GENETIC VARIATION IN THE TERRESTRIAL ORCHID *TIPULARIA DISCOLOR*

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ABSTRACT — *Tipularia discolor* is a terrestrial woodland orchid of the eastern United States that exhibits variable spatial patterns. The genetic diversity of several populations in Maryland, Delaware, and Virginia was examined using ISSR markers. Low levels of genetic variation were detected, yet four distinct genotypes were determined. A Mantel test revealed that genetic diversity did not correlate with spatial distance (Mantel t-test = -0.349; p = 0.364). An Analysis of Molecular Variance (AMOVA) showed genetic differences between and within populations (p = <0.01). Our data suggest that gene flow has occurred between the four populations of *Tipularia discolor*, although field studies have documented that populations are maintained through clonal growth. This study illustrates that ISSR markers can be used to detect genetic diversity at a population level.

INTRODUCTION

Tipularia discolor (Pursh) Nutt. (Fig. 1), commonly known as the Crane-fly Orchid, is a wintergreen orchid native to the Eastern United States that occupies a singular niche in the understory of successional or mature forests. It is found from Massachusetts west to Michigan and south to Texas and Florida (Brown 1997). The green leaves, which have a bright purple underside, are present in the winter and absent in the summer. This wintergreen strategy is shared with another temperate orchid species, *Aplectrum hyemale* (Muhl.) Torr., which flowers with the spring ephemeral species. *Tipularia discolor*, however, delays its flowering until mid- to late summer when few other woodland species are in bloom.

Tipularia discolor is the only North American representative of the genus, which contains four disjunct species. The remaining members of the genus, *T. josephi* Rchb. f. ex Lindl., *T. japonica* Matsum., and *T. camtschatica* Spreng., are all found in Asia. The advanced floral features and diminished vegetative characters of *Tipularia* Nutt. make this group of terrestrial orchids difficult to classify. However, the classification of the genus *Tipularia* as a member of the subfamily Epidendroideae, tribe Calypsoeae, is supported by both morphological

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(Dressler 1993) and genetic evidence (Cameron et al. 1999). *Tipularia* is clearly a member of the Epidendroideae, yet it is one of four genera that represent a reversal from an epiphytic habit (Neyland and Urbatsch 1995).

Its unique pattern of development has made *T. discolor* the subject of many studies. Most investigations have focused on this species' reproductive biology (Coke 1990; Efird 1987; Neiland and Wilcock 1998; Rasmussen 1995; Rasmussen and Whigham 1993, 1998a, 1998b; Snow and Whigham 1989; Stoutamire 1978; Whigham and McWethy 1980; Whigham and O'Neill 1991), physiology (Tissue et al. 1995, Whigham 1984), and ecology (Whigham 1989, Whigham and O'Neill 1988, Zimmerman and Whigham 1992), while only one has focused on its population biology (Frye 1993).

As a terrestrial woodland herb, *T. discolor* often germinates on decomposing wood and may have more than one fungal symbiont unique to different life stages (Rasmussen and Whigham 1998a). Individuals typically consist of one to five shallow-rooted corms and one leaf, which emerges in the early fall (Efird 1987, Snow and Whigham 1998, Whigham 1984). *Tipularia discolor* is photosynthetic over a wide range of conditions, as the wintergreen leaves are present during conditions of relatively high light and low temperatures (Tissue et al. 1995). The leaves senesce in spring and an inflorescence of 20 to 30 flowers appears in summer. *Tipularia discolor* is unique

Figure 1. *Tipularia discolor*. Left, leaves in winter. Right, flowers in summer (no leaves present during flowering). Photographs by Julie Smith.

from all other North American orchids in its asymmetric flowers that allow placement of pollinia on the compound eyes of Noctuid moths. If the flowers were symmetric, as in other temperate orchid species, the pollen masses would align only with body parts covered with scales, to which the pollinia cannot adhere (Stoutamire 1978). Though nectar-rewarding, it flowers at a time when pollinator resources are limited in the herb layer (Neiland and Wilcock 1998, Whigham and McWethy 1980). *Tipularia discolor* is also self-compatible, although selfing appears to be rare (Whigham and McWethy 1980). Whigham and O'Neill (1988) found no seedling recruitment near mature plants, though high germination rates and seedling survival were observed using aseptic germination techniques in a lab setting (Coke 1990).

Clonal propagation in *T. discolor* can occur by fragmentation and branching of the corm system (Efird 1987). In general, corms are retained year after year, with only the newest corm producing leaves and flowers. However, older corms can initiate new leaves and corms if severed from the rest of the plant. In addition, branching can occur when two new corms are produced in the same year. It has been estimated that 10% of *T. discolor* plants undergo branching annually (Efird 1987). Since the longevity of individual corms has not been determined, it is reasonable to assume that clusters of seemingly distinct plants may actually be clones. Allozyme studies on several populations showed each dense cluster was a distinct genet (Frye 1993).

Molecular markers are useful in studies of clonality (Camacho and Liston 2001, Mayes et al. 1998, Parker and Hamrick 1992, Parks and Werth 1993, Peakall and Beattie 1991). Markers that are generally used in population studies are allozymes, isozymes, random amplified polymorphic DNA (RAPD), and, more recently, inter-simple sequence repeat (ISSR) techniques. ISSR markers are generated from single primer PCR reactions, much like RAPD markers. The longer primers used with ISSR result in more stringent annealing temperatures and increased reproducibility (Wolfe and Liston 1998). The hypervariability of ISSR markers enables small differences to be detected, even at the subspecies level (Wolfe et al. 1998).

Populations of *T. discolor* exhibit variable spatial patterns, ranging from dense clusters of plants to widely spaced populations where individuals are several meters apart. It was the goal of this study to examine the genetic diversity of several populations using ISSR markers and to determine what relationship exists between geographic location and genetic diversity.

METHODS

We studied four populations of *T. discolor* in Maryland, Delaware, and Virginia (Table 1). One, in Salisbury, MD consisted of three subpopulations of densely clustered plants. Ten individuals were sampled from each of the three apparently clonal clusters. Seven to ten individuals were also sampled from more widely spaced populations in Felton, DE; Edgewater, MD; and Nethers, VA. Partial leaf samples were taken from each individual, a non-destructive sampling method. Total genomic DNA was extracted from fresh leaves using the method of Doyle and Doyle (1989), with the volume of the extraction increased.

DNA amplification was performed in a Thermolyne Temp-Tronic (model #DB66925) with 35 cycles of 30 s at 94ºC, 30 s at 55ºC, and 30 s at 72º C. Amplification was in 25 FL volumes consisting of 8 µl of 25 mM $MgCl_2$, 0.5 µl dNTP, 1.5 µl primer, 0.2 µl taq, 0.5 - 1.0 µl DNA, and 9.8 - 10.3 µl distilled water. ISSR primers were obtained from the University of British Columbia Biotechnology Laboratory. Sixty primers were scanned for their ability to amplify with multiple samples of *T. discolor*. Four of the primers produced interpretable and consistent banding patterns (UBC-840, $[GA]_8YT$; UBC-846, $[CA]_8RT$; UBC-858, $[TG]_8RT$; UBC-861, $[ACC]_6$).

PCR reactions were characterized on a 1.5 % agarose gel in 1X TBE buffer. ISSR bands, stained with ethidium bromide, were visualized on a UV transilluminator, documented digitally, and analyzed

Table 1. Locations of *Tipularia discolor* used in this study.

Site Name	Latitude	Longitude	State	County	Samples
Salisbury	38°20.843'N 38°20.925'N 38°21.106'N	75°38.503'W 75°38.219'W 75°38.569'W	MD	Wicomico	30
Felton	38°43'N	75°05'W	DE	Kent	10
Edgewater	38°53'N	76°33'W	MD	Anne Arundel	9
Nethers	38°34'N	78°17'W	VA	Madison	7

Table 2. Measures of genetic diversity in four populations of *Tipularia discolor.*

Site	Genotype	% polymorphic loci	Gene diversity (*h*)
Salisbury, MD	A & B	4.5	0.0217
Felton, DE	C & D	18.2	0.0687
Edgewater, MD	C & A	9.1	0.0333
Nethers, VA	A	0.00	0.0000

using the BioMax 1D image analysis software (Eastman Kodak Company). Fragment sizes were estimated based on 1-kb ladder standards according to the algorithm provided in the BioMax 1D software. ISSR markers are inherited in a dominant or codominant Mendelian fashion (Gupta et al. 1994). For data analyses, they were interpreted as dominant markers and were scored as diallelic with band present or absent. Allele frequency, percentage of polymorphic loci, and Nei's gene diversity were calculated using Tools for Population Genetic Analysis (TFPGA) 1.3 (Miller 1997) and POPGENE 1.2 (Yeh et al. 1997). Analysis of Molecular Variance (AMOVA) was performed using AMOVA 1.55 to detect variation within and among populations (Excoffier et al. 1992). Finally, NTSYSpc 2.1 was used to generate a Neighbor Joining tree using the Jaccard coefficient, as well as a Mantel test to determine the correlation between geographic distance and genetic diversity (Rohlf 2000).

RESULTS

For the 56 samples the four primers produced 22 scorable bands, five of which were polymorphic. Low levels of genetic variation were detected, yet four distinct genotypes were determined. Population level genetic diversity statistics are summarized in Table 2.

Genotype A occurred in the Salisbury, Edgewater, and Nethers populations with a maximum distance between the sites of 256 km. Genotype C was found in the Felton and Edgewater populations with a distance between the sites of 142 km. Genotypes B and D occurred at one site each. A Mantel test was employed to test for correlations between the matrix of genetic diversity and the spatial distance between populations (Mantel t-test = -0.349; p = 0.364). Genetic diversity did not correlate with spatial distance.

The AMOVA analysis showed genetic differences between and within populations (Table 3). Although the genetic diversity values are low overall, there was detectable variability across the species' range and within individual populations. However, the Neighbor Joining tree did not segregate the individuals by populations (not shown).

Table 3. Analysis of Molecular Variance (AMOVA) for 56 individuals in 4 populations of *Tipularia discolor* using 22 ISSR markers.

Source of variation	*df*	Variance component	%Total variance	P-value	Bartlett's statistic
Among populations	3	0.28	41.50	<0.0099	0.415
Within populations	52	0.40	58.50	<0.0099	

At the Salisbury site where three subpopulations were sampled, some structure was observed. Subpopulation 1 occurred over a 0.36 m^2 area, and was comprised of eight individuals of genotype A and two of B. Subpopulation 2 consisted of 10 genotype A individuals, on a similar scale. Subpopulation 3 was composed of one genotype A individual and nine genotype B individuals within 0.16 m^2.

DISCUSSION

This study is the first to utilize ISSR markers to characterize genetic variation in *T. discolor*. There were multiple genotypes in three populations, while the Nethers, VA, population was monomorphic. Two unique genotypes were found, one in Salisbury, MD and the other in Felton, DE. Significant variation was found among and within populations (AMOVA), although the overall genetic diversity was low. The only other population genetic study of *T. discolor* was conducted by Frye (1993). This study encompassed populations in Virginia, North Carolina, and Massachusetts and spanned a distance of 965 km. These populations represented three different habitats occupied by *T. discolor*: piedmont, mountain, and island. Frye's (1993) allozyme study of *T. discolor* revealed high genetic variation within populations, but little difference between populations. Frye reported mean genetic identity of 0.986, mean genetic distance of 0.076, and 0.75% polymorphic loci at all three habitats. Our study revealed low population level variation, and similar levels of variation between populations. An observable difference between our study and Frye's (1993) is that our study shows lower population level variation. This difference may be the result of three factors. The Frye (1993) study encompassed a large geographic range, while this study sampled within the midrange of the distribution and had a maximum distance between populations of 308.5 km. There could also be a sampling effect as Frye sampled every member of a population while we subsampled the populations. Lastly, increased genetic diversity might have been detected in our study with the use of more polymorphic primers.

On a landscape scale, there was no correlation between geographic and genetic distance, which indicates gene flow has occurred. However, it is unclear when the gene flow occurred. The patterns that are seen now may be remnants of a larger historical distribution. If clonal reproduction is important for population maintenance (Whigham and O'Neill 1988), variation may take a long time to accumulate in populations that are now well separated. However, seed dispersal is evident as new plants are found on top of decaying stumps, which cannot be the result of corm fragmentation (Rasmussen and Whigham 1998b; pers. observ.). As a fungal symbiont is necessary and the timing of the

protocorm stage is relatively unknown, colonization is hard to observe and is often considered a rare event. With the unknown time frame of colonization events, genetic variation may take much longer to accumulate in populations.

Tipularia discolor maintains populations through clonal growth, but our data suggest that new habitats are colonized through seed dispersal. These results are consistent with a simulation model on the clonal plant, *Asarum canadense* (Damman and Cain 1998). Gene flow between well-separated populations has been documented in other orchid species using molecular techniques (Aagaard et al. 1999, Case 1994, Chung and Chung 2000). The method of gene flow that most likely occurs in *Tipularia* is long distance dust seed dispersal. Our technique is suggestive, but more promising genetic approaches for estimating long-distance seed dispersal are being developed (Cain et al. 2000).

Although there was low genetic variation, different genotypes were detected within subpopulations. By using the ISSR markers, we were able to detect variation within the three dense clusters at the Salisbury, MD site. This is contrary to earlier work done by Frye, who found clumps within populations to be uniform (Frye 1993). The overall trend was for each subpopulation to be dominated by one genotype, consistent with the reported clonal growth pattern of *Tipularia* (Efird 1987, Frye 1993). Other clonal plants have shown that variation within clumps may be the result of intermingled genets (Kudoh et al. 1999), which may explain the apparent contrast of our study with the results of Frye. In addition, it is difficult to identify the genet in the field, as corm fragmentation and disintegration occurs in this species.

This study illustrates that ISSR markers can be used to detect genetic diversity at a population level. This relatively new technique may be especially useful in studies of plants with unique reproductive strategies. Using molecular markers in novel ways in combination with detailed field work can lead to more comprehensive population studies in the future.

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