanta Botanical Garden (*Zomlefer 2419*, GA).

Genetic Diversity—Table 3 summarizes indices of genetic diversity for all 16 populations sampled for this study. Analyses of AFLP data recovered 1269 loci and did not differ significantly when the Missouri data (population 16) were included (n = 216) or excluded (n = 200, not shown). Private bands (those occurring only in a single population) were present at ca. 10% frequency in Georgia populations 1 and 3 (Dade and Walker Counties, respectively). The highest genetic diversity (heterozygosity, $H_e = 0.110$) was found in Polk Co., Georgia (population 4), and the lowest diversity ($H_e = 0.025$), in Missouri (population 16). Georgia and Florida populations, on average, were three times more diverse than the Missouri population. There was no correlation between estimated population size and mean H_e (not shown; r = 0.044; p = 0.871). The number of private bands varied from 0 (population 9, Franklin County, Georgia) to 36 (population 3, Walker County, Georgia) and 39 (population 1, Dade County, Georgia). The number of detected genotypes was lowest for population 12 (2; Decatur County, Georgia) and population 2 (3; Dade County, Georgia). Genotypic diversity (for a sample size of 15 individuals) was generally higher in northern populations 4, 5, and 8 (0.971, 0.981, 1.000) than for southern populations 12 (0.133) and 14 (0.629). Besides the lowest H_e, the Missouri population (16) was characterized by the lowest level of genetic diversity according to several other indices: 108 observed number of bands, 7.9% polymorphic loci, and one private band.

Population Structure—Table 4 summarizes the results of the hierarchical AMOVA for the 15 Georgia and Florida populations, partitioned according to watershed (Fig. 2A) and analyzed to infer the amount of variation among watersheds, among populations within watersheds, and within populations. The overall among population differentiation (F_{ST}) from the AMOVA analysis was 0.244. The among watershed

component (F_{SW}) of the total F_{ST} was 0.142 (i.e. 58% of the among population F_{ST}), whereas the among populations within watershed component (F_{SP}) of the total F_{ST} was 0.102 (i.e. 42% of the total F_{ST}).

Table 5, the matrix of pairwise F_{ST} (F-statistics) values between all 16 populations sampled for this study, also includes the F_{ST} population means. The average pairwise F_{ST} (average of population means) was 0.204. Population 5 (Paulding County, Georgia) had the highest mean F_{ST} (0.490, more than two times the average pairwise F_{ST}), followed by populations 12 (0.250; Decatur County; Georgia), 16 (0.243; Missouri), and 4 (0.242; Polk County; Georgia). Populations 4 and 5 are more similar to each other (average pairwise F_{ST} = 0.152) than to any other populations, and population 4, in general, has relatively low F_{ST}'s with most other Georgia populations. Weighted means (weighted by number of pairs) for within watersheds was 0.132, and 0.215 for among watersheds (Table 6). To determine whether this difference was a function of geographical distance between populations or due to a watershed effect, an additional analysis was performed comparing mean F_{ST} values of the disjunct Chattahoochee–Apalachicola populations (Table 6), partitioned into the north Georgia populations (6 and 7) and the south Georgia-Florida populations (11–15): the mean F_{ST} for the north vs. south populations was 0.136, whereas the mean F_{ST} for the all seven of the Chattahoochee–Apalachicola populations with populations outside of that watershed was 0.215.

Simulations from STRUCTURE, when harvested, resulted in an optimal $\Delta K = 4$ (Fig. 3). Populations 4 (Polk County) and 5 (Paulding County) had distinct multi-locus genotypes (orange). Population 4 varied from population 5 by having five individuals identical to the most common genotype (blue) of all the populations in Georgia and Florida and seven individuals (only four visible in Fig. 3) that combined the orange and blue genotypes. Populations 1 (Dade County) and 3 (Walker County) also showed evidence of reciprocal admixture, probably with population 4. One genotype (green) primarily occurred in the Florida (13–15), Missouri (16), and Gwinnett County, Georgia (6) populations, with some introgression into population 8 (Hall County). Another distinct genotype (purple) was most common in individuals in Missouri (population 16) with some admixtures as well in south

TABLE 3. Indices of genetic and clonal diversity for the 16 populations of *Veratrum woodii* sampled for this study. See Table 1 for voucher specimen information. H_e = expected heterozygosity. Number of detected loci was 1269.

Population number	Sample size (n)	Observed number of bands	Percent polymorphic loci	Number of private bands	Mean H_e	Number of detected genotypes	Genotype diversity	Effective number of genotypes
1	15	399	31.1	39	0.095	11	0.933	7.76
2	5	184	11.5	3	0.043	3	0.800	2.78
3	15	377	29.6	36	0.086	10	0.933	7.76
4	15	422	32.9	14	0.110	12	0.971	10.71
5	15	339	23.3	15	0.073	13	0.981	11.84
6	17	338	26.6	4	0.081	6	0.831	4.59
7	15	322	25.3	4	0.081	9	0.914	6.82
8	15	322	24.6	4	0.078	15	1.000	15.00
9	6	236	17.7	0	0.066	5	0.933	4.50
10	15	391	30.7	14	0.091	12	0.962	9.78
11	7	309	23.9	15	0.086	7	1.000	7.00
12	15	189	14.6	1	0.042	2	0.133	1.14
13	15	363	28.5	13	0.093	10	0.943	8.33
14	15	289	21.4	5	0.068	5	0.629	2.42
15	15	275	21.4	3	0.067	9	0.924	7.26
16	16	108	7.9	1	0.025	6	0.783	3.77
Population Means	216	298	23.2	11	0.074	—	—	—
Overall	—	1269	65.0	—	0.098	—	—	—

TABLE 4. Summary of AMOVA results with the 15 Georgia and Florida populations in 5 groups, partitioned according to watershed (see Table 1; Fig. 2A). Population 16 (from Missouri) was not included in these analyses. The overall F_{st} among population differentiation was 0.244 ($p \leq 0.001$).

Source of variation	Degrees of freedom	Sum of squares	Variance components	Proportion of variation
Among watersheds (F_{SW})	4	2073.395	9.54048	0.142
Among populations within watersheds (F_{SP})	10	1384.096	6.88138	0.102
Within populations	184	9368.629	50.91646	0.756

Georgia (11, 12), Florida (14), and one Dade County, Georgia, population (1).

The unrooted neighbor-joining tree (Fig. 4), based on Nei's (1972) genetic distance matrix, had condensed (almost collapsed) nodes, reflecting the low amount of genetic differentiation between populations. However, the phylogram segregated three main groups: a Coosa–Tallapoosa watershed group (populations 4 and 5), a northern Georgia group (except for population 11), and a Florida–southern Georgia group (except for population 10) plus Missouri (population 16).

Mantel Tests (not shown) were conducted with and without Missouri population 16. Neither analysis showed a significant relationship ($p > 0.10$) between geographic and genetic distances for populations of *V. woodii*. An additional analysis was run excluding outlier populations 4 and 5, as these two populations were distinct and similar to each other (STRUCTURE and neighbor-joining analyses) supported by their average pairwise F_{ST}. The relationship with distance was stronger and also significant ($p = 0.020$, $r^2 = 0.0533$).

Discussion

Genetic Variation of Veratrum woodii—The *Veratrum woodii* populations examined in this study had relatively low levels of genetic variation compared to other species with similar life history characteristics. The mean percent polymorphic loci (23.2%; Table 3) and H_e values (pooled H_e 0.098 and mean H_e 0.074) are much lower than mean H_e values for rare, perennial, outcrossing species (mean H_e 0.12–0.19) in AFLP-based studies assessed by Reisch and Bernhardt-Römermann (2014), or outcrossing, long-lived perennial species with wind-dispersed seeds, narrow geographic ranges, and late successional habitats (mean H_e 0.25–0.30) for studies reviewed by Nybom (2004). However, differentiation among populations of *V. woodii* ($F_{ST} = 0.244$; Table 4) is comparable to values for species with similar life history traits. For example, Φ_{ST} values ($\approx F_{ST}$; Ma et al. 2015) range from 0.20–0.27 for AFLP studies reviewed by Reisch and Bernhardt-Römermann (2014). Likewise, values of G_{ST} (= F_{ST}; see Nei 1973, 1977) range from 0.10–0.25 in papers surveyed by Hamrick and Godt (1989, 1996) and Nybom (2004). The significantly lower amounts of intrapopulation variation and somewhat elevated F_{ST} values for a long-lived perennial (compared to the survey means) may be due to the naturally fragmented range of *V. woodii* (Hamrick 2004), and its extant populations may also have been derived from different refugial populations that have become monomorphic for different loci. This interpretation is supported by the low intrapopulation percent polymorphic loci (23.2%; Table 3) relative to the high overall value (65.0%).

Data from the one Missouri population included in the study (population 16) may provide some additional insight into patterns of variation in *V. woodii*, although more sampling from the northern range of the species is needed to corroborate these inferences. This population had by far the lowest level of genetic diversity (7.9% polymorphic loci; mean $H_e = 0.025$) compared to the other 15 populations (Table 3), and even populations with a smaller sample size (populations 2, 9, and 11), generally had greater variation. Populations 2 (11.5% polymorphic loci, $H_e = 0.043$) and 12 (14.6% polymorphic loci; $H_e = 0.042$) also had low levels of genetic diversity; however, population 2 comprised only five individuals, and population 12 had only two multi-locus genotypes. The pattern of variation loss exhibited by population 16 is consistent with sequential founder effects: as a species moves out of refugia, variation is lost as connecting areas disappear (Honnay and Jacquemyn 2007; Eckert et al. 2008), and for rare species, the areas with the most contemporary populations may have the least genetic variation (Godt and Hamrick 1998). A good example is *Helonias bullata* L., a rare perennial herb also in the

TABLE 5. Matrix of average pairwise F_{ST} (F-statistics) values between the 16 populations of *Veratrum woodii* sampled for this study and the F_{ST} population means. Significant values ($p < 0.05$) in **boldface**.

Population number	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16
1	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
2	**0.117**	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
3	**0.112**	**0.085**	—	—	—	—	—	—	—	—	—	—	—	—	—	—
4	**0.195**	**0.233**	**0.212**	—	—	—	—	—	—	—	—	—	—	—	—	—
5	**0.446**	**0.557**	**0.484**	**0.152**	—	—	—	—	—	—	—	—	—	—	—	—
6	**0.131**	**0.167**	**0.111**	**0.235**	**0.471**	—	—	—	—	—	—	—	—	—	—	—
7	**0.120**	**0.215**	**0.138**	**0.242**	**0.493**	**0.061**	—	—	—	—	—	—	—	—	—	—
8	**0.214**	**0.207**	**0.176**	**0.283**	**0.525**	**0.197**	**0.253**	—	—	—	—	—	—	—	—	—
9	**0.121**	**0.084**	**0.129**	**0.231**	**0.517**	**0.086**	**0.170**	**0.136**	—	—	—	—	—	—	—	—
10	**0.154**	**0.159**	**0.113**	**0.258**	**0.495**	**0.050**	**0.125**	**0.125**	**0.061**	—	—	—	—	—	—	—
11	**0.102**	**0.160**	**0.098**	**0.177**	**0.462**	**0.078**	**0.160**	**0.078**	**0.054**	**0.052**	—	—	—	—	—	—
12	**0.178**	**0.316**	**0.177**	**0.299**	**0.573**	**0.147**	**0.103**	**0.290**	**0.241**	**0.231**	**0.219**	—	—	—	—	—
13	**0.132**	**0.136**	**0.102**	**0.244**	**0.491**	**0.040**	**0.138**	**0.158**	**0.061**	**0.022**	**0.043**	**0.199**	—	—	—	—
14	**0.224**	**0.259**	**0.189**	**0.318**	**0.562**	**0.150**	**0.281**	**0.165**	**0.101**	**0.087**	**0.121**	**0.335**	**0.066**	—	—	—
15	**0.123**	**0.118**	**0.106**	**0.272**	**0.531**	**0.094**	**0.166**	**0.194**	**0.043**	**0.095**	**0.084**	**0.187**	0.040	**0.111**	—	—
16	**0.147**	**0.381**	**0.121**	**0.281**	**0.584**	**0.102**	**0.172**	**0.303**	**0.233**	**0.196**	**0.242**	**0.260**	**0.158**	**0.323**	**0.144**	—
Population mean	0.168	0.213	0.157	0.242	0.490	0.141	0.192	0.220	0.151	0.151	0.142	0.250	0.135	0.219	0.154	0.243

TABLE 6. Within and among watershed mean F_{ST} values, with focus on partitioning the Chattahoochee–Apalachicola watershed. See Table 2 and Fig. 2A for population watershed assignments. Watershed abbreviations: C–A = Chattahoochee–Apalachicola, C–T = Coosa–Tallapoosa, MT–C = Middle Tennessee–Chickamauga, O–A = Oconee–Altamaha, S = Savannah. Weighted means are weighted by the number of pairs.

Watershed	Watershed subgrouping	Number of populations	Number of pairs within watershed	Mean F_{ST} within watershed	Mean F_{ST} among watersheds
C–A	—	7	21	0.139	0.215
	populations 6 + 7 (north)	2	1	0.061	0.188
	populations 11−15 (south)	5	10	0.150	0.192
	populations 6 + 7 vs. 11−15	—	10	0.136	0.192
C–T	—	2	1	0.152	0.405
MT–C	—	3	3	0.105	0.188
O–A	—	1	—	—	0.220
S	—	2	1	0.061	0.170
—	Missouri, population 16	1	—	—	0.243
Weighted means	—	—	—	0.132	0.215

Melanthiaceae and with specialized habitat (Godt et al. 1995). The populations of this species are scarce in the southern part of its range (southern Appalachians; H_e = 0.061), and the genetic diversity is much lower where the species is more common in the northern part of its range (New Jersey; H_e = 0.033) and intermediate for the Virginia populations (H_e = 0.045).

Curiously, in the STRUCTURE analyses of our study, the Missouri population appeared most similar to the geographically distant southern Georgia–Florida populations (green and purple bands in Fig. 3), which may infer a shared refugium and loss of variation northwards. However, these results (based on our limited collections) may not infer a shared refugium: alternatively other small, now extinct, southern refugial populations located further to the west may have retained these widespread alleles that migrated northward following the last glacial epoch.

Distribution and Genetic Variation of Veratrum woodii Populations in Georgia—Bellemare and Moeller (2014) found that some small-ranged herbaceous species with distributions extending beyond the southern boundary of the eastern temperate deciduous forest biome tended to occupy patchy habitats in the southeastern coastal plain which mimic a cooler and more mesic temperate environment. The markedly disjunct, northern–southwestern distribution of *V. woodii* populations in Georgia (Fig. 2) is a result, in part, of the availability of temperate-like microhabitats in the southern part of the state, and is shared by a suite of plant species (many perennial), such as *Actaea pachypoda* Elliott, *Claytonia virginica* L., *Erythronium umbilicatum* C. R. Parks & Hardin, *Hepatica americana* (DC.) Ker Gawl., *Podophyllum peltatum* L., *Polygonatum biflorum* Elliott, *Schisandra glabra* (Brickell) Rehder, and *Silene ovata* Pursh (Thorne 1949; Wharton 1978; Chafin 2007). These herbaceous species are typically associated with higher elevations in the southern Appalachian−piedmont region in the northern part of the state and are not usually found in the coastal plain. They survive in southwestern Georgia area due

to the relatively cool, humid, and sheltered understory conditions along ravines in the Southeastern Plains mesic slope forest (Wharton 1978; Edwards et al. 2013).

This general area, the Apalachicola River region (Florida panhandle, adjacent Georgia and southeastern Alabama), is well-known as a biodiversity hotspot, haven for rare species, and Pleistocene refugium (Braun 1950; Edwards et al. 2013; Bellemare and Moeller 2014). The mesic slope forests inhabited by *V. woodii* as southern disjuncts in Georgia may have served as refugia for mixed mesophytic vegetation with northern affinities: these species may have persisted during a series of glaciations and migrated northward during multiple interglacial periods (Thorne 1949; Batista and Platt 1997; Delcourt 2002).

As outlined in the results, measures of genetic diversity (such as mean H_e and genotypic diversity) and neighbor-joining analysis of pairwise genetic distance values showed genetic variation of populations having some general partitioning between the disjunct northern and southern Georgia (and Florida) populations. Exceptions were 1) the distinct group comprising northern Georgia populations 4 and 5, and 2) southern Georgia population 11 (clustered with northern populations) and northern population 10 (clustered with southern populations). Average pairwise F_{ST} values and results of STRUCTURE and neighbor-joining analyses corroborate populations 4 and 5 as similar to each other (F_{ST} = 0.152; Table 5) but distinct from other populations in northern Georgia. The two populations also share the Coosa–Tallapoosa watershed (Fig. 2A) that is distinct from the adjoining Chattahoochee–Apalachicola watershed: these two main river systems are separated by ca. 330 km where each enters the Gulf of Mexico. Therefore, populations 4 and 5 probably represent a different refugium than other populations in Georgia.

In the neighbor-joining tree, northern population 10 and southern population 11 are nested within the southern and
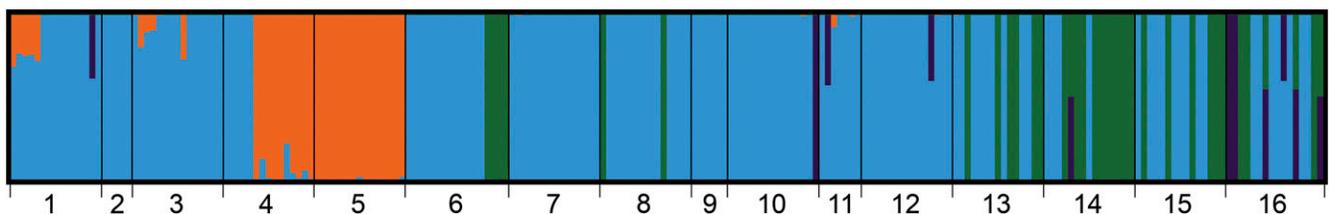

FIG. 3. Genetic structure diagram across 216 individuals (populations 1–16; see Table 2) of *Veratrum woodii* using the model-based Bayesian algorithm in STRUCTURE, corresponding to optimal ΔK = 4.
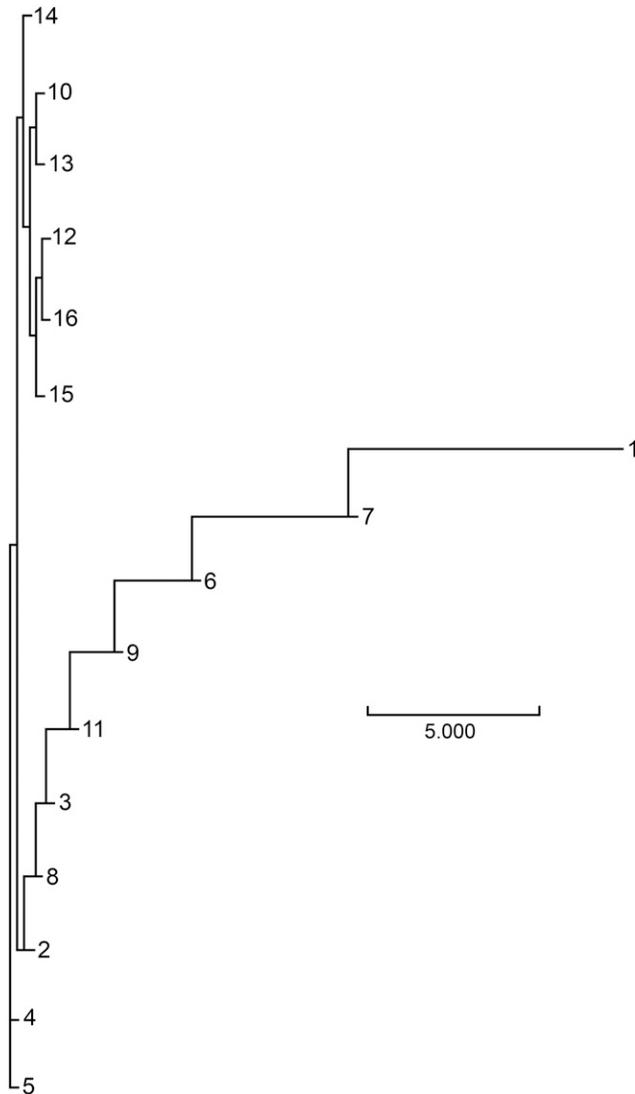
Fig. 4. Unrooted neighbor-joining tree of all 16 populations of *Veratrum woodii* (Table 2), based on Nei's (1972) genetic distance and generated by PHYLIP.

This hypothesized watershed effect is further supported by comparison of the north–south disjunct populations of the Chattahoochee–Apalachicola watershed (Table 6; Fig. 3A). Northern populations 6 + 7 vs. southern populations 11–15 have a lower weighted mean F_{ST} within watershed value (0.136) than the Chattahoochee–Apalachicola watershed populations vs. other watersheds (0.192), indicating a watershed effect rather than geographic distance correlation. Thus, the southern Georgia populations may have dispersed through the Chattahoochee–Apalachicola drainage system, and once populations were established in the Georgia piedmont, the species may have spread into the Middle Tennessee–Chickamauga watershed (accounting for the purple band in STRUCTURE for population 1; Fig. 3), and subsequently northward from there. Movement between watersheds likely would have been limited by the lack of suitable habitats connecting them.

Implications for Conservation Efforts—*Veratrum woodii* is well-known among the natural heritage community as an unusual, rare, and infrequently flowering species with unknown recovery potential (Ohio Department of Natural Resources 2017). Conservation recommendations have focused primarily on preserving its habitat by preventing disturbance (e.g. Ward 1979; Chafin 2007; Kentucky State Nature Preserves Commission 2013). However, anecdotal evidence (Ebinger 1993, 1996; G. Yatskievych pers. obs.) indicating the positive effects of ecological disturbance (fire and/or canopy removal) on flowering and fruiting of *V. woodii* is supported by studies on other rare herbaceous perennials in eastern temperate deciduous forests (e.g. Gill 1996; Bourg et al. 2015). Therefore, measures to counteract the consequences of fire suppression should be considered in management plans for *V. woodii* populations.

The results of our study suggest that this species, as a whole, has relatively low levels of genetic diversity. Since seed-set is low and long-distance seed dispersal events are unlikely, an increase in numbers of individuals and genotypes for declining populations (or colonization of new sites) is very limited under natural conditions. Our results suggest that preservation of the southernmost populations (likely representing a refugium) may be crucial to protect the genetic diversity of this species. Our findings also highlight the importance of watershed assignment for targeting populations to best preserve genetic variation of *V. woodii* in Georgia, a consideration for restoration efforts beyond the basic geographic distance between populations. However, our study is based on the assumption of selectively neutral AFLP markers, and these may not have detected the distribution of genes influencing adaptive phenotypic traits. Our results may be best corroborated by an extensive reciprocal transplant study, a difficult challenge for species such as *V. woodii*.

northern Georgia groups, respectively. The pairwise F_{ST} for these two populations is low (0.052; Table 5), and the F_{ST} values for population 10 with the southern populations is slightly lower (0.097) than those of population 11 with southern populations (0.117). This anomaly may account for assignment outside of their respective north–south grouping.

These data indicating north-south partitioning provide some evidence for one refugium that may have been the source for northward spread of the species in Georgia. When genetically distinct populations 4 and 5 were omitted from the Mantel tests, the results showed a strong positive relationship ($p = 0.020$) between geographic distance and genetic differentiation between populations, but the r^2 value indicated that only 5.33% of the variation was explained by this relationship. Our analyses suggest that watershed assignment provides a better explanation for variation between populations and population structure, as indicated by the distinctive populations 4 and 5 in the Coosa–Tallapoosa watershed in northern Georgia (discussed above) and by the weighted mean pairwise F_{ST} (Table 6) showing populations more similar within a watershed (0.132) than among watersheds (0.215).

Author Contributions

WBZ conceived the study and provided funding, conducted the fieldwork, made the voucher specimens, wrote the manuscript, and prepared the figures. JRC assisted with fieldwork, conducted the laboratory work, scored the data, prepared the data matrices, and provided preliminary data analyses. RDL analyzed the data, added references, and contributed significantly to the narrative on methodology and results and to initial interpretations of the data on a landscape scale. JLH interpreted the results, analyzed the data via alternative approaches, provided crucial references, and was coauthor of the results and discussion sections. The field expertise of JRA was invaluable for locating populations of *Veratrum woodii* in Georgia, which he had discovered decades previously as a botanist for the Georgia Department of Natural Resources.

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