Genetic variation in *Trillium erectum* (Melanthiaceae), a widespread forest herb in eastern North America

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Abstract: *Trillium erectum* L. is an insect-pollinated understory herb widespread in forests of eastern North America. Marker gene studies indicate that the species has a mixed mating system, but aspects of population genetic structure have not been investigated. Using 10 allozyme loci, we measured genetic variation within and among 23 populations sampled from throughout the species' range. Overall, *T. erectum* displayed moderate levels of genetic diversity in comparison with other herbaceous plants. The percentage of loci that were polymorphic was 52%, with average values (±SE) of 1.20 ± 0.02, 0.08 ± 0.01, and 0.13 ± 0.01 for the number of alleles per locus (A), observed heterozygosity (H_o), and expected heterozygosity (H_e), respectively. There was evidence of inbreeding within populations (F_{is} = 0.39, 95% CI 0.26–0.55) and significant population differentiation (F_{st} = 0.16, 0.05–0.24). Analysis of genetic data provided no evidence of isolation by distance, and together with the occurrence of population subdivision, this suggests that there is relatively limited contemporary gene flow among populations. Northern populations of *T. erectum* tended to have less genetic variability than southern populations, probably as a result of historical factors associated with postglacial migration. Limited opportunities for gene dispersal as a result of low plant densities, the capacity for self-fertilization, and local seed dispersal by ants are likely to be the main factors maintaining contemporary patterns of genetic variation in *T. erectum*.

Key words: allozymes, genetic diversity, gene flow, population genetic structure, *Trillium*.

Résumé : Le *Trillium erectum* L. est une plante herbacée de sous-bois pollinisée par les insectes, qui est très répandue dans les forêts de l'est de l'Amérique du Nord. Des études de gène marqueur indiquent que l'espèce possède un système de croisement mixte, mais les aspects de la structure génétique des populations n'ont pas reçu d'attention. En utilisant 10 lieux allozymiques, les auteurs ont mesuré la variation génétique entre, et au sein de 23 populations échantillonnées sur l'ensemble de l'aire de distribution de l'espèce. Dans l'ensemble, le *T. erectum* affiche un degré modéré de diversité génétique, en comparaison avec d'autres plantes herbacées. Le pourcentage de lieux polymorphiques est de 52 %, avec des valeurs moyennes (± erreur-type) de 1,20 ± 0,02, 0,08 ± 0,01 et 0,13 ± 0,01 pour le nombre d'allèles par lieu (A), l'hétérozygocité observée (H_o) et l'hétérozygocité attendue (H_e), respectivement. Il y a des signes évidents d'auto-croisement au sein de la population (F_{is} = 0,39, 95 % intervalle de confiance 0,026–0,55) et une différenciation significative des populations (F_{st} = 0,16, 0,05–0,24). L'analyse des données génétiques ne prouve pas qu'il y aurait isolation par la distance et, conjointement avec la présence de subdivision dans la population, ceci suggère qu'il existe un flux génétique contemporain relativement limité entre les populations. Les populations nordiques du *T. erectum* ont tendance à montrer moins de variabilité génétique que les populations méridionales, probablement dû à des facteurs historiques liés à la migration postglaciaire. Les opportunités limitées pour la dispersion des gènes résultant de la faible densité des plantes, la capacité d'autofécondation et la dispersion locale des graines par les fourmis sont vraisemblablement les facteurs qui maintiennent les patrons contemporains de variation génétique, chez le *T. erectum*.

Mots clés : allozymes, diversité génétique, flux génétique, structure génétique des populations, *Trillium*.

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Introduction

Genetic variation and population structure reflect both the influence of present-day evolutionary forces such as natural selection, genetic drift, and gene flow, as well as historical processes associated with patterns of colonization and migration (Wright 1951; Slatkin 1985; Barrett and Husband 1990). Features of life history, such as growth form, mating system, and seed dispersal mechanism, are also associated with differences in the patterns of genetic variation among angiosperm species (Hamrick and Godt 1996). In temperate regions, many plant species that now occupy previously glaciated regions migrated from southern refugia following glacial retreat (Davis 1983; Taberlet et al. 1998). Migration models predict the loss of genetic variation in more recently colonized derived populations (Ibrahim et al. 1995), and re-

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duced allozyme diversity compared with refugial populations has indeed been reported in several plant species (Schwaegerle and Schaal 1979; Loveless and Hamrick 1988; Dolan 1994; Lewis and Crawford 1995; Broyles 1998).

Trillium erectum L. (Melanthiaceae) is a long-lived perennial herb common in the understory of eastern North American forests (Case and Case 1997). The species is pollinated primarily by beetles and small flies (Irwin 2000) and has a weak self-incompatibility system resulting in moderate seed set following self-pollination (Sage et al. 2001). Marker gene studies indicate that natural populations practice a mix of self-fertilization and outcrossing (Broyles et al. 1997; Sage et al. 2001), a condition referred to as mixed mating. Like other members of the genus, *T. erectum* seeds are adapted for ant dispersal (Gates 1941; Case and Case 1997; Kalisz et al. 1999), although dispersal of *Trillium* seeds by wasps (Jules 1996) and in the droppings of white-tailed deer have also been reported (Vellend et al. 2003); these agents could also be occasionally involved in *T. erectum* seed dispersal.

Palynological studies of the migration of tree species in eastern North America suggest that associated forest herbs, such as *T. erectum*, likely survived the last glacial cycle in refugia in the southern United States before migrating northwards (Davis 1983). Depending on the patterns of colonization and migration, northern migration might be expected to result in the erosion of genetic diversity in populations. Although two studies have examined allozyme variation within *T. erectum* populations (Broyles et al. 1997; Irwin 2001), the distribution of genetic variation throughout the glaciated and unglaciated portions of the range of this species has not been examined.

Here, using polymorphisms at allozyme loci, we investigate the genetic diversity of *T. erectum* populations throughout the species' geographical range, to address the following specific questions: (1) What are the patterns of genetic variation within and among populations of *T. erectum*, and is there evidence of inbreeding and population differentiation? (2) Because *T. erectum* likely migrated northwards from southern refugia following glacial retreat, is there evidence of reduced genetic diversity in northern populations compared with southern populations? Following the presentation of our results, we discuss the relative importance of contemporary and historical factors on the structuring of genetic diversity in *T. erectum*.

Materials and methods

Sampling

Trillium erectum is near-continuously distributed within a roughly triangular area bounded by New Brunswick to the northeast, Michigan to the northwest, and Tennessee and North Carolina to the south (Fig. 1; Case and Case 1997). In April–May 2000, we collected leaf samples from 23 *T. erectum* populations from throughout this area (Table 1). We sampled flowering individuals only, and these were collected at 2- to 3-m intervals along transects within populations. *Trillium erectum* does not propagate clonally and therefore each individual is a separate genet. We sampled leaves of *T. erectum* from up to 30 individuals at flowering from each population, and these were stored on ice for up to 10 d before being returned to the laboratory.

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 dithiothreitol, and 3 mmol/L polyvinylpyrrolidone-40. The extract was adsorbed onto Whitman No. 3 wicks and frozen at –80 °C for later analysis. Following Broyles et al. (1997), we were able to assay 10 loci for *T. erectum*: 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 phosphoglucose isomerase (*Pgi*), alcohol dehydrogenase (*Adh*), and glutamate oxaloacetate transaminase (*Got*-1 and *Got*-2). All allozymes were resolved on 11% starch gels. On average, we assayed 26.3 ± 0.8 (mean ± SE) individuals per population (Table 1).

Analysis of allozyme variation

To analyze genetic diversity at allozyme loci, 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 prediction that northern populations should have less genetic diversity than southern populations, we used Spearman correlations in JMP (SAS Institute Inc. 2000) to investigate the relations between H_o, H_e, and A with latitude.

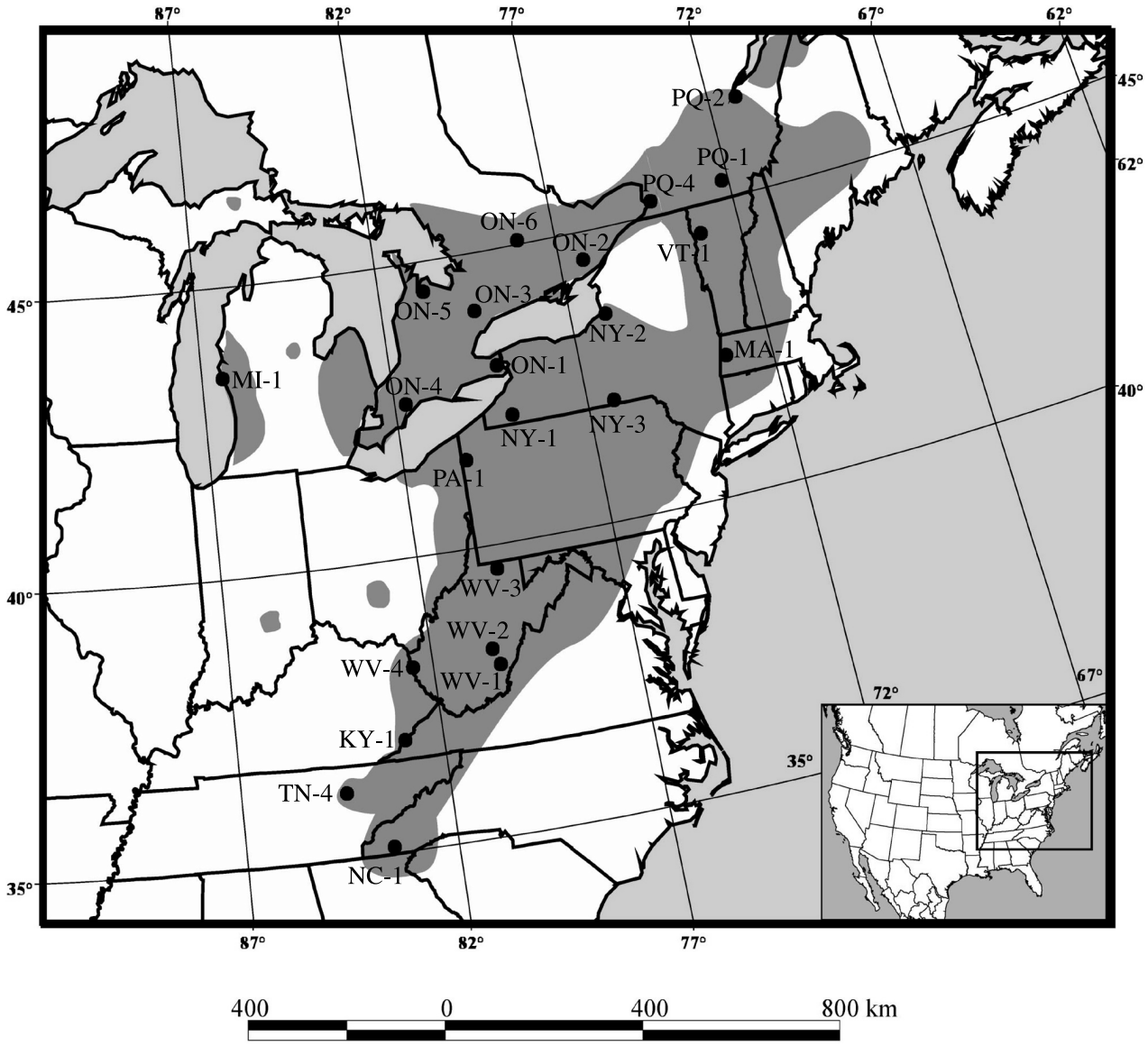
We used FSTAT (Goudet 1995, 2000) to calculate f, θ, and F, which are analogous to Wright's (1951) measures of F_{is}, F_{st}, and F_{it} (Weir and Cockerham 1984). FSTAT calculates mean values for f, θ, and F by jack-knifing over loci and calculates 95% confidence intervals (CI) for these measures by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

To assess genetic similarity between populations, we used POPGENE to construct a UPGMA (unweighted pair group method with arithmetic mean) dendrogram (Sneath and Sokal 1973) based on Nei's genetic distance, calculated from the population allele frequencies (Nei 1972). To detect whether 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. Distances between populations were calculated using ArcView GIS (ESRI 1998).

Results

Trillium erectum displayed moderate amounts of genetic diversity (Table 1). In most populations approximately 5 of the 10 allozyme loci assayed were polymorphic. One locus (*Got*-1*)* was monomorphic in all populations. All F-statistics were significantly different from zero (f = 0.39 (95% CI 0.26–0.55), θ = 0.16 (0.05–0.24), and F = 0.50 (0.36–0.65)),

Fig. 1. The geographical distribution of *Trillium erectum* (after Case and Case 1997), and the location of the 23 populations sampled in the present study. Population names are given next to each location; see also Table 1.



indicating significant genetic structure both within and among populations.

No clear geographical pattern was evident in the UPGMA dendrogram of *T. erectum* populations, with neighbouring populations showing relatively little clustering (Fig. 2). Similarly, there was no correlation between F_{st} and geographic distances between populations (data not shown; $F_{st}/(1 - F_{st}) = 0.040 - 0.000\,55[\ln(\text{distance})]$, $P = 0.46$), and therefore no evidence of an isolation-by-distance pattern. Although all three measures of genetic diversity (Table 2) were negatively correlated with latitude (i.e., decreased diversity in northern populations), none of these correlations was significant when the total sample was analyzed. However, in these correlations one population from Michigan (MI-1) was an outlier exhibiting exceptionally high diversity compared with most other northern populations. When MI-1 was excluded from our analysis, H_e and A exhibited a significant negative correlation with latitude (Table 2).

Discussion

The present study investigated genetic diversity at allozyme loci in the forest herb *T. erectum* from a sample of populations throughout the species' range. This species commonly occurs at low density, flowers in early spring when pollinator service is unreliable, and has a mixed mating system. As mating patterns should strongly influence levels of heterozygosity, we were interested in whether measurements of population genetic parameters would indicate significant inbreeding within populations of *T. erectum*. Our results provide evidence that inbreeding in populations of *T. erectum* affects both heterozygosity and population genetic structure.

With one exception, our results were in general accord with previous studies of *T. erectum*. Irwin (2000) reported values of $H_o = 0.324$ and $H_e = 0.326$ for a population of *T. erectum* from Vermont, USA. In contrast, values for H_o and H_e reported by Broyles et al. (1997) from a population

Table 1. Location and measure of genetic diversity based on 10 allozyme loci from 23 populations of *Trillium erectum* from eastern North America, ordered by latitude.

Population*	Lat. (°N)	Long. (°W)	*N*	PL	*A*	H_o	H_e
NC-1	35.124 75	83.539 65	25	0.50	1.22	0.055	0.14
TN-4	36.136 42	84.498 50	13	0.40	1.20	0.056	0.13
KY-1	36.924 90	82.947 07	27	0.50	1.21	0.081	0.13
WV-1	37.976 97	80.378 72	28	0.60	1.29	0.122	0.18
WV-4	38.147 57	82.522 33	25	0.80	1.37	0.118	0.24
WV-2	38.255 22	80.515 72	26	0.60	1.20	0.081	0.14
WV-3	39.603 60	80.073 85	25	0.40	1.15	0.062	0.10
PA-1	41.524 53	80.396 82	25	0.50	1.07	0.059	0.06
NY-3	42.074 55	76.370 25	22	0.50	1.27	0.118	0.16
NY-1	42.162 30	79.010 77	26	0.55	1.21	0.104	0.13
MA-1	42.391 45	73.227 80	23	0.50	1.17	0.072	0.12
ON-4	42.633 37	81.720 47	23	0.40	1.22	0.092	0.14
ON-1	43.039 83	79.185 98	18	0.40	1.18	0.082	0.11
MI-1	43.459 02	86.454 77	20	0.40	1.35	0.086	0.21
NY-2	43.550 12	76.092 65	20	0.50	1.23	0.127	0.14
ON-3	44.029 73	79.527 15	26	0.60	1.21	0.063	0.14
ON-5	44.520 10	81.126 00	23	0.40	1.09	0.076	0.08
VT-1	44.530 23	73.097 42	26	0.50	1.19	0.063	0.12
ON-2	44.536 20	76.373 98	28	0.60	1.17	0.089	0.11
ON-6	45.082 10	78.035 77	26	0.50	1.12	0.055	0.09
PQ-4	45.259 87	74.230 12	26	0.60	1.22	0.058	0.14
PQ-1	45.320 98	72.199 05	24	0.50	1.13	0.077	0.10
PQ-2	46.651 03	71.245 73	21	0.50	1.12	0.039	0.09
Average (± SE)			26.3 ± 0.8	0.52 (0.45–0.57)†	1.20 ± 0.02	0.080 ± 0.010	0.13 ±0.01

Note: *N*, sample size; PL, percent polymorphic loci; *A*, effective number of alleles per locus; H_o, observed heterozygosity; H_e, expected heterozygosity.
*The first two letters of each population name refer to the province or state in which the population was found.
†95% CI.

in New York State, USA, are comparable to our own findings. Irwin's (2000) heterozygosity values are well outside the range recorded in our study and are unexpected because *T. erectum* should experience significant self-fertilization (*t* ranges from 0.42 to 0.52; Broyles et al. 1997; Sage et al. 2001), leading to a reduction in heterozygosity. Therefore, the discrepancy between studies is difficult to reconcile unless the mating system of Irwin's population was largely outcrossing. Fukuda (1989) reported a value of $F_{is} = 0.23$ from a chromosome analysis of 10 populations of *T. erectum*. No range is given for this value, and it falls just outside the 95% CI for our estimate of F_{is} (0.26–0.55). Thus, with the exception of Irwin's (2000) study, the evidence suggests that significant inbreeding occurs in most *T. erectum* populations (and see below).

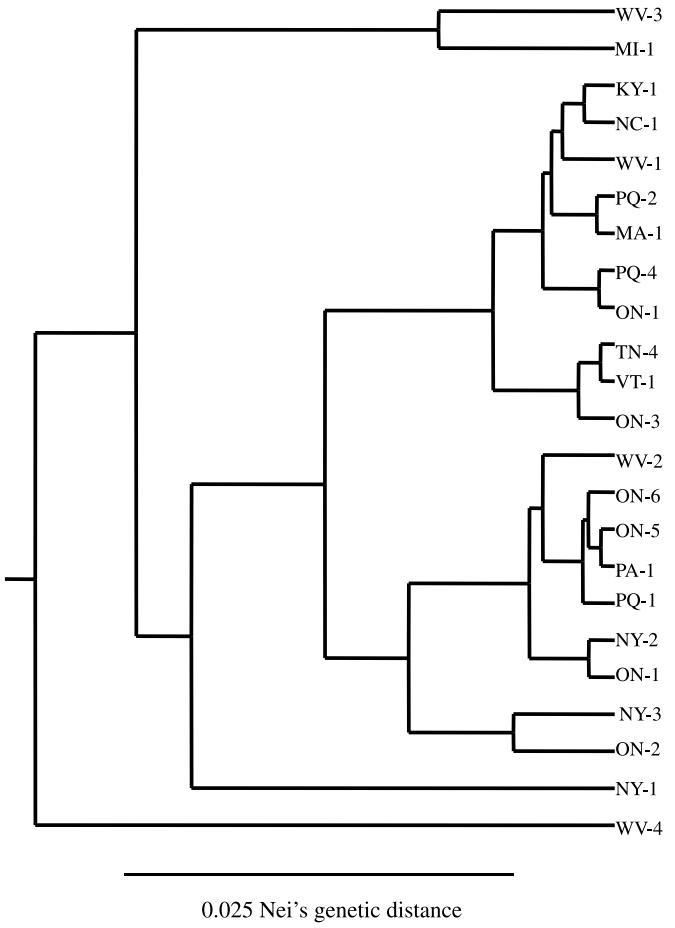
How do the patterns of genetic variation in *T. erectum* compare with those in other *Trillium* species and with angiosperm species in general? Unfortunately, few species of *Trillium* have been examined for allozyme diversity, so comparisons are limited. A geographical survey of *T. grandiflorum*, a predominantly outcrossing species (Broyles et al. 1997; Kalisz et al. 1999; Sage et al. 2001) with a similar range to *T. erectum*, found similar overall levels of genetic diversity but less inbreeding (Griffin and Barrett 2004). In contrast, *T. erectum* has considerably more diversity at allozyme loci than *Trillium nivale* (Bayer et al. 1987) and *Trillium sessile* (Whitkus et al. 1987), two species with much smaller geographical ranges.

In a survey of over 1400 studies of plant allozyme diversity, Hamrick and Godt (1996) identified mating system and geographic range as important correlates of the levels of genetic variation. Considering both its wide range in eastern North America and mixed mating system, *T. erectum* shows somewhat greater than average genetic variation. For example, $A = 1.20$ in *T. erectum* versus 1.12 for mixed-mating angiosperms (Hamrick and Godt 1996). Additionally, mating system was the principal determinant of population genetic structure (Hamrick and Godt 1996). In this regard, *T. erectum* exhibits lower F_{st} but higher F_{is} values than other plant species with mixed mating systems.

Higher than expected levels of inbreeding in *T. erectum* may be associated with the pollination biology and demography of the species. Flies and beetles pollinate *T. erectum*, and visitation rates are generally very low (Irwin 2000; S.R. Griffin and S.C.H. Barrett, unpublished data). Pollen dispersal by these pollinators is likely restricted in addition to the obvious reductions caused by self-fertilization. The densities of plants within populations are low throughout the range of *T. erectum*, particularly in comparison with *T. grandiflorum* (S.R. Griffin, personal observation), and thus the capacity for self-fertilization may provide some reproductive assurance. Restricted pollen dispersal and low plant density seem likely to limit opportunities for outcrossing and consequently contribute to high levels of F_{is}.

Our analysis of population structure in *T. erectum* revealed considerable population differentiation at allozyme loci. How-

Fig. 2. UPGMA (unweighted pair group method with arithmetic mean) dendrogram of allozyme variation in 23 populations of *Trillium erectum*. The letters of each name refer to the province or state of the population sampled; see Table 1 and Fig. 1 for population locations.



ever, there was no clear geographical clustering of populations in the UPGMA analysis (Fig. 1) or evidence for isolation by distance. However, the scale of our sampling may have been too large to detect localized patterns of isolation by distance. Contemporary gene flow between the populations in our sample seems unlikely, since even the closest pair of populations was separated by at least 50 km. Thus, a smaller scale study of *T. erectum* might reveal different patterns of population structure and evidence for contemporary gene flow. At least some of the genetic differentiation that we observed in our study seems likely to have arisen during postglacial migration, perhaps from multiple refugia, and this may have prevented us from detecting isolation by distance.

Our inability to detect isolation by distance could also be explained by the overall rarity of contemporary gene flow among populations. Given that there is likely to be restricted pollen dispersal within populations, pollen dispersal among populations should be even more limited. *Trillium* seeds are adapted for ant dispersal (Gates 1941; Case and Case 1997; Kalisz et al. 1999), and this mechanism seems unlikely to be very effective in promoting gene flow among populations. However, other mechanisms could conceivably contribute to long-distance dispersal. For example, Vellend et al. (2003) recently showed that the seeds of *Trillium grandiflorum* can be dispersed long distances via ingestion and defecation by white-tailed deer, and there is molecular evidence to support the role of long-distance dispersal in this species (Griffin and Barrett 2004). Nonetheless, long-distance seed dispersal leading to establishment is likely a very rare event, and limited opportunities for pollen and seed dispersal in *T. erectum* may explain the apparently restricted gene flow among populations.

Table 2. Spearman correlation coefficients of genetic variation and latitude showing two analyses: in the first, all populations are included; in the second, population MI-1 was removed.

	All populations (N = 23)		Outlier (MI-1) excluded (N = 22)	
Diversity measure	r	P	r	P
A	–0.39	0.063	–0.44	0.039
H_o	–0.18	0.400	–0.19	0.400
H_e	–0.41	0.055	–0.45	0.034

Note: A, effective number of alleles per population; H_o, observed heterozygosity per population; H_e, expected heterozygosity per population.

Decreased genetic variation in derived populations following postglacial recolonization is expected from migration models with restricted gene flow (Ibrahim et al. 1995). In eastern North America, it is likely that most forest herbs migrated northwards from southern refugia, although there is surprisingly limited genetic evidence to support this assumption. We detected a weak trend towards decreased allozyme variation in northern compared with southern populations of *T. erectum*. However, this trend was only statistically significant after removal of the outlier population, MI-1 (Table 2). This small population occupies a disjunct fragment of the range on the eastern shore of Lake Michigan (see Case and Case 1997). It might be expected that such peripheral populations would show decreased diversity. Paradoxically, MI-1 displayed some of the highest levels of allozyme variation of all the populations that we sampled in our study (Table 1).

The absence of a strong historical signal in our geographical survey of genetic variation, in contrast with several previous studies (e.g., Schwaegerle and Schaal 1979; Loveless and Hamrick 1988; Dolan 1994; Lewis and Crawford 1995, Broyles 1998), may reflect the limited role that long-distance dispersal has played in the northward migration of *T. erectum*. Pervasive contemporary gene flow homogenizing the allelic diversity of populations seems an unlikely explanation, given the reproductive biology of the species. Future studies of the geographical patterns of genetic diversity in the guild of widespread forest herbs of eastern North America that often co-occur with *T. erectum* (e.g., *Aralia nudicaulis* L., *Asarum canadense* L., *Clintonia borealis* (Ait.) Raf., *Maianthemum canadense* Desf.) would be valuable in assessing the role of historical and contemporary forces in structuring genetic diversity.

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