

Genetic Structure in a Heterocyanic Population of *Trillium sessile* (Liliaceae)

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Abstract Species of *Trillium* in the subgenus *Phyllantherum* are either polymorphic for flower color, or monomorphic for flower color and related to a polymorphic species. This leads to the suggestion that polymorphic species may be the progenitors for monomorphic ones. For this to be true, it must be demonstrated that genetic divergence among flower morphs can occur within polymorphic populations. Genetic structure was assessed in a population of *T. sessile* that contains a polymorphism for flower color. A survey of 11 enzyme systems using starch gel electrophoresis revealed three polymorphic loci: 6PGD-1, AAT-1 and AAT-2. Analysis of large and small scale spatial structure, stage classes, and flower color classes revealed significant genetic divergence in all instances. Spatial structure in the population is likely a result of genetic neighborhoods which can maintain populational variation via random genetic drift. Genetic divergence of the yellow flower color morph was probably initiated through genetic drift since the morph occurs in low frequencies. The results imply that the initial genetic divergence of species in the subgenus can arise within polymorphic populations.

Key words: allozyme, flower polymorphism, genetic structure, *Trillium sessile*.

In the subgenus *Phyllantherum* of the genus *Trillium*, nine species are recognized in which floral color polymorphism exist (Freeman, 1975). Within each species, flower color morphs have been recognized taxonomically such as *T. sessile* f. *viridiflorum* for the yellow-petalled morph of this predominately red-petalled species. In addition, four pairs of morphologically similar, presumed sister species are distinguished by flower color, with one species displaying a polymorphism, whereas the related species is monomorphic. Thus, *T. cuneatum* ranges from purple to yellow in flower color, while the related *T. luteum* is predominately yellow (Freeman, 1975).

Although conspicuous polymorphisms are not considered to be typical of the variation on which evolution depends (Wright, 1978), it is tempting to deduce from the above observations that flower color polymorphism within species of *Trillium* can become the basis for species differences. To strengthen this hypothesis, genetic divergence among flower color morphs within a polymorphic population

would have to be demonstrated.

We have studied a population of *Trillium sessile* with a polymorphism for flower color that results from the differential expression of anthocyanin pigment in the petals, giving rise to three phenotypic classes: red, intermediate and yellow-petalled flowers. We attribute these heterocyanic differences to the action of one gene with incomplete dominance acting at the locus that regulates the conversion of dihydroflavonol precursors into anthocyanin pigments (Bryan et al., 1985; Les et al., unpublished). Although genetic investigations of flower color have shown the existence of serial gene systems and epistasis (Grant, 1975), presence or absence of a particular floral pigment is usually a one-gene difference (Ennos and Clegg, 1983; Epling et al., 1960; Kamsteeg et al., 1980). Using a model of a single gene system, we showed that the phenotypic classes in the population deviate significantly from Hardy-Weinberg equilibrium and that the deviation was attributable to several parameters of selection (Bryan et al. 1985; Les et al., unpublished).

If differential selection acts upon the flower color morphs, there should be evidence of genetic differentiation as well. We initiated the present study to determine whether differentiation exists in the population for isozyme genotypes and gene frequencies, and if so, to ascertain how the variation is apportioned.

Materials and Methods

The study population is located on an 11 hectare woodlot on the northwest corner of The Ohio State University campus in Columbus. A complete description and history of the area are given by Horn (1985). Plants polymorphic for flower color are restricted to a portion of the woods bounded on the east and south by footpaths and on the north and west by an area where *Trillium* plants are absent for a distance of several meters. The study area extends 35 meters on a side and includes over 18,000 individuals with 93.8% reds, 5.1% intermediates and 1.1% yellows.

The population was sampled for spatial, stage class and flower color class components of enzyme polymorphism. Two sampling methods were employed to assess spatial polymorphism. For large scale structure (across entire population), a 0.5 × 35 meter N-S transect was established and 0.5 × 8 meter sections were located at three intervals. The three sections were chosen to approximately equalize the number of plants in each section and exclude large areas with no plants. To assess small scale structure a 2 × 2 meter plot was established in an area of high plant density (38 plants/m²) and divided into four 1 m² plots. Additionally, the 35 meter transect was divided into six pairs of adjacent 0.5 × 2 meter sections. Since gene flow distance in herbs is generally on the order of 1 to 4 meters (Levin, 1981) the establishment of 1 m² plots should adequately detect small scale structure. All plants were sampled in each plot.

Plants collected in the spatial sampling were scored for stage class (flowering or large non-flowering three-leaved plants = adult, single-leaved or small three-leaved plants = juvenile) and flower color class (red, intermediate). Due to their rarity in the population, no yellow-petalled morphs occurred in the spatial sample plots, so a random sample of 27 yellow morphs was taken from areas adjacent to the plots. In total, 413 plants were sampled and used in 11 comparisons: A. small scale. i) four 1 m² plots in 2 × 2 meter plot (comparison 1); ii) six pairs of adja-

cent 0.5 × 2 meter transect sections (comparisons 2-7); B. large scale. i) three 0.5 × 8 meter transect sections (comparison 8); ii) three transect sections and 2 × 2 meter plot (comparison 9); C. stage class (comparison 10) and D. flower color class (comparison 11).

Leaf samples for electrophoresis were collected from each plant in the field and transported to the laboratory on ice and stored at -60°C. Material was ground in Gottlieb's (1981a) extracting buffer and PVP to make a thick slurry. Samples were centrifuged for 2 minutes and the supernatant blotted onto paper wicks for application to starch gels. Samples were run on 12% starch gels using two buffer systems. The lithium borate system (pH 8.3; Gottlieb, 1981a) was used for the enzyme systems ADH (EC 1.1.1.1), GDH (EC 1.4.1.2), AAT (EC 2.6.1.1), PGI (EC 5.3.1.9), and TPI (EC 5.3.1.1). A histidine citrate system (pH 6.5, Cardy et al., 1981) was used for the systems 6PGD (EC 1.1.1.44), IDH (EC 1.1.1.42), MDH (EC 1.1.1.37), PGM (EC 5.4.2.2), SKDH (EC 1.1.1.25) and SOD (EC 1.15.1.1). Gels using the lithium borate system were run at 75 milliamps and those using the histidine citrate system at 250 volts. Gel were run until a bromphenol blue marker reached the opposite wick. After staining, gels were scored when the bands were most intense. Genetic interpretation of the banding patterns were inferred from the known isozyme number for diploid plants and the active subunit structure for these enzymes (Crawford, 1983; Gottlieb, 1981b, 1982).

Statistical analyses performed to investigate population structure were: i) a chi-square analysis of deviations of genotypic frequencies from Hardy-Weinberg expectations within samples; ii) a chi-square contingency analysis of heterogeneity of gene frequencies among samples; iii) the F-statistics of Wright (1951, 1965). Expected genotypic arrays of samples were obtained from Levene's (1949) formula for exact distributions to accommodate small sample size. Sums of observed and expected frequencies were made across samples and a chi-square value obtained for each polymorphic locus. Degrees of freedom for a comparison were calculated as the number of genotypic classes minus the number of alleles.

Chi-square contingency values were computed using the formula of Snedecor and Irwin (1933), with degrees of freedom equal to the number of alleles minus 1 times the number of samples minus 1. Workman and Niswander (1970) have shown that

the chi-square contingency value provides a significance test for one of the F-statistics (i.e., F_{ST}).

Wright's (1951, 1965) F-statistics can be interpreted as measures of deviations from values expected under Hardy-Weinberg equilibrium within subpopulations (or samples; F_{IS}), between subpopulations (or samples; F_{ST}), or within the total population (over all samples; F_{IT}). F_{ST} can also be viewed as a measure of genetic divergence among subpopulations and F_{IS} a measure of inbreeding within subpopulations (Wright, 1965). Values of F_{IS} and F_{IT} are negative if an excess of heterozygotes is present, and F_{ST} is positive and approaches 1 if subpopulations are fixed for different alleles. The three statistics are related to one another as $1 - F_{IT} = (1 - F_{IS})(1 - F_{ST})$. From this formula, the relative contribution of F_{IS} and F_{ST} to F_{IT} can be assessed. F-statistics for the present study were estimated using formulae 1-4 and 10 of Weir and Cockerham (1984).

Results

From the eleven enzyme systems assayed, the pro-

ducts of 19 presumable genetic loci were consistently recorded. Of these, three loci were polymorphic at the 95% level: 6PGD-1 with population frequencies of 0.161, 0.103, 0.575, 0.149, and 0.012 for alleles *a* through *e*, respectively; AAT-1 with frequencies of 0.738 and 0.262 for alleles *a* and *b*, respectively, and AAT-2 with alleles *a* and *b* at an average frequency of 0.716 and 0.284, respectively. Variational patterns from these loci were evident at all levels of population structure.

Small scale spatial structure is evident from analysis of gene frequency heterogeneity and F-statistics (Table 1 and 2). Additionally, in three of the seven comparisons, significant deviations from Hardy-Weinberg expected genotypic frequencies are obtained. For the AAT-1 locus, this is due to a deficiency of heterozygotes (caused by inbreeding within subpopulations) as evidenced by the large positive F_{IS} values (see comparisons 1, 5 and 6). Significant heterogeneity for gene frequencies at the 6PGD-1 and AAT-1 loci, as well as over all three loci, are obtained in four of the seven comparisons. These data indicate that F_{ST} values for these comparisons are significant as well (negative values of estimates of F_{ST}

Table 1. Chi-square values of within sample genotypic frequency deviations from Hardy-Weinberg expectations and chi-square contingency values of gene frequency heterogeneity among samples. Numbers in parentheses below reported values indicate degrees of freedom.

Comparison	Genotypic Frequencies			Gene Frequencies			Total
	6PGD-1	AAT-1	AAT-2	6PGD-1	AAT-1	AAT-2	
Small scale 1 (n=152)	3.50 (1)	15.86*** (1)	1.36 (1)	18.93 (12)	28.35*** (3)	4.96 (3)	52.24*** (18)
2 (n=23)	3.53 (1)	0.09 (1)	0.41 (1)	2.52 (2)	0.60 (1)	0.05 (1)	3.17 (4)
3 (n=55)	1.20 (2)	0.00 (1)	0.85 (1)	12.10** (3)	5.05* (1)	0.18 (1)	17.33*** (5)
4 (n=65)	1.67 (4)	0.00 (1)	0.09 (1)	2.88 (4)	0.00 (1)	3.00 (1)	5.88 (6)
5 (n=28)	0.89 (2)	15.77*** (1)	3.39 (1)	7.59 (3)	0.04 (1)	3.57 (1)	11.20* (5)
6 (n=22)	0.33 (1)	8.16*** (1)	0.05 (1)	6.27* (2)	0.49 (1)	0.26 (1)	7.02 (4)
7 (n=21)	1.78 (1)	2.02 (1)	3.74 (1)	21.29*** (3)	1.20 (1)	0.22 (1)	22.71*** (5)
Large scale 8 (n=166)	3.32 (1)	9.50*** (1)	1.39 (1)	89.07*** (8)	73.90*** (2)	2.45 (2)	165.42*** (12)
9 (n=318)	4.84* (1)	28.05*** (1)	2.70 (1)	196.25*** (12)	119.09*** (3)	7.60 (3)	322.94*** (18)
Stage class 10 (n=413)	6.99 (7)	98.77*** (1)	0.47 (1)	22.68*** (4)	2.52 (1)	0.35 (1)	25.55*** (6)
Color class 11 (n=323)	1.23 (1)	64.18*** (1)	0.32 (1)	87.47*** (8)	16.52*** (2)	33.71*** (2)	137.70*** (12)

* $P < 0.05$; ** $P < 0.01$; *** $P < 0.005$

Table 2. F-statistics for individual loci and over all loci.

Comparison		Locus	F_{IT}	F_{ST}	F_{IS}	
Small scale	1	6PGD-1	-0.074	0.008	-0.083	
		AAT-1	0.529	0.120	0.465	
		AAT-2	-0.105	0.006	-0.112	
		all	0.087	0.039	0.050	
	2	6PGD-1	-0.327	-0.013	-0.310	
		AAT-1	-0.148	-0.048	-0.095	
		AAT-2	-0.179	-0.038	-0.136	
		all	-0.360	-0.113	-0.222	
	3	6PGD-1	-0.010	0.037	-0.048	
		AAT-1	0.020	0.039	-0.020	
		AAT-2	-0.136	-0.025	-0.065	
		all	-0.039	0.025	-0.065	
	4	6PGD-1	-0.092	0.004	-0.096	
		AAT-1	0.000	0.000	0.000	
		AAT-2	-0.025	0.008	-0.033	
		all	-0.076	0.005	-0.081	
	5	6PGD-1	-0.034	-0.009	-0.025	
		AAT-1	0.603	-0.062	0.626	
		AAT-2	0.359	0.014	0.349	
		all	0.112	-0.013	0.123	
	6	6PGD-1	0.113	0.025	0.090	
		AAT-1	0.702	-0.093	0.727	
		AAT-2	-0.089	-0.040	-0.047	
		all	0.212	-0.014	0.223	
	7	6PGD-1	0.469	0.054	0.439	
		AAT-1	0.236	-0.035	0.262	
		AAT-2	-0.435	-0.023	-0.403	
		all	0.241	0.026	0.222	
Large scale	8	6PGD-1	0.048	0.067	-0.018	
		AAT-1	0.482	0.216	0.339	
		AAT-2	-0.090	-0.001	-0.089	
		all	0.069	0.069	0.000	
	9	6PGD-1	0.089	0.105	-0.018	
		AAT-1	0.600	0.222	0.486	
		AAT-2	-0.089	0.007	-0.096	
		all	0.154	0.108	0.051	
	Stage class	10	6PGD-1	0.036	0.005	0.032
			AAT-1	0.566	-0.002	0.567
			AAT-2	-0.039	-0.003	-0.036
			all	0.123	0.002	0.121
Color class	11	6PGD-1	0.089	0.112	-0.026	
		AAT-1	0.557	0.072	0.523	
		AAT-2	0.066	0.124	-0.066	
		all	0.174	0.107	0.075	

indicate true values are close to or actually zero). In all comparisons, however, F_{IS} values over all loci contribute most to the total within sample deviation from Hardy-Weinberg equilibrium (Table 2).

Similar results are seen for the large scale comparisons. The AAT-1 locus again provides large and significant values for genotypic frequencies (Table 1), and is accompanied by a large positive F_{IS} value (Table 2), again indicting a deficiency of heterozygotes. Both the AAT-1 and 6PGD-1 loci produce significant gene frequency heterogeneity (Table 1), indicating significant divergence among the three plots in the transect and between the transect and 4 m² plot. The values of F_{ST} over all loci show most of the deviation from random mating in the population comes from differences among samples rather than within (Table 2).

In the stage class comparison, genotypic frequencies for the AAT-1 locus are significantly different from equilibrium values. Unlike the other large sample comparisons, there is no significant heterogeneity of gene frequencies at the AAT-1 locus among the stage classes (Table 1) and it is reflected in the small F_{ST} value. However, divergence between the stage classes is taking place, as evidenced by the significant differences in the frequency of 6PGD-1 alleles (Table 1).

In the flower color class comparison, the AAT-1 locus again provides a significant value for the genotype frequencies, while all three loci show significant amounts of genetic divergence among the color classes (Table 1). Although most of the F_{IT} value over all loci comes from the contribution of F_{ST} , 41% of the total is contributed by F_{IS} (Table 2).

Discussion

The low percentage (15.8%) of polymorphic loci found in this population concurs with the conclusion of Fukuda (1987) that populations of North American *Trillium* species are predominately self-pollinating. However, within this population, the limited amount of variation is expressed at all levels of population structure. The most likely source of this variation may be the presence of genetic neighborhoods. Although our sampling was not oriented for estimating neighborhood size, it is likely our samples picked up differences in neighborhoods. Using total heterogeneity values in the small sample comparisons, a rough estimate of neighborhood size may be made. The smallest sample size with a significant value is comparison 7 with $n=21$, while

the largest non-significant sample is comparison 4 with $n=65$. Therefore, as a first estimate, genetic neighborhood sizes may fall somewhere within this range. As noted by Wright (1946), populations with neighborhoods on the order of less than 100 can produce and maintain a substantial amount of local differentiation via random genetic drift. Although there is significant divergence among samples in these comparisons, F_{ST} values over all loci range from 10 to 44% of their corresponding F_{IT} values. A similar situation was seen by Schaal (1975) in a population of *Liatris cylindracea* where F_{ST} values were relatively small in comparison to F_{IT} values, but they nonetheless represented significant divergence among samples in the population.

The observed spatial variation may have a hierarchical component. In the large scale comparison, values of F_{ST} over all loci represent 68 to 100% of their corresponding F_{IT} values with a corresponding decrease in F_{IS} (Table 2). Therefore, increasing sampling area results in a concomitant increase in divergence and decrease in inbreeding effects. This would result if a population consisted of neighborhoods more similar to those close by than to more distant ones. The alternative condition of neighborhoods equally diverged no matter what distance from each other would not produce the results obtained here. In that instance, comparing large areas of the population to one another should homogenize the differences between their included groups of neighborhoods and thus result in lowering the values of F_{ST} .

The stage class comparison is the only large-sample comparison where the AAT-1 locus does not produce a significant heterogeneity value. Thus whatever force is acting on the population to reduce the frequency of heterozygotes, it is acting equally between the stage classes. This result could be obtained if the juvenile stage class consisted of both juveniles and adults that had reverted to the single-leaf juvenile stage. Several plant species are known to grow vegetatively for a season to store up energy for the next bout of flowering (Harper, 1977), and sterile adults of *T. sessile* remain in the three leaf stage class (Kawano et al., 1986). Therefore it was not expected that the juvenile class represented a mixture. This was checked in our population by inspecting rhizomes on large, non-flowering individuals, flowering individuals, and small, non-flowering individuals. In all cases, rhizomes of large non-flowering individuals and flowering individuals are noticeably larger and contain more rings or constrictions.

tions, which may be used to approximate the age of *Trillium* plants (Brandt, 1916; Davis, 1981; Nesom and LaDuke, 1985), than single-leaf or small, three-leaved individuals (unpublished). We can conclude our stage classes represent a minimum of two generation classes that show no generation to generation change in gene frequency for the AAT loci. The 6PGD-1 locus, however, indicates a change in gene frequency between the stage classes has occurred. Either the alleles have not yet reached equilibrium values, or the presence of five alleles at this locus makes it a more sensitive indicator of random generation to generation fluctuation in gene frequencies. Since the F_{ST} value of the locus is close to zero, the latter situation appears more likely.

The color class comparison is the only one in which all three loci produce significant heterogeneity. Values of F_{ST} for individual loci as well as over all three loci are large, with the value over all three loci being 59% of the F_{IT} value. Since the red and intermediate morphs come from the spatial sample, it is likely that some part of the amount represents the spatial variation. However, neither of the two large-scale spatial comparisons produces a significant heterogeneity value for the AAT-2 locus and so some additional variation is unique to the color classes. Since the red and yellow morphs have different frequencies of alleles between them, they are genetically differentiated, yet they still share the same genotypes at all three loci. Although the yellow morph occurs in low frequency in the population, it has a greater selective advantage in being preferentially pollinated over the red morph, produces significantly larger ovules and more ovules per ovary (Bryan et al., 1985; Les et al., unpublished). Low numbers indicate this morph represents a small sample of the population gene pool, suggesting genetic drift may account for the initial genetic divergence of the yellow-petalled morph. Being selectively favored, the yellow morph should increase in frequency and cross more often with the red morph, resulting in the two morphs eventually sharing similar genotypes and gene frequencies. However, if the yellow morph were to become spatially separated from the red morph, a new population of yellow-petalled individuals would arise that was genetically diverged from the parental population through simple genetic drift. Long periods of isolation could then drive divergence to the point where the two morphs become reproductively isolated.

We have shown that a population of *T. sessile* is

genetically subdivided, both spatially and with regards to flower color. The spatial substructure likely represents the presence of genetic neighborhoods that can maintain genetic variation within the population via drift (Wright, 1946, 1951). Genetic differentiation between the red and yellow flower morphs has probably arisen by drift as well and is expected to eventually disappear. However, we have demonstrated the possibility that flower color polymorphisms in the subgenus *Phyllantherum* can be the basis for species differences since the initial genetic divergence arises within a polymorphic population. A more extensive survey of isozymes in the subgenus would be needed to see if those species monomorphic for flower color are genetically similar to their related, polymorphic species.

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