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POST-GLACIAL HISTORY OF *TRILLIUM GRANDIFLORUM* (MELANTHIACEAE) IN EASTERN NORTH AMERICA: INFERENCES FROM PHYLOGEOGRAPHY[1]

STEVEN R. GRIFFIN AND SPENCER C. H. BARRETT[2]

Department of Botany, University of Toronto, 25 Willcocks St., Toronto, Ontario, Canada M5S 3B2

Dispersal and migration are important processes affecting the evolutionary history and genetics of species. Here we investigate post-glacial migration and gene flow in *Trillium grandiflorum* (Melanthiaceae), a wide-ranging, forest herb from eastern North America. Using phylogeographic approaches, we examined cpDNA and allozyme diversity in 35 populations of *T. grandiflorum* sampled from throughout the geographic range of the species. Nested clade analysis (NCA) of cpDNA haplotypes indicated that *T. grandiflorum* likely survived in two refugia in the southeastern US during the last glaciation and that long-distance dispersal characterized the post-glacial recolonization of northern areas. There was no evidence for reduced allozyme diversity in populations from glaciated compared to ice-free regions, probably because of the greater abundance and larger effective size of populations in the north. An analysis of isolation-by-distance based on the allozyme data suggested a pattern of population differentiation consistent with restricted gene flow. Notwithstanding the significance of rare seed dispersal events for migration, a comparison of allozyme and cpDNA genetic structure indicates that pollen flow between populations is more likely than seed dispersal. These results for *T. grandiflorum* represent the first phylogeographic analysis of a temperate woodland herb in eastern North America and support the importance of occasional long-distance dispersal events in the post-glacial migration of plants.

Key words: allozymes; cpDNA; Melanthiaceae; phylogeography; pollen: seed flow; post-glacial migration; Reid's Paradox; *Trillium*.

Dispersal is a central process affecting the ecology, genetics, and geographical distribution of species (Ridley, 1930; Van der Pijl, 1969; Jaquard et al., 1984; Sauer, 1988; Dingle, 1996). In temperate regions, dispersal ability has directly influenced response to glacial cycles. During the Pleistocene (2.4×10^6 yr(myr)–10 000 yr BP), at least six glacial advances affected the physical and biological environments of the Northern Hemisphere (Cox and Moore, 2000). In North America, the Wisconsin glaciation began 120 000 yr BP and ended approximately 8000 yr BP (Davis, 1983). At its maximum, the ice sheet extended as far south as 40° N in eastern North America. As the Wisconsin ice sheet began to retreat approximately 18 000 yr BP, species that survived in ice-free refugia migrated north to previously glaciated habitats.

In plants, post-glacial colonization is especially paradoxical because observed average seed dispersal distances cannot account for the rapid northward migration that occurred in many species (Reid's Paradox; Clark et al., 1998). For example, Cain et al. (1998) modeled migration of ant-dispersed *Asarum canadense* L. and concluded that long-distance seed dispersal, by unknown mechanisms, was necessary to explain its present-day distribution in eastern North America. Similarly, analyses of preserved pollen samples suggest that *Acer* spp. migrated nearly 2000 km in approximately 10 000 yr, a rate of 200 m/yr (Davis, 1983). Recent modeling studies also indicate that modes of colonization, particularly rare long-distance dispersal events, influence genetic diversity in glaciated regions (Ibrahim et al., 1995). Indeed, reduced genetic diversity has been documented in glaciated ranges of several European and North American species (for a review see Hewitt [2000]), although in most cases the occurrence and mechanisms of long-distance dispersal are not known.

Phylogeographic methods can be used to investigate post-glacial migration and dispersal and are especially useful for the study of herbaceous species that do not preserve well in the palynological record (Cruzan and Templeton, 2000; Hewitt, 2000). Early studies used intuitive approaches to decipher phylogeographic data (Avise, 2000); subsequently, Templeton and colleagues (Templeton et al., 1995; Templeton, 1998; Posada et al., 2000) developed nested clade analysis (NCA), a procedure that statistically assesses the process of migration and dispersal resulting in observed phylogeographic patterns. Recent concerns that NCA may lead to erroneous conclusions (Knowles and Maddison, 2002) suggests that both intuitive and NCA approaches should be used in phylogeographic studies. Since in most angiosperms chloroplast DNA (cpDNA) is maternally inherited through seed alone, phylogeographic analysis of cpDNA haplotypes can provide an unambiguous marker for the study of seed dispersal. In addition, surveys of allozymes can provide insights into the genetic consequences of post-glacial migration (e.g., Broyles, 1998). Furthermore, when used in combination, comparisons of the genetic structures of cpDNA and allozymes can allow estimates of rates of dispersal via pollen and seed (Ennos, 1994).

Despite a wealth of palynological studies of the post-glacial history of eastern North America (Davis, 1983), there is much less phylogeographical data from plant species from this region (Sewell et al., 1996; Echt et al., 1998; Tremblay and Schoen, 1999; Maskas and Cruzan, 2000; Walter and Epperson, 2001). Furthermore, most phylogeographic studies of plant migration in North America and elsewhere have dealt

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[2] E-mail: barrett@botany.utoronto.ca.

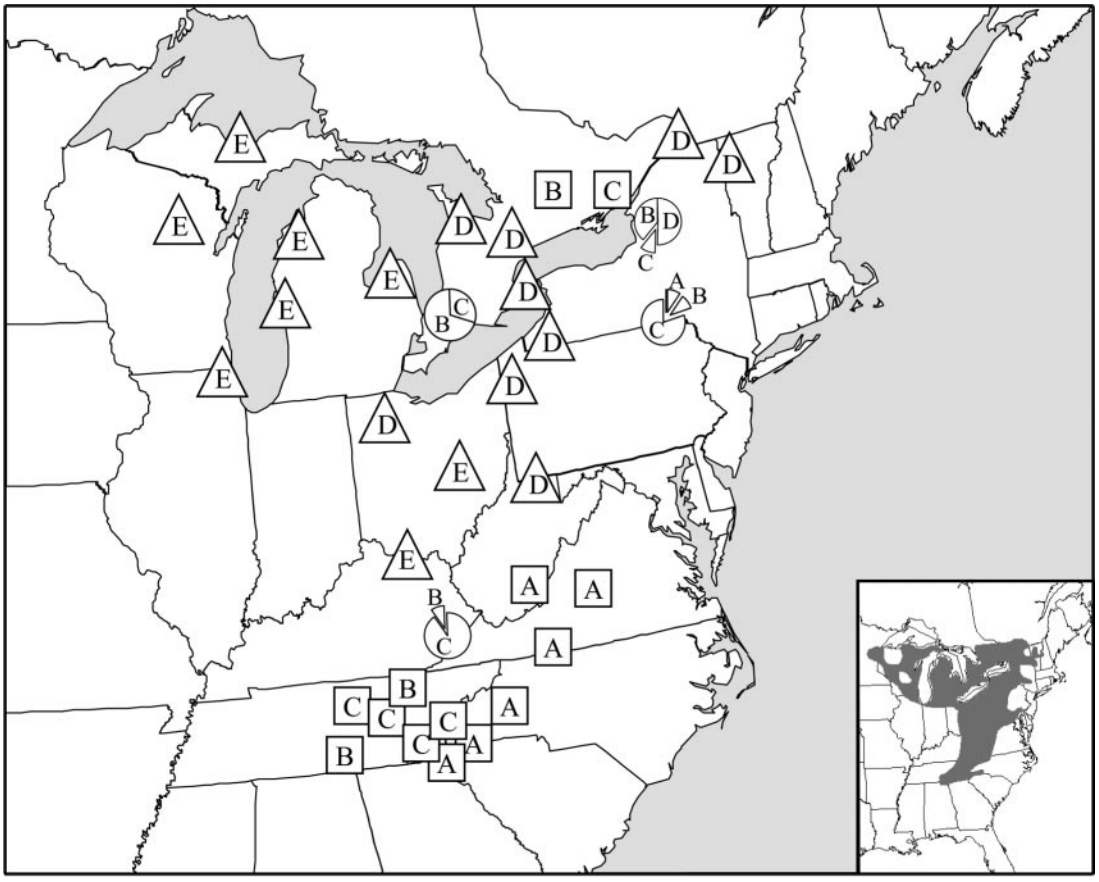

Fig. 1. Distribution of five cpDNA haplotypes in *Trillium grandiflorum*. Sample sizes ranged from 8 to 10 individuals per population. Populations indicated by the symbol for a haplotype were monomorphic for that haplotype. Squares represent the 1-1 clade and triangles the 1-2 clade. Four populations were polymorphic and these are marked with pie diagrams indicating haplotype frequencies. See Table 1 for exact locations of populations and Fig. 3 for phylogenetic relationships among haplotypes. Inset: the present distribution of *Trillium grandiflorum* in eastern North America, after Case and Case (1997).

with tree species (reviewed in Taberlet et al., 1998). To our knowledge, no phylogeographic study to date has used molecular markers to investigate angiosperm species whose ranges span both glaciated and ice-free portions of eastern North America. Therefore, the principal goal of this study was to examine the phylogeography of a wide-ranging woodland herb to evaluate the role of long-distance dispersal in the migratory history of this species.

Trillium grandiflorum (Michx.) Salisb. (Melanthiaceae), a long-lived perennial that is widespread as an understory woodland herb in eastern North America (Fig. 1 inset; Case and Case, 1997), is a useful candidate for phylogeographic studies of post-glacial migration. The species is predominately bumble bee pollinated and self-incompatible. Marker gene studies of mating patterns indicate that most populations are predominantly outcrossing (Broyles et al., 1997; Kalisz et al., 1999; Sage et al., 2001). It is not known to hybridize with other members of the genus (Case and Case, 1997). All *Trillium* species have ant-dispersed seeds (i.e., myremecochory: Gates, 1941; Case and Case, 1997) and ant-mediated dispersal of up to ~2 m in *T. grandiflorum* has been documented (Kalisz et al., 1999). Seed dispersal by wasps of up to several hundred meters has also been reported in the genus (Jules, 1996). These reports suggest limited dispersal range and raise the question of how *T. grandiflorum* could have achieved its current widespread distribution following glacial range contraction. Vellend et al. (2003) recently demonstrated that dispersal of *T. grandiflorum* seeds can occur when white-tailed deer ingest the fruit and excrete them in their stool. They suggest that deer could have provided an important mechanism for long-distance dispersal in *T. grandiflorum*. In principle, phylogeographic approaches should enable long-distance dispersal to be distinguished from other modes of colonization.

What evidence is there to evaluate whether *Trillium* species may have survived glaciations in the southeastern United States? Higher intra- and interspecific diversity might be expected in glacial refugia. No previous comparisons have assessed geographical patterns of genetic diversity of widespread *Trillium* species. However, of the 45 species in the genus approximately 30 are found in the southeastern United States, with only seven inhabiting glaciated areas to the north (Case and Case, 1997). Moreover, the pollen record indicates that refugial populations of tree species (*Acer* spp., *Fagus* spp.) associated with *Trillium* and the guild of woodland herbs with which it occurs existed in Alabama and Arkansas and migrated northwards from this southern refugium following the retreat of the Wisconsin glacier (Davis, 1983). *Trillium* species may have also survived in multiple refugia during the Wisconsin glaciation. For example, separate refugia could have existed in the Appalachians, along the Atlantic coast and elsewhere. To evaluate these ideas we used nested clade analysis in com-

TABLE 1. Population locations of *Trillium grandiflorum*, ranked by latitude for reference.

Code	County	Latitude (°N)	Longitude (°W)
NC-1	Macon Co., NC	35.12475	83.53965
NC-3	Clay Co., NC	35.23713	83.13412
NC-4	Jackson Co., NC	35.30633	83.64775
TN-1	Grundy Co., TN	35.46870	85.59683
TN-2	Sevier Co., TN	35.63883	83.49338
NC-2	Burke Co., NC	35.92607	81.93808
TN-4	Morgan Co., TN	36.13642	84.49850
TN-3	Putnam Co., TN	36.19372	85.38337
TN-5	Campbell Co., TN	36.41283	84.09843
VA-2	Patrick Co., VA	36.74408	80.20822
KY-1	Harlan Co., KY	36.92490	82.94707
VA-1	Nelson Co., VA	37.91768	79.19535
WV-1	Greenbrier Co., WV	37.97697	80.37872
KY-2	Lewis Co., KY	38.61663	83.21778
WV-3[1]	Monongolia Co., WV	39.60360	80.07385
OH-1	Brown Co., OH	40.06463	81.48470
OH-2	Hancock Co., OH	41.13823	83.55307
PA-1	Crawford Co., PA	41.52453	80.39682
NY-4	Tioga Co., NY	42.07732	76.34417
NY-1	Cattarugus Co., NY	42.16230	79.01077
IL-1	Lake Co., IL	42.29318	87.84328
ON-4	Elgin Co., ON	42.63337	81.72047
ON-1	Welland Co., ON	43.03983	79.18598
MI-1	Oceana Co., MI	43.45902	86.45477
NY-5	Jefferson Co., NY	43.72318	76.07603
MI-2	Huron Co., MI	44.01137	83.04937
ON-3	York Co., ON	44.02973	79.52715
ON-5	Grey Co., ON	44.50000	81.00000
VT-1	Chittendon Co., VT	44.53023	73.09742
ON-2	Leeds Co., ON	44.53262	76.37398
MI-3	Kalkaska Co., MI	44.77987	85.29752
ON-6	Haliburton Co., ON	45.08210	78.03577
WI-1	Oconto Co., WI	45.20165	88.50903
PQ-3	Les Coteaux, Vandreuil-Soulanges, PQ	45.27783	74.20093
MI-1	Alger Co., MI	46.43760	86.55287

Note: 1 = Not included in the allozyme analysis.

bination with allozyme data in an effort to discriminate between the single vs. multiple refugia hypotheses.

This investigation examines the migratory history and mechanisms of gene flow in *Trillium grandiflorum* through a geographical survey of contemporary patterns of genetic diversity at cpDNA and allozyme loci. In our study, we addressed the following specific questions: (1) Using both NCA and intuitive approaches, is there a phylogeographic signal in the molecular data from which we can reconstruct the postglacial history of this species? (2) What are the genetic consequences of glacial range contraction and subsequent recolonization in *T. grandiflorum*? In particular, is there evidence of reduced genetic diversity in northern population of the species? (3) What is the relative importance of gene flow via pollen vs. seeds in *T. grandiflorum* and has long-distance dispersal contributed towards northern migration?

MATERIALS AND METHODS

Tissue sampling—*Trillium grandiflorum* is distributed within a roughly triangular area bounded by Quebec to the northeast, Wisconsin to the northwest, and Tennessee/North Carolina to the south (Fig. 1 inset; Case and Case, 1997). In April–May 2000, we collected leaf samples from 35 *T. grandiflorum* populations throughout most parts of this range (Table 1). The mean distance between the 35 populations was 660 km (range 33–1460 km). We sampled flowering individuals only and these were collected at 2–3 m intervals within populations. *Trillium grandiflorum* does not propagate clonally and therefore each individual is a separate genet. We sampled a single leaf from up to 30 individuals from each population and leaf samples were subsequently stored on ice for up to 10 d.

cpDNA analysis—Small pieces of leaf tissue (1 cm^2) were placed in 1.5-mL microcentrifuge tubes and frozen at −80°C for later analysis. We extracted total DNA following a simplified version of Doyle and Doyle (1987). We used polymerase chain reaction (PCR) and restriction fragment length polymorphism (RFLP) analysis to detect intraspecific cpDNA variation. First, we screened 20 universal cpDNA primer pairs (Taberlet et al., 1991; Demesure et al., 1995; Dumolin-Lapegue et al., 1997). Ten primer pairs yielded reliable PCR fragments (TFab, TFcd, TFef, TC, VL, HK, K1K2, CS, ST, ML). One primer pair, CS, could not be optimized to eliminate nonspecific amplification. For the remaining nine primer pairs, we screened each PCR fragment with 17 restriction enzymes (four-, five-, and six-base pair cutters). All PCR reactions were carried out on a Hybaid PCRexpress (Thermo Electron Corp., Mississauga, Ontario, Canada) under the following conditions: 1.5 mmol/L $MgCl_2$, 200 μmol/L of each dNTP, 0.00625 units/μL Taq, and 1 × PCR buffer; 1 min at 94°C, 1 min at the optimized annealing temperature (Tfab −57°C, TFcd −50°C, Tfef −61°C, TC −59°C, VL −57°C, HK −60°C, K1K2 −60°C, CS −60°C, ST −61°C, ML −61°C) and 2 min at 72°C for 35 cycles. The PCR fragments were assayed on 1.6% agarose gels stained with ethidium bromide.

Through preliminary screening, we were able to resolve three sequence polymorphisms (gain/loss of a restriction site) in *T. grandiflorum*. Fragment VL exhibited a sequence polymorphism when cut by *MspI*. Fragment HK showed two sequence polymorphisms when cut by *MspI* or *AluI*. Thus, all cpDNA analyses are based on these three characters. To assay the extent of

polymorphism, we assayed 8–10 individuals of *T. grandiflorum* from 35 populations for each polymorphic fragment/restriction enzyme combination. In total, 345 individuals were assayed for cpDNA polymorphisms.

Statistical analysis of cpDNA variation—We used HAPLODIV (Pons and Petit, 1995) to calculate the within-population diversity (h_S), which is the probability that two randomly chosen haplotypes in a population are different, and total gene diversity (h_T), which is the probability that any two haplotypes are different. We used ARLEQUIN (Schneider et al., 2000) to calculate the percentage of among- and within-population differentiation of the cpDNA genome with analysis of molecular variance (AMOVA) and to calculate the haploid equivalent of F_{ST}.

We used maximum parsimony to construct an intraspecific phylogeny of haplotypes as described by Templeton et al. (1995). To justify the use of parsimony, we followed the procedure outlined by Templeton et al. (1992) to calculate *H*, the probability that a restriction site change has been caused by more than one mutation. So long as $H < 0.05$, then the use of maximum parsimony is justified. First, we estimated θ, which is the probability that two alleles chosen at random differ at a single nucleotide (Ewens, 1983). In this case, $\theta = k/\{2\ rm\ [\ln(n)]\}$, where *k* is the number of polymorphic sites, *r* is the length of the restriction enzyme recognition sequence, *m* is the total number of cut sites and *n* is the sample size. For *T. grandiflorum*, after the initial screen, $k = 3$, $r = 5$ (on average), $m = 105$ and $n = 12$. Substituting θ into Equation one of Templeton et al. (1992) yields $H = 0.011$, well below the 0.05 criterion. Therefore, the use of parsimony was justified for this analysis.

To analyze the historical signal in *T. grandiflorum* cpDNA we used GeoDis (Posada et al., 2000), which implements the nested-clade analysis (NCA) designed by Templeton et al. (1995). We assessed the significance of dispersion (D_C) and displacement (D_N) values by permutation tests of the data, based on 1000 permutations. To assess historical processes within each level of the nested cladogram we used the dichotomous key developed by Templeton (1998).

Allozyme analysis—In the laboratory, we ground small pieces of leaf tissue in an extraction buffer consisting of 0.7 mmol/L Borax, 4 mmol/L sodium metabisulfite, 40 mmol/L sodium diethyldithiocarbonate, 50 mmol/L sodium ascorbate, 0.2 mol/L Tris-HCl, 11 mmol/L DTT, and 3 mmol/L PVP-40. The extract was adsorbed onto Whitman #3 wicks and frozen at −80°C for later analysis. Following Broyles et al. (1997), we were able to assay eight loci for *T. grandiflorum*: a morpholine-citrate buffer system (pH 6.1; 50 mA) was used for isocitric dehydrogenase (*Idh*), malate dehydrogenase (*Mdh*-1 and *Mdh*-2), phosphoglucomutase (*Pgm*), 6-phosphogluconate dehydrogenase (*Pgd*), and shikimic dehydrogenase (*Skdh*). A discontinuous system consisting of lithium-borate electrode buffer (pH 8.3; 60 mA) and tris-citrate gel buffer was used to resolve glutamate dehydrogenase (*Gdh*) and phosphoglucose isomerase (*Pgi*). On average, we assayed 28.5 individuals (range 17–30) of *T. grandiflorum* per population.

Statistical analysis of electrophoretic data—To analyze genetic diversity, we used POPGENE (Yeh et al., 1997) to calculate observed heterozygosity (H_o), expected heterozygosity (H_e) and the effective number of alleles per locus (*A*). To test the expectation that there is reduced genetic diverisity in glaciated vs. nonglaciated parts of the range, we used Pearson correlations in JMP (SAS, 2000) to investigate the relations between *A*, H_o, and H_E with latitude.

We used FSTAT (Goudet, 1995, 2000) to calculate *f*, θ, and *F*, which are analogous to Wright's (Wright, 1951) measures F_{IS}, F_{ST}, and F_{IT} (Weir and Cockerham, 1984). FSTAT calculates mean values for *f*, θ, and *F* by jackknifing over loci and calculates 95% confidence intervals (CI) for these measures by bootstrapping over loci. Standard errors (SE) and CIs were calculated by 1000 permutations of the data.

To assess genetic relatedness between populations, we used POPGENE to construct UPGMA dendrograms (Sneath and Sokal, 1973) based on Nei's genetic distance calculated from population allele frequencies (Nei, 1972). To detect if there was a significant isolation-by-distance effect, we used the ISOLDE program in GENEPOP (Raymond and Rousset, 1995) to perform a Mantel test of $[F_{ST}/(1 - F_{ST})]$ against the natural logarithm of geographic distance. To determine if the variance in genetic distance increased with geographic distance, we performed a Mantel test on the residual of $[F_{ST}/(1 - F_{ST})]$ against the natural logarithm of geographic distance (Hutchinson and Templeton, 1999). Distances between populations were calculated with ArcView (ESRI, 1998).

TABLE 2. Genetic variation and hierarchical fixation indices for both allozyme and cpDNA loci in 35 populations of *Trillium grandiflorum*.

	Allozyme	cpDNA
Genetic variation	$A = 1.28 \pm 0.03$ $H_o = 0.12 \pm 0.03$ $H_e = 0.17 \pm 0.01$	$H_S = 0.05 \pm 0.02$ $H_T = 0.81 \pm 0.02$
Population structure	$f = 0.29$ (0.22–0.36) $\theta = 0.35$ (0.23–0.36) $F = 0.53$ (0.37–0.70)	$F_{ST} = 0.95$

Note: For allozymes, A = number of effective alleles per locus per population (mean ± 1 SE); H_o = observed heterozygosity per population (mean ± 1 SE); H_e = expected heterozygosity per population (mean ± 1 SE); *f* (95% CI), θ (95% CI), and *F* (95% CI) are analogous to F_{IS}, F_{ST}, and F_{IT} (Weir and Cockerham, 1984). *F*, θ, and *f* are unbiased jackknife estimates (and the bootstrap-generated 95% confidence interval) based on 1000 jackknife or bootstrap permutations of the data. For cpDNA, H_S = within-population diversity (mean ± 1 SE), H_T = between population diversity (mean ± SE), and F_{ST} as calculated for haplotype data (note that error estimates for F_{ST} from Arlequin/AMOVA are not calculated; see Excoffier et al. [1992] for further details).

As the results of cpDNA analysis for *T. grandiflorum* (see Results) revealed a striking pattern of haplotype distribution, we used population haplotype information to further analyze allozyme structure that could be related to cpDNA structure. First, we coded each population by cpDNA haplotype or clade (see Results); three polymorphic populations were coded by the predominant haplotype or clade while one population (NY-5) consisted of equal numbers of 1-1 and 1-2 clade haplotypes and was excluded from these analyses. We then used POPGEN (Yeh et al., 1997) to perform hierarchical *F*-statistic analyses at two levels for consistency with nested clade analysis (NCA): populations grouped by haplotypes within one-step clades and populations grouped by one-step clade (1-1 or 1-2) within the two-step clade. Thus, F_{PT} represents the differentiation between haplotypes within a one-step clade (at the 1-1 and 1-2 levels) or the differentiation between one-step clades at the two-step clade level.

Relative rates of gene flow via pollen and seed—We used equations developed by Ennos (1994) to estimate the relative rates, *r*, of gene flow via pollen and seed. This method assumes that cpDNA is maternally inherited in *Trillium*, as it is in most angiosperms (Palmer, 1987). The relative rate of pollen to seed migration, *r*, is r = pollen/seed = $(A - 2C)/C$, where $A = 1/F_{ST(b)} - 1$ and $C = 1/F_{ST(m)} - 1$ subscript *b* denotes a biparentally inherited marker and *m* a maternally inherited marker. However, this should be considered a special case if inbreeding is zero. As there is some biparental inbreeding in *Trillium* (see Results), we used the modified form of the equation that takes into account the inbreeding coefficient F_{IS} as follows (see Ennos, 1994 for details), where pollen/seed = $(A[1 + F_{IS}] - 2C)/C$.

RESULTS

Geographic structuring of genetic variation—*Trillium grandiflorum* displayed variation at allozyme and chloroplast loci (Table 2). All eight assayed allozyme loci were polymorphic, although *Idh* was polymorphic in only one population (see Supplemental Data accompyaning the online version of this article for population allozyme allele and cpDNA haplotype frequencies). We found no evidence to support an a priori

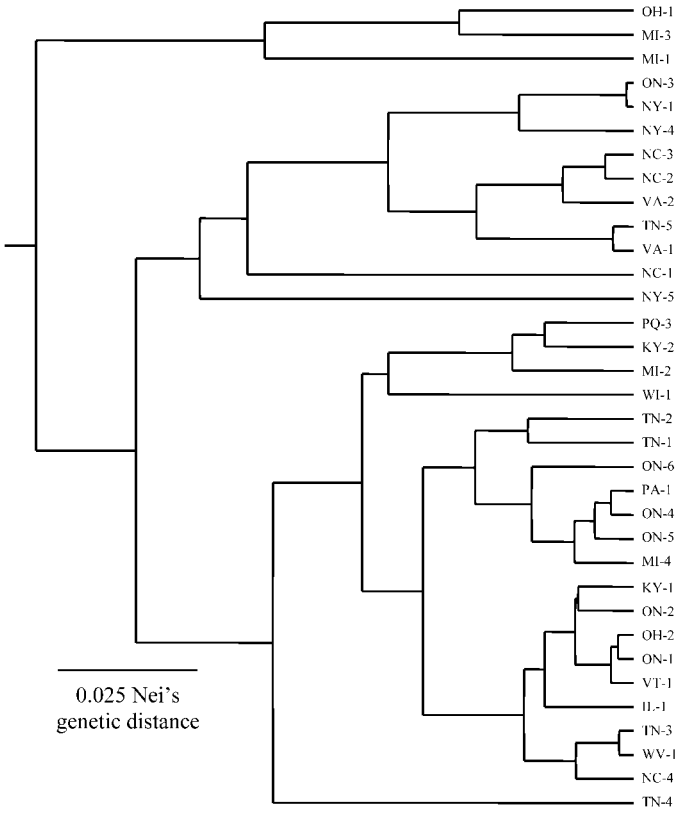

Fig. 2. Genetic relationships among 34 populations of *Trillium grandiflorum*. The UPGMA dendrogram of Nei's (1972) genetic distance is based on eight allozyme loci. Population codes indicate the state or province where collected (see Table 1 for locations).

expectation of reduced nuclear genetic diversity in populations from glaciated compared to nonglaciated regions. None of the correlations of nuclear genetic diversity against latitude was significantly different from zero; however, all three correlations were positive (*A*: $r = 0.22$, $P = 0.20$; H_e: $r = 0.10$, $P = 0.54$; and H_o: $r = 0.14$, $P = 0.45$). There was a reduction in haplotype diversity in the northern part of the range of *T. grandiflorum* (Fig. 1). All five haplotypes were found in populations from the nonglaciated portions of the range; however, haplotype A was absent from the glaciated region with the exception of one individual from New York.

Trillium grandiflorum exhibited significant between-population structuring of genetic diversity at allozyme loci (Table 2). However, between-population structuring of cpDNA haplotypes was much stronger than for allozymes (Table 2). The high F_{ST} value for cpDNA occurred because most populations were fixed for a single haplotype and only four populations were polymorphic (Fig. 1). Accordingly, AMOVA revealed that most of the genetic variation was distributed among populations (94.8%) rather than within populations (5.8%; AMOVA: within populations, df = 310, SS = 10.4, among populations df = 34, SS = 209.2, $P < 0.0001$).

No clear spatial patterns were evident in the UPGMA dendrogram of allele frequencies at allozyme loci (Fig. 2). Furthermore, there was a significant although weak effect of isolation-by-distance (Mantel test; slope of regression = 0.028, $P = 0.01$) and the variance in genetic distance increased significantly with geographic distance (slope of regression = 0.17, $P = 0.012$).

Nested clade analysis—We infer two distinct historical processes that have shaped the geographic distribution of cpDNA haplotypes in *T. grandiflorum* based on nested clade analysis (NCA; Table 3): fragmentation and range expansion via long-distance colonization. The construction of a one-step phylogeny (Fig. 3) of cpDNA haplotypes required the removal of one closed haplotype loop (i.e., BCDE). Closed loops are a common problem in intraspecific phylogenies (Templeton et al., 1992). With five haplotypes and a closed loop, there are four possible networks. There is no phylogenetic rationale to choose among the four equally parsimonious trees. Caution in the following analysis is clearly warranted. While NCA of the four alternate trees were mostly consistent, in that long-distance range expansion and fragmentation were detected for all, two trees did yield inferences of contiguous range expansion. However, coalescent theory can be used to choose the most

TABLE 3. Inference of historical processes shaping the geographic distribution of *Trillium grandiflorum* cpDNA haplotypes. Inferences are made from relative values of dispersion distance (D_C) and displacement distances (D_N) of tip and interior haplotype/clades (see Fig. 1, Fig. 3 and text for further details). I-T refers to the difference between tip and interior clade/haplotypes. Haplotype/clade frequencies are given both within a clade (f_C) and globally (f_G). Inferences for each clade are based on Templeton's (1998) inference key. The inference chain based on the key for each clade is given in parentheses.

Nested clade	Clades/ haplotypes	Position	*N*	f_C	f_G	D_C	D_N	Inference chain	Conclusion
1-1								(1, 2, 3, 4, 9)	Fragmentation
	A	Tip	58	0.34	0.17	180^S	247^S		
	B	Interior	42	0.25	0.12	410	439		
	C	Tip	71	0.42	0.21	509^L	488^L		
	I–T					39	52		
1-2								(1, 2, 11, 12, 13)	Range expansion—long distance colonization
	D	Interior	96	0.55	0.28	274^S	345^S		
	E	Tip	78	0.45	0.23	311^S	407^L		
	I–T					-38	-78^S		
2-1								(1, 2, 11, 12, 13)	Range expansion—long distance colonization
	1–1	Interior	171	0.50	0.50	392^S	507^L		
	1–2	Tip	174	0.50	0.50	380^S	443^S		
	I–T					11	64^S		

Note: S = D_C or D_N values that are significantly smaller than expected at the 5% level based on 1000 permutations. L = D_C or D_N values that are significantly larger than expected at the 5% level based on 1000 permutations.

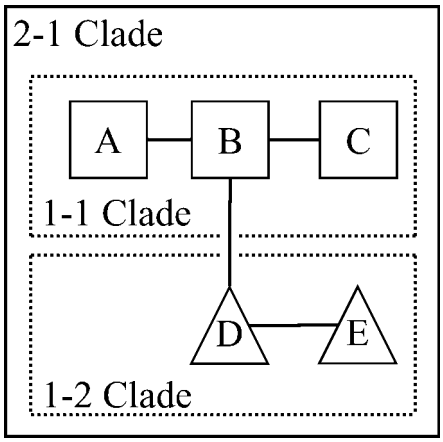

Fig. 3. Nested cladogram of *Trillium grandiflorum* haplotypes. Each line connecting one haplotype to another indicates one mutational step (i.e., the gain/loss of a restriction site). There were two one-step clades (1-1 and 1-2 clades) and one two-step clade (2-1 clade) following the nesting rules of Templeton et al. (1992).

likely tree topology (Crandall and Templeton, 1993). Therefore, the intraspecific phylogeny and interpretation we present below are the most likely based on coalescent predictions (and see Discussion).

Coalescent theory predicts that older (interior) haplotypes should be more common than derived (tip) haplotypes. Indeed, there is empirical evidence to support this claim (see Crandall and Templeton, 1993 and references therein). Following Crandall and Templeton (1993), tip haplotypes are defined as those connected to only one other haplotype, whereas interior haplotypes are connected to multiple haplotypes. The D haplotype is the most common (Table 3), suggesting that it is an interior haplotype and that C and E are tip haplotypes (Fig. 3, other networks not shown).

Further explanation is required for the designation of tip and interior clades at the two-step level. Inferences from NCA usually only apply to haplotypes within a species or species group (see Templeton et al., 1995; Golden and Bain, 2000). A recent phylogeny of *Trillium* by Osaloo and Kawano (1999) placed the morphologically similar *T. ovatum* as the sister species of *T. grandiflorum* with very little molecular divergence between the two. Therefore, we argue that information from this closely related taxon could be used to infer the age of the *T. grandiflorum* one-step clades. Clade 1-1 is closer to the *T. ovatum* cpDNA haplotype than clade 1-2 (S. R. Griffin and S. C. H. Barrett, unpublished data). Presumably, older clades have had more time to accumulate mutations and the 1-1 clade comprises three haplotypes whereas the 1-2 clade comprises two haplotypes. Thus, we argue that clade 1-1 warrants interior status. Nevertheless, it is important to note that the qualitative interpretation using Templeton's (1998) key for the 2-1 nesting level is the same (long-distance colonization; Table 3) regardless of whether clade 1-1 or 1-2 is designated as the interior clade.

Long-distance colonization and fragmentation can explain the distribution of haplotypes and clades in *T. grandiflorum* (Table 3). Within the 1-1 clade, past fragmentation has resulted in the separation of haplotype A from haplotypes B and C. Range expansion through long-distance colonization can be invoked to explain the distribution of haplotypes E and D (though see Discussion). Long-distance colonization is also inferred at the 2-1 clade level. Hence, NCA indicates that long-distance dispersal events characterize the post-glacial migration of *T. grandiflorum* in eastern North America.

TABLE 4. Three-level hierarchical *F* statistics (Weir, 1990) of allozyme structure for populations of *Trillium grandiflorum*. Populations were grouped by cpDNA haplotype and clade (see Figs. 1 and 3 and text for details). The analyses were performed independently at two levels: populations within the 1-1 clade (excluding populations in the 1-2 clade); populations grouped by haplotype within the 1-2 clade (excluding the 1-1 clade); and all populations grouped by clade within the 2-1 clade. F_{IT}, F_{ST}, and F_{PT} are analogous to Weir's (1990) F, θ_S, and θ_P.

Nested clade	Haplotypes/ clade (*N*)	F_{IS}	F_{ST}	F_{PT}	F_{IT}
1-1 Clade	A (6)				
	B (4)	0.30	0.33	0.11	0.54
	C (7)				
1-2 Clade	D (8)	0.27	0.31	0.05	0.50
	E (8)				
2-1 Clade	1-1 Clade (17)	0.28	0.35	0.07	0.54
	1-2 Clade (16)				

Hierarchical *F* statistics of populations based on haplotypes/clades (Table 4) indicated little differentiation between haplotypes or clades. When analyzed at the one-step clade level (1-1 or 1-2), the differentiation between haplotypes (i.e., F_{PT}) was small compared to the differentiation among all populations. Similarly, at the two-step clade level (2-1), there was little differentiation between the 1-1 and 1-2 clades (F_{PT}) compared to differentiation among all populations (F_{ST}).

Rates of gene flow via pollen and seed—Using the F_{ST} values for allozymes and cpDNA, the ratio of pollen flow to seed flow for *T. grandiflorum* is 33.6, without adjustment for inbreeding. However, some inbreeding was detected in *T. grandiflorum* and taking the value of 0.29 for F_{IS} (*f* in Table 2) into account, the ratio of pollen to seed flow is 45.6.

DISCUSSION

Geographic patterns of genetic variation—There have been relatively few allozyme studies of widespread plant species in glaciated and nonglaciated regions of eastern North America. Those that have been conducted, including *Sarracenia purpurea* L. (Schwaegerle and Schaal, 1979), *Polygonella* spp. (Lewis and Crawford, 1995), and *Asclepias exaltata* L. (Broyles, 1998), typically report reduced genetic variation in glaciated regions compared to regions that remained ice-free during the Wisconsin glaciation. In contrast, we found no significant correlation of allozyme diversity against latitude in *T. grandiflorum*, and indeed all three estimates of diversity exhibited weak positive trends (i.e., increased diversity) in higher latitudes. Several factors may account for the lack of correlation between nuclear genetic diversity and latitude in *T. grandiflorum*.

First, the southern populations of *T. grandiflorum* that we sampled may not have been growing in the refugial areas where the species survived during the Wisconsin glaciation. Based on the analysis of pollen cores (Davis, 1983), refugial deciduous forests existed to the southwest (i.e., Alabama and Arkansas) of the current southern margins of the range of *T. grandiflorum*. Therefore, it is possible that all contemporary populations of this species are the result of post-glacial expansion and thus show reductions in genetic diversity relative

to now-extinct refugial populations. Nonetheless, even if all present-day populations are indeed derived, both long-distance and stepping-stone models of migration predict a loss of genetic diversity in the most recently founded (i.e., northern) populations (Hewitt, 2000). Second, if the current range does include some refugial populations (as our cpDNA haplotype analysis suggests; see below), contemporary processes may have obscured historical signals at allozyme loci. For example, while the center of origin for *Trillium* may be in the southeastern United States, the current center of abundance for *T. grandiflorum* lies much further north (e.g., Michigan, Ontario, New York, Quebec; Case and Case, 1997). Population sizes in the north are typically much larger than in the south (S. R. Griffin, unpublished data), thus buffering populations against the effects of genetic drift. Moreover, contemporary gene flow between populations can also obscure historical signals, especially when populations are abundant and of large effective size, as they are in many northern locales.

Isolation-by-distance analysis indicates that gene flow occurs among populations of *T. grandiflorum*. Hutchinson and Templeton (1999) developed a graphical model to estimate the relative influence of drift and gene flow on population genetic structure. Local gene flow between populations leads to a positive relation between genetic and geographic distance. On the other hand, drift will tend to increase the variance in allele frequencies and therefore increase the variance in genetic distance. Thus, if there is little gene flow at large distances, drift will lead to increasing variance in genetic distance as geographic distance increases. Our isolation-by-distance analysis of allozyme data indicates that *T. grandiflorum* displays this pattern: both genetic distance and the variance in genetic distance increased with the geographic distance between populations (i.e., Case I sensu Hutchinson and Templeton, 1999). This result raises the question of what mechanisms might account for gene flow among populations of *T. grandiflorum*.

One possible explanation is that bumble bees, the predominant pollinators of *T. grandiflorum*, mediate pollination between populations. Although pollen flow between the particular populations we sampled seems unlikely because of the large geographical scale of our study, pollinator-mediated gene flow among unsampled populations may account, in part, for the pattern of isolation-by-distance we observed. An alternative mechanism of gene flow between populations has recently been suggested by Vellend et al. (2003) who demonstrated that *T. grandiflorum* seeds could be dispersed via ingestion and defecation by deer. Indeed, our analysis of cpDNA diversity using NCA provided evidence that long-distance seed dispersal contributed to post-glacial recolonization in *T. grandiflorum* (Table 3 and see below). Nevertheless, our estimates of rates of gene flow via pollen vs. seed using the Ennos method (1994) implicates pollinators as the primary agents of between-population gene flow in *T. grandiflorum*. A third possibility is that the findings from our analysis of isolation-by-distance are unrelated to current evolutionary processes involving gene flow. The genetic structure at allozyme loci revealed in our geographical survey may have largely arisen during post-glacial range expansion and contemporary rates of pollen and seed dispersal have not had a strong impact in altering these historical patterns.

In contrast to the patterns observed at allozyme loci, cpDNA haplotype diversity in *T. grandiflorum* displays much greater population structure (Table 2). The high $F_{ST\text{-}cpDNA}$ value (0.95) agrees with theoretical predictions and most empirical data from other plant species (Ennos, 1994). Indeed, the differences in population structure between the allozyme and cpDNA markers suggest that gene flow via pollen is 45× higher than that via seed.

Phylogeographic patterns in Trillium grandiflorum—Despite considerable interest in the effects of past climate change on plant migration (reviewed in Comes and Kadereit, 1998), relatively little is known about the historical processes that have shaped present-day genetic diversity in eastern North American plants, especially in glaciated regions. The geographical structuring of cpDNA haplotypes provided insights into the post-glacial history of *T. grandiflorum*. Given the recent concerns about NCA (Knowles and Maddison, 2002), we evaluated our results from *T. grandiflorum* using both NCA-based and intuitive approaches. For the most part, NCA concurs with visual inspection of our data set, and our comparison strengthens our phylogeographic inferences for *T. grandiflorum*.

The haplotypes within the 1-1 clade (i.e., haplotypes A, B, and C; Fig. 3) are primarily limited to the ice-free portion of the range of *T. grandiflorum*, though some are found in populations from glaciated regions (Fig. 1). Furthermore, haplotype A is further restricted from the rest of the clade occurring largely along the eastern side of the Appalachian Mountains. This suggests a past fragmentation into two refugia on either side of the Appalachians during the Wisconsin glaciation. Indeed, NCA inferred a fragmentation event in the 1-1 clade (Table 3). The restriction of haplotype A to the eastern side of the Appalachians suggests that these mountains may have restricted this haplotype from dispersing from its refugium. Thus, most of the recolonization of glaciated regions (through long-distance dispersal; see below) may have occurred from a refugium west of the Appalachian Mountains, containing haplotypes B and C. In Europe, the Alps and Pyrenees are also thought to have blocked the dispersal of some animal and tree species from Italian and Iberian refugia, respectively (reviewed in Taberlet et al., 1998; Hewitt, 2000). However, haplotype C is found in two populations from North Carolina east of the Appalachians (Fig. 1) suggesting that there has been some secondary contact between the putative refugia. If this occurred, it implies that the barrier imposed by the Appalachians may not have been an absolute one. In contrast, Sewell et al. (1996) found one common cpDNA haplotype in *Liriodendron tulipifera* L. spanning the area covered by the *T. grandiflorum* clade 1-1 (i.e., both east and west of the Appalachians) while another less common haplotype was restricted to populations in Florida. Thus, in this case the Appalachians did not restrict the migration of *L. tulipifera* in this region. *Liriodendron tulipifera* has wind-dispersed seeds that can presumably travel greater average distances than *Trillium* seeds and that may have facilitated *L. tulipifera*'s dispersal across the Appalachians.

Haplotypes B and C occur in two separate clusters in Ontario and New York that are geographically separated by regions occupied by other haplotypes (Fig. 1). This pattern is consistent with models of range expansion where occasional long-distance dispersal results in a patchy distribution of genotypes in glaciated regions (Hewitt, 2000) and has been observed in European oaks (Petit et al., 1997). The results of our NCA indicate that long-distance colonization has likely shaped the distribution of clades at the 2-1 level (Table 3).

Long-distance dispersal can also be inferred from NCA

within the 1-2 clade (Table 3). However, this conclusion is at odds with an intuitive interpretation of the geographical distribution of haplotypes D and E (Fig. 1). The limited distribution of these haplotypes suggests a history of fragmentation, with the D and E haplotypes restricted to separate refugia during the last glaciation. These two haplotypes are restricted to the northeast and northwest corners of the range, respectively, with minimal geographical overlap between them. Alternatively, NCA inferred long-distance dispersal within this clade. It is it difficult to reconcile the NCA inference with our intuitive assessment of the geographic distribution of these haplotypes. Thus, caution in the use of Templeton's key (1998) seems warranted, as it may lead to suspect conclusions, especially for limited data sets as are commonly found in plant phylogeographic studies.

To attempt to discriminate among the different phylogeographic inferences of long-distance dispersal and fragmentation, and especially for clade 1-2, we examined hierarchical allozyme structure in our data set (Table 4). If *T. grandiflorum* was restricted to geographically separate refugia during glaciation and if different haplotypes/clades occurred in these refugia, then populations grouped by cpDNA haplotype should exhibit genetic differentiation between haplotype groups. On the other hand, if all haplotypes/clades within a nesting level originated from a single refugium, then relatively little genetic differentiation between haplotype/clades would be predicted. Our results revealed little differentiation in allozymes between haplotype/clade groups at all three nesting levels (i.e., F_{PT}, Table 4). Hence, this analysis was equivocal with respect to the multiple refugia hypothesis. Elsewhere, Soltis et al. (1997) reported similar findings for several plant species from the Pacific Northwest. Despite cpDNA structure that indicated distinct northern and southern refugia, allozymes and ribosomal DNA were not associated with the two refugia in this region. Discordance among nuclear and cytoplasmic loci largely reflects differences in the ability of these markers to detect historical events. In addition, such differences may also reflect contrasting patterns of gene dispersal via pollen or seed.

A closed haplotype loop presented an additional dilemma for the use of NCA, as different interpretations of the loop may affect the interpretations of NCA. As discussed above (see Results), we used coalescent predictions to choose the most likely haplotype network. Analyses of the other three networks yielded similar, though not identical, results. One consistent result was that one-step clades containing the B haplotype all showed a pattern of past fragmentation. This finding strengthens the argument that the Appalachians may have served as a barrier to recolonization. Long-distance colonization was also inferred in one-step clades from two of three alternate networks. A significant discrepancy, however, was that NCA inferred contiguous range expansion at the two-step clade level in two of the alternate trees. A conclusion of contiguous range expansion is difficult to reconcile with either the NCA analysis discussed above or an intuitive assessment of the distribution of haplotypes. If the phylogenetic relationships among haplotypes are ignored, the restricted distributions (Fig. 1) of haplotypes B, D, and E suggest fragmentation events and the disjunct populations containing B and C suggest long-distance seed dispersal. Resolution of additional haplotypes would likely refine our phylogeographic analysis of *T. grandiflorum* but, unfortunately, this species appears to have limited cpDNA variability.

In conclusion, our study initiates research on the post-glacial migration of woodland herbs in eastern North America. Notwithstanding the caveats of our phylogeographic analyses, fragmentation and range expansion, likely by long-distance dispersal, have been important factors in the post-glacial history of *T. grandiflorum* and the present-day distribution of genetic variation. Future work investigating phylogeographic patterns in the guild of woodland herbs commonly associated with *T. grandiflorum* (e.g., *Asarum canadense* L., *Clintonia borealis* (Ait.) Raf., *Erythronium americanum* Ker-Gawl., *Sanguinaria canadensis* L.) would be valuable to determine if these species also display similar migratory histories. Studies of this type will undoubtedly lead to a better understanding of the biogeographic history of this wide-ranging plant community that characterizes the forested regions of many parts of eastern North America.

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