03-051W

Genetic diversity of *Dioscorea oppositifolia,* an invasive exotic in Southern Illinois: A preliminary report

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Report prepared for
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Illinois Wildlife Preservation Fund
Small Project Proposal FY01

January 9, 2003

Introduction

Invasive exotic species make up 28% of the Illinois flora and are one of the most pernicious threats to the integrity of natural areas in the state (Illinois Department of Energy and Natural Resources 1994). Understanding their basic population biology is a first step for developing methods of control and integrated pest management for these species. A fundamental part of obtaining this basic information is information concerning the area of origin and genetic diversity (Pappert et al. 2000; Meekins et al. 2001). Such information will allow informed decisions to be made in planning control measures for introduced invasive species.

Here we report our preliminary findings concerning the genetic diversity of *Dioscorea oppositifolia* L., a native of Asia that was introduced into the mainland U.S. in the late 19th century (Hawley 1956), and that has spread invasively in 23 states including Illinois (USDA 2001). Populations of *D. oppositifolia* have been documented in 13 counties in southern Illinois, and putatively reported in an additional 8 counties of southern IL (Basinger 2001).

Dioscorea oppositifolia is a dioecious perennial vine with oppositely-arranged, halberd-shaped leaves (Gleason and Cronquist 1991). These populations apparently reproduce only via asexual axillary bulbils. The species is dioecious and no female flowers have been observed in any U.S. population; therefore, it has been postulated that *D. oppositifolia* may have invaded the U.S. from a single, or small number, of introduced propagules.

Thus, there is an expectation of low genetic diversity, perhaps even monomorphy, within and among U.S. populations.

Obtaining information on the genetic diversity and origin of *D. oppositifolia* populations in southern Illinois will be of value in developing management plans for this invasive species. If the plants have negligible or low genetic diversity (our expectation) then management plans will not need to address the possibility of highly divergent genotypes responding differently to management strategies. Alternatively, if *D. oppositifolia* exhibits high levels of genetic diversity, and/or was introduced from multiple source populations, then managers must be prepared to deal with, for example, the responses of different genotypes to herbicides or biological control agents.

Our overall objective was to quantify the genetic diversity of *D. oppositifolia* populations in southern Illinois, and to make a comparison with populations in nearby surrounding states (Missouri, Kentucky, Tennessee). We also proposed to compare our results with collections from native Asian populations. Because US mainland populations may have originated from a single source population, we expect *D. oppositifolia* to display little to no variation in genetic diversity either within or among U.S. populations.

In this report, we present preliminary results addressing our most fundamental question: did *D. oppositifolia* invade the U.S. from a single genetic individual? We also discuss future plans to provide a more detailed analysis of population diversity.

Materials and Methods

Sampling scheme: We collected leaf samples from XXX *D. oppositifolia* populations in southern Illinois (e.g., sites reported in Basinger 2001; Beyerl 2001), plus XX samples in X other states. In order to assess the amount of genetic diversity in D. oppositifolia populations from within its native Asian range, we obtained samples from XX sites in Japan. Finally, we sampled three congeners: the Illinois native *D. quaternata*, The Asian native *D. bulbifera* (exotic in Florida and Texas), and the Japanese species *D. japonica*. Details of our sampling plan is outlined in Table 1. When possible, we obtained leaf samples from ten to 25 ramets, sampled at intervals of 10 m to avoid resampling of genets. For some populations, less than ten ramets were sampled. Approximately 2 g of leaf material from a single plant was collected and returned to SIU either fresh or dried on silica gel. Voucher specimens from sampled populations are deposited in the SIUC Plant Biology Herbarium. Comparative samples of closely related *Dioscorea* species, including *D. bulbifera* (exotic in FL and TX) and *D. quaternara* (native to southern Illinois) were also obtained.

Samples were stored either fresh in refrigeration, frozen at -80C, or dried on silica gel until DNA was extracted; we found that all three storage methods yielded DNA of sufficient quality for our analyses.

Choice of molecular markers: We originally proposed to use ISSR (inter simple sequence repeats) markers to assess population genetic structure and levels of variability. ISSR markers are hypervariable regions of the genome that are amplified using primers designed from microsatellite (simple sequence repeats) regions. However, preliminary tests showed that AFLP markers (amplified fragment length polymorphisms; Vos et al. 1995) yielded better results. AFLP is a PCR-based fingerprinting technique in which random fragments of the genome are amplified and visualized. In the AFLP protocol, genomic DNA is first digested with restriction enzymes. The resulting random array of fragments are ligated to adaptors (short pieces of DNA that are complimentary to the restriction sites). One or more selective PCR reactions using primers based upon the adaptor produce amplifed products from a random subset of the fragments. These fragments are separated and visualized on a gel via electrophoresis, most commonly using fluorescent labeling and an automated DNA sequencer.

Molecular methods: We extracted DNA from *Dioscorea* leaves following standard CTAB protocols (Doyle and Doyle 1987). Preliminary trials showed that fresh, frozen, or dried leaf samples produced satisfactory DNA; bulbils were unsatisfactory due to their high starch content.

AFLP protocol was as follows: DNA from samples were digested with restriction enzymes *Eco*RI and *Mse*I under the following conditions: 1X restriction-ligation buffer (10 mM Tris-Hac, 10 mM MgAc, 5 mM

DDT, pH 7.5), 5 units each *Eco*RI and *Mse*I, 50-100 ng DNA, 0.5 mg/ml BSA, water to final volume 50 µl. Digestion was carried out at 37C for 3 hours. All thermal cycles were carried out in a Perkin-Elmer GeneAmp 2700 thermocycler.

Ligations of adaptors to the restriction digest products were carried out under the following conditions: 1X restriction-ligation buffer, 5 pmols each of 4 oligonucleotide adaptors (Table 2), 1.2 mM ATP, 1 unit T4 DNA Ligase. Ten µl of this mixture were added to the entire volume of the restriction digest products. Ligation was carried out at 16C for 4 hrs.

PCR-amplification consisted of two steps: a 1+1 PCR, using primers with 1 selective nucleotide, and a 3+3 PCR, using primers with 3 selective nucleotides. 1+1 reactions were as follows: 1X Fisher PCR buffer, XX MgCl2, 0.8 mM (total conc.) dNTP's, 0.1 µM Eco+X primer, 0.1 µM Mse+X primer (see below and table 2 for specific primers used), 1 unit Fisher *Taq* DNA polymerase, 5 µl of diluted restriction-ligation products (diluted 0.25 X to 0.1X), 2 µl deionized formamide, final volume 50 µl. Thermocycler conditions were: 72C for 2 min.; 30 cycles of 94C for 30 s., 50C for 30 s, 72 for 1 min.

The products of the 1+1 PCR reactions were used as templates for the 3+3 PCR reactions using the following conditions: 1X Fisher PCR buffer, 0.8 mM (total conc.) dNTP's, 0.05 µM Eco+XXX+6-fam primer (dye-labeled) 0.2 µM Mse+XXX primer, 0.5 units Taq, 0.2 µl deionized

formamide, 2.5 µl of diluted 1+1 PCR products (diluted 0.25X to 0.1X), total volume 10 µl. Thermocycler conditions for 3+3 reactions were: 94C for 2 min.; "touchdown" using 94C denature for 30 s, 65C anneal for 30 s, 72 extension for 1 min: this sequence was repeated 9 times, each time reducing the annealing temperature by 1C; 30 cycles of 94C for 30 s, 56C for 30 s, 72C for 30 s.

Several 3+3 primer pairs have been screened; however, the pair that has, to date, produced the clearest banding pattern is EcoRI+ACG and MseI+AGT (Table 2).

The final dye-labeled PCR products were cleaned of unincorporated primers using spin columns of G-50 sephadex, and dried down to await electrophoresis. Fragments were visualized and sized on an ABI 377 automated DNA sequencer with GeneScan software. GeneScan 500XL ROX internal size standards were used to size the fragments.

Results and Discussion

We have obtained preliminary AFLP fingerprints for representative individuals from several of the *D. oppositifolia* populations for which we sampled DNA, plus three samples of the native *D. quaternata* for comparative purposes. The two species display highly dissimilar fingerprints, with few bands (interpreted as alleles) in common (Fig. 1). Among the seven populations of *D. oppositifolia* in this initial survey

(from 1 site in TN, and sites from 5 IL counties), genetic variation was observed. As shown in the fluorescent gel image (Fig. 1) at least 11 polymorphic bands can be observed in the 200-300 size range for the primer pair EcoRI+ACG and MseI+AGT. Though preliminary, these data strongly suggest that multiple genets of *D. oppositifolia* have invaded the U.S. These data contradict the hypothesis that the species' current distribution in the U.S. arose from the introduction of a single genet that subsequently spread clonally. Moreover, the existence of multiple genotypes in southern IL alone not only suggests multiple introductions, but also a high ability to disperse clonally.

With our current sampling and these preliminary results, we are now poised to collect more detailed information that will allow us to quantify genetic diversity within populations as well as between populations. Moreover, we will be able to assess the genetic similarity of *D. oppositifolia* populations in the U.S. to populations from at least a portion of its native Asian range (Japan). This information may cast new light on both the history of this invasive species, and of its future control.

Acknowledgements

We thank the Illinois Wildlife Preservation Fund for support of this project, and J. Shimp, S. Chandy, N. Okagami, A. Townsend, and J. Beeler for assistance in the collection of plant material.

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Table 1 Localities and species of *Dioscorea* sampled for genetic diversity study.

Species	Location	Date	Collector	# sampled ramets
D. oppositifolia				
	TN: Sevier Co., Gatlinburg. disturbed roadside			
	08/31/02	J. Beeler	16	
	IL: Jackson Co., Giant City State Park, along tributary of Drury Creek			
	09/07.02	J. Thomas	20	
	IL: Jackson Co., SIUC campus, Thompson Woods			
	09/18/02	D. Gibson	20	
	IL: Pope Co., Lusk Creek Canyon		08/31/02	J. Thomas 15
	IL: Edwards Co., Albion, on roadside		09/24/02	J. Thomas 20
	IL: Pope Co., Spiney Valley Glade Nature Preserve			
	09/23/01	J. Shimp	9	
	IL: Pope Co., Upper Lusk Creek Watershed, Delwood, on roadside			
	0920/01	D. Gibson	15	
	IL: Saline Co., Church Road, Harrisburg		09/20/02	D. Gibson 5
	IL: Pope Co., Upper Lusk Creek Watershed, Hay's Creek, on junk pile			
	09/20/01	D. Gibson and S. Chandy		11
	IL: Jefferson Co., Mount Vernon		10/02/02	J. Shimp 9
	IL: Johnson Co., on roadside	10/03/02	J. Shimp	9
	IL: Lawrence Co., Red Hills State Park		10/04/02	J. Shimp 10
	JAPAN:, Morioka City	09/09/03	Terui	3
	JAPAN: Miyagi County	09/10/03	Okagami	2
	JAPAN: Sendai City	09/11/03	Okagami	6
	JAPAN: Aobayama	09/12/03	Okagami	2
D. japonica				
	JAPAN: Ohkubo, Aira County, Kagoshima site			
	09/12/03	Okagami	1	
	JAPAN: Suzaki, Shimoda City, Shizuoka site			
	09/12/03	Okagami	1	
	JAPAN: Nenoshiroishi, Sendai City, Miyagi site			
	09/12/03	Okagami	2	
D. quaternata				
	IL: Williamson Co., Atwood Ridge			S. Chandy 4
	IL: Jackson Co., Giant City State Park, on roadside			
	09/16/03	S. Chandy	14	

	IL: Pope Co., Bell Springs State Park		09/20/03	S. Chandy 7
D. bulbifera	Houston TX, Ornamental in private residence			
	09/29/03	A. Townsend		1

Table 2 Oligonucleotides used in AFLP fingerprinting of *Dioscorea*.

Adaptors:		
	EcoRI.1 adaptor	5' CTC GTA GAC TGC GTA CC 3'
	EcoRI.2 adaptor	5' AAT TGG TAC GCA GTC TAC 3'
	MseI.1 adaptor	5' GAC GAT GAG TCC TGA G 3'
	MseI.2 adaptor	5' TAC TCA GGA CTC AT 3'
1+1 PCR primers		
	EcoR1+A	5' GAC TGC GTA CCA ATT CA 3'
	MseI+A	5' GAT GAG TCC TGA GTA AA 3'
3+3 PCR primers		
	EcoRI+ACG (6-fam labeled)	5' /6-fam/ GAC TGC GTA CCA ATT CAC G 3'
	MseI+AGT	5' GAT GAG TCC TGA GTA AAG T 3'

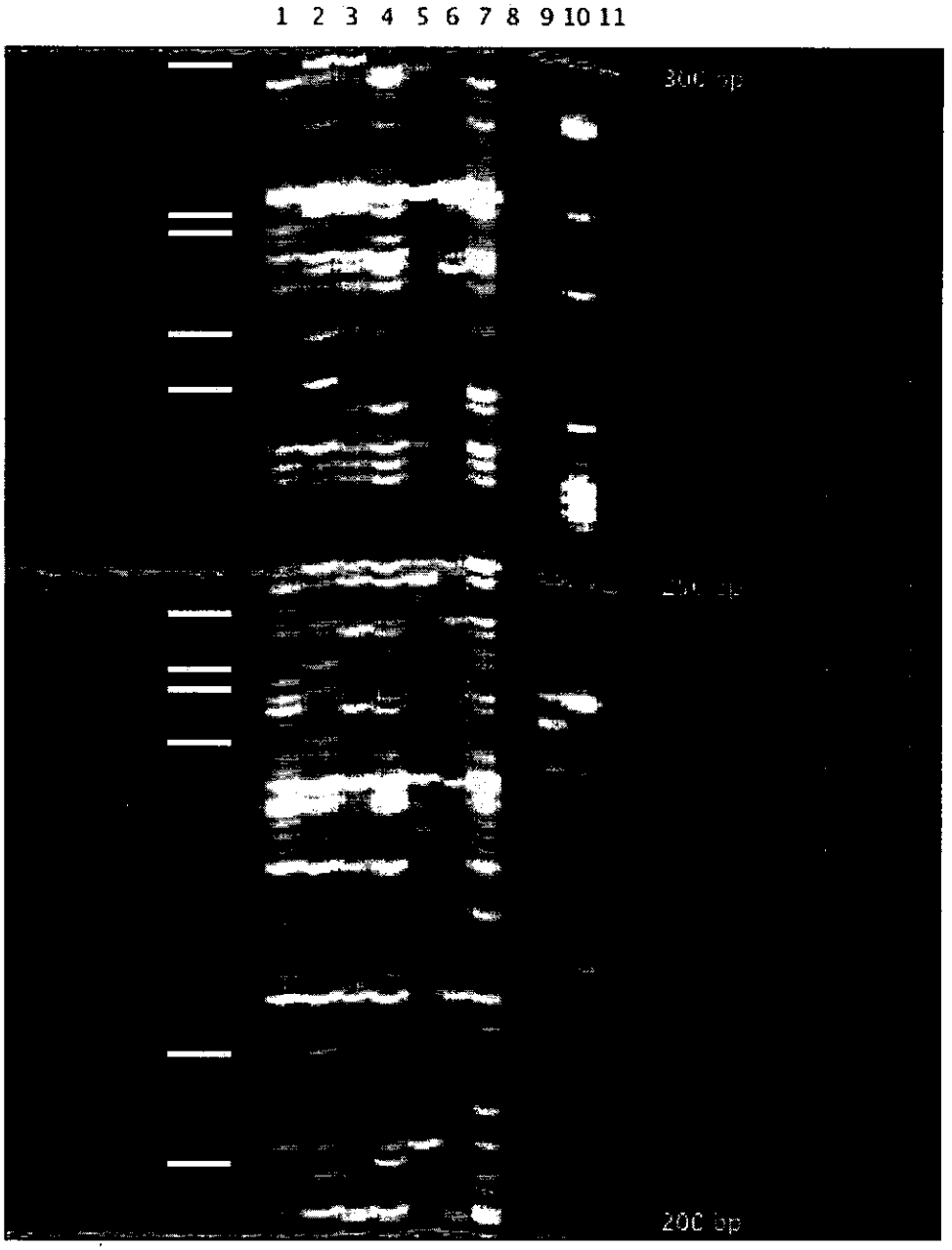
1 2 3 4 5 6 7 8 9 10 11
300 bp
200 bp

Figure 1. Fluorescent gel image of AFLP fingerprints (blue bands) for 7 populations of *D. oppositifolia* (lanes 1-7) and 3 populations of *D. quaternata* (lanes 8-10). Red bands are internal size standards indicating 200, 250, and 300 base pairs. Yellow lines indicate sites that are polymorphic among the *D. oppositifolia* populations. (positions in which some samples have a band and others do not). The polymorphic sites indicated here are those which have shown repeatability through several repetitions. Sample lanes are as follows: 1 = Gatlinburg TN; 2 = Albion IL; 3 = Spiney Valley Glade Nature Preserve IL; 4 = Harrisburg IL; 5= Upper Lusk Creek, IL; 6 = Mt. Vernon, IL; 7 = Red Hills State Park IL; 8 = Bell Springs State Park, IL (*D. quaternata*); 9 = Atwood Ridge Research Natural Area, IL (*D. quaternara*); 10 = Lusk Creek Canyon, IL (*D. quaternara*); 11= negative control. See Table 1 for complete locality information.