DNA Fingerprinting in *Hydrastis canadensis* Using RAPD Analysis

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Abstract

***Hydrastis canadensis* L. (goldenseal) is an endangered perennial wildflower species native to eastern North America. In this study, we analyze several populations of *Hydrastis canadensis* for genetic diversity within and among populations. The samples are from cultivated populations in Massachusetts and Canada and wild populations from Ohio, West Virginia and New York. The RAPD analysis technique was used to generate DNA profiles from individual plants and to estimate genetic relatedness within and between populations. Ready to Go RAPD Analysis Bead Kits were used for the amplifications to help ensure consistency and reproducibility. The results for the cultivated material show 72-86% similarity among the four populations tested and the wild populations 20-67% similarity. These numbers are in line with what is expected when comparing a cultivated group with the wild populations. An interesting result was the similarity between one Ohio population and the New York populations. These results imply that the New York population may have originated from cultivated material. Because there are disadvantages to having limited genetic variability, greater knowledge about the levels variation within the populations could assist in crop production and reintroduction strategies for this species.**

INTRODUCTION

Hydrastis canadensis L. (goldenseal) is a perennial herb native to eastern North America, with a general distribution across the northern states from Vermont to Nebraska and to the south, through the Ohio River Valley to Arkansas (Mitchell and Dean, 1982). This under-story species prefers the rich moist soil found in arboreal forests, with partial shade and cool temperatures (Davis, 2001). Used for its medicinal properties by the Native Americans, goldenseal was introduced to the European settlers and gained commercial interest in the 1800's when it became a favored treatment for eye and throat infections, as well as urinary tract infections and gonorrhea. Harvested for its root that contains the alkaloids berberine, hydrastine and canadine, this herb continues to be popular; and studies have shown it to have anti-inflammatory, antibiotic and anti-pyretic effects (Murray and Pizzorono, 1991).

Reproduction, Cultivation and Genetic Diversity

Today, the numbers of wild *Hydrastis* populations are on the decline, to the point of extirpation in some states (Massachusetts Division of Fisheries & Wildlife 2001). It is thought that this is largely due to habitat loss and over-harvesting in the wild, but this may be exacerbated by the plant's reproductive strategy. Goldenseal reproduces by both vegetative and sexual means, with juvenile plants reproducing clonally until they reach sexual maturity at two to three years of age. At this stage the root is considered large enough to harvest (Massachusetts Division of Fisheries & Wildlife 2001). A mature plant only produces one flower per year with eight to 15 carpels. The number of ovules produced per carpel can range from as few as two, one fertile and one abortive, to occasionally three or four (Tobe and Keating, 1985).

With so few seeds being produced per year, *Hydrastis* may tend toward being more clonal in its reproductive strategy. This, in combination with the fact that collectors tend to harvest the mature plants, would serve to further reduce genetic diversity within wild populations that are subject to harvesting on a regular basis. A long-term decrease in

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genetic diversity may lead to an inability to cope with changing environmental conditions and an increased susceptibility to pathogens, thereby putting entire populations of goldenseal at risk (Mayr, 2001).

We would also expect to find low levels of genetic variation in cultivated populations of goldenseal due to difficulties involved in seed propagation. The seeds cannot be stored and must be kept moist in order to germinate, so the standard method of cultivation currently involves creating clones through root cuttings (Davis 2001). Thus, the gene pool for goldenseal would appear to be limited, both in the wild and in cultivation. The degree to which this is the case can be investigated using the molecular techniques of RAPD analysis and DNA fingerprinting.

DNA Fingerprinting and RAPD Analysis

For the purposes of estimating levels of genetic variability, differences can be detected among individuals' DNA sequences. There are several methods now in use, each with its own advantages and drawbacks: restriction fragment length polymorphisms (RFLP), random amplified polymorphic DNA (RAPD), microsatellites, and amplified fragment length polymorphisms (AFLP) (Weising et al., 1995).

The RAPD technique was chosen over RFLP and AFLP for our analysis for a number of reasons. RFLP analysis does not reveal as many polymorphisms as RAPD and requires larger amounts of DNA (Bowditch et al., 1993). AFLPs, while offering a large number of polymorphic bands and high reproducibility, has the drawback of requiring two rounds of DNA amplification combined with the use of polyacrylamide sequencing gels and radioactive isotopes or an automated sequencer for nonradiolabeled fragments (Wolf and Liston, 1998). The RAPD technique was selected over microsatellites, due to the large investment in time and expense necessary in locus development for microsatellites, especially in a genetically unknown species such as goldenseal.

RAPD analysis has the following advantages: the overall simplicity of the technique, the fact that numerous sites within the genome can be analyzed with just a few primers when no specific nucleotide sequence information of the genome is available, and that the amplification strength tends to be consistent in flowering plants (Rieseberg, 1993).

In this study we use Ready to Go RAPD Analysis Beads (Amersham Pharmacia) to assess the genetic diversity within and among cultivated and wild populations of *Hydrastis canadensis* by generating polymorphic DNA profiles for cladistic and statistical analysis.

MATERIALS AND METHODS

Plant Material

Leaf samples were collected from plants and kept on ice until extraction. The plants used for testing were cultivated plants collected from nurseries in Framingham, MA, Shelburne, MA, and Ontario, Canada, and wild plants sampled from various locations throughout Ohio, West Virginia and New York State (Tables 1, 2). Two cultivated out-groups were selected for comparison, tomato (*Lycopersicon esculentum*) and a member of the Ranunculaceae, *Helleborus viridis*. Eight to ten plants were sampled from each goldenseal population and 6 samples per population were chosen for amplification based on the quality and quantity of DNA extracted from each.

DNA Isolation

Approximately 2 cm^2 of leaf tissue from each sample was processed for DNA extraction using the microprep procedure (Fulton et al, 1995). The resulting DNA pellet was resuspended in 50 µl TE buffer (10 mM Tris, 1 mM EDTA pH 8) at 65 °C for 30 minutes and checked for quality by electrophoresis on a 1% agarose mini gel prepared in 1x TAE (Trisbase, glacial acetic acid, 0.5 M EDTA) buffer. Undigested and *Eco*RI digested aliquots

(3 µl) of each DNA sample were electrophoresed alongside uncut λ Phage DNA standards (50 and 100 µg) in order to assess DNA quality and quantity. DNA samples were stored at –20 °C until needed for RAPD analysis.

Amplification

Dilutions (1/10 and 1/5) were prepared of the DNA samples to check for the optimal amount of template DNA to use with the Ready to Go RAPD Analysis Bead Kit (Amersham Pharmacia) primers. The 1/10 and 1/5 dilutions were amplified using primer 2 per package instructions from the manufacturer. The 1/10 DNA dilution was chosen for both the cultivated and the wild samples. Approximately 5-50 ng of template DNA was used for each reaction (1 µl). The reactions were run in a Perkin Elmer Cetus Thermocycler 480 using a cycle profile of 1 cycle at 95 °C for 5 minutes followed by 45 cycles at 95 °C for 1 minute, 36 °C for 1 minute and 72 °C for 2 minutes. The amplified DNA was separated by electrophoresis along with 100 Kbp and 100 bp (base pair ladder) standards on 2 % agarose gels prepared in 1x TAE buffer and visualized under UV light after ethidium bromide staining. The resulting banding patterns were photographed with Polaroid 680 film for analysis.

Polymorphic Band Analysis

Base pair sizes of DNA fragments amplified from the RAPD primers were calculated by reference to known base pair ladders (100 Kbp and 100 bp). Bands above 2800 bp or below 270 bp were not included, as these size fragments tend to be unreliable for assessment. Amplified fragments were characterized by their size and intensity. The presence or absence of fragments was recorded as either 0 (absent) or 1 (present) characters. All resulting characters were analyzed using three different statistical techniques (Weising et al., 1995).

RESULTS

Cultivated Populations

Optimization of DNA template concentration was performed prior to RAPD analysis on all the samples using 1/5 and 1/10 dilutions of the DNA samples and primer two. The results of the optimization showed comparable results between the two concentrations. The 1/10 dilution was selected over the 1/5 dilution due to the consistent banding for that concentration and used as the template for all subsequent RAPD reactions.

The six RAPD primers yielded 76 different characters (bands) from the 24 samples of the cultivated populations and a distinctive banding pattern was generated by each of the primers for all the populations. A second run of all the primers and samples was performed in order to test for reproducibility of the banding patterns.

The bands of the first run were analyzed via statistical methods to perform pair-wise comparisons within populations (S_1 values), to determine the percentage of shared bands per population (F_6 values) and to compare populations (S_2 values). The data yielded by the second run are not included in this report due to the replication they showed of the results from the first run.

The S_1 values varied for each population and ranged from a low of .669 to a high of 1 (a value of 1 is equal to 100 % fragments are shared in common between two individual samples). The F_6 values indicated a high percentage of shared bands within each population as the values ranged from 97 % to 100 %. The seed grown Garden in the Woods showed a very high similarity to the field grown Garden in the Woods samples, surprising for offspring generated via sexual reproduction (Table 3).

The S_2 values indicated the degree of similarity of the populations to one another via pair-wise comparison of shared bands and allowed the populations to be ranked according to their degree of relatedness (Figure 1). The S_2 values and ranking of populations indicate that the Garden in the Woods seed population is the most related to

the Canada population at 87 %, the plants from Canada are next most closely related to those at Mount Holyoke College (86 %). There was 83 % similarity between the Garden in the Woods field and seed grown plants. The greatest differences were found between the Garden in the Woods field population and the Mount Holyoke College (78 %) and Canada populations (72 %). Not surprisingly, Garden in the Woods seed is related to Mount Holyoke College by the same amount of relatedness as the Garden in the Woods field population at 78 %.

Wild Populations

Optimization tests were not performed for the samples prior to RAPD analysis in an attempt to conserve Ready to Go Beads. The 1/10 dilution was chosen for the reactions, as it had been so successful for the cultivated populations, but was replaced by the 1/5 for primers 3-6 and for the second run of primer one.

It was observed after the run of primer two that some of the samples appeared weaker after visualization than those of the cultivated samples. Another issue was that some of the samples were not producing bands at all. Due to the somewhat lower concentrations of the DNA extracted from the wild samples, which also had a greater probability of DNA degradation after long distance shipment, a higher concentration was tried (1/5) to determine if it would increase the efficiency of amplification. The banding pattern remained essentially the same, but the concentration was maintained at 1/5 throughout the rest of the primers. The problem with poor amplification of some samples was resolved by eliminating entire populations from statistical consideration if more than 50 % of the samples did not produce bands.

The results from the wild populations showed 102 different characters (bands) from the six RAPD primers over a total of 86 individuals. A distinctive banding pattern was obtained from each of the primers. No second run was made for the wild samples, due to a lack of time to complete another set of reactions for all the primers and funding limits, except in the case of primer one, which yielded no bands whatsoever for any of the populations during the first run of PCR. However, the second run was successful, yielding scorable bands.

The RAPD fragment patterns for all wild populations and the outgroups were analyzed using the same statistical methods as those applied to the cultivated populations. In addition, F_6 values were averaged for all the populations within each state, due to the larger number of groups involved from Ohio and West Virginia.

The F_6 values indicated that the nine Ohio populations shared the lowest percentage of bands in common, the seven West Virginia populations shared more bands and the two New York populations shared the greatest number (Table 3).

The S_2 values, which indicated the degree of similarity of the populations to one another in a pair-wise comparison of shared bands, allowed the ranking of the highest to lowest, between populations for degree of relatedness (Figure. 2). Not surprisingly, the New York to New York populations were ranked the highest at 67 %, but the relatedness of New York to Ohio (51 %) exceeded the ranking of Ohio to Ohio populations (44 %) and the West Virginia to West Virginia populations (43 %). West Virginia was least related to both Ohio and New York at 20 % each.

The S_1 values were varied and ranged from a low of .154 to a high of 1, with the out groups being valued at zero.

DISCUSSION

A clone is considered an exact copy of the original material it is derived from, but the use of the term clonal populations in plant studies may not be so clear-cut as it would seem on the surface. If one were speaking about root cutting propagation, then one would be inclined to expect identical plants to result. But if one is speaking about vegetative self-propagation by the plant, what degree of similarities might one expect? If some sexual reproduction is occurring between clonal offshoots, how does that affect the genetic structure of the population?

Cultivated Populations

As was expected, cultivated goldenseal plants (many of which are derived from root cuttings) proved to be highly similar, as the mean (.985) of shared characteristics (F_6 values) was well above the mean within the Ranunculaceae, (.799) and clearly indicates a high degree of relatedness within each population (Van Buren et al., 1994). This is in contrast with recent findings for *Rosa* where a higher degree of genetic variability was found in the cultivated species over the wild species. These findings were unexpected as the long accepted idea for *Rosa* was that there would be less variability in such a highly cultivated species (Debener et al., 1996).

Not as expected was the high degree of similarity of the Garden in the Woods seed grown plants and the field plants. The field plants are most likely either the parents or grandparents of the seed plants and are thought to have been propagated via root cuttings originally from New Hampshire. The high level of similarity may be due to increased levels of inbreeding within closely neighboring populations and/or excessive clonal off-shoot propagation at this location. Studies have indicated that the closer plants are to one another, the higher the incidence of shared DNA banding patterns; the Garden in the Woods populations were found growing within half an acre of each other (Weising et al., 1995).

The pair-wise character similarity that was found between the Canadian and the Mount Holyoke College samples, as seen in the S_2 value of 86 %, was unexpected given the original source of the population on the Mount Holyoke College campus. Records indicate the stock originated from wild collected plants in Tennessee. The question arises, are the Canadian samples originally from Tennessee stock? The Canadian origin was a grower in Ontario, who claimed to have acquired root cuttings from Wisconsin; could they really have originated in Tennessee? Herein lies the general problem with *Hydrastis*, it has been of interest for so long, with people trading in root cuttings for decades, that clones may have been planted over the entire range of habitat. At this point, reintroduction could be so widespread that genetic similarities in *Hydrastis* may be more common than is normal for clonal plants in general.

Wild Populations

In light of the high degree of genetic similarity shown by the cultivated populations, expectations for finding greater diversity within the wild populations were high. The results were not disappointing and the New York populations, which showed the highest percentage of shared bands at 82 % (F_6 values), were still lower than those values for any of the cultivated populations.

The S_2 values revealed some interesting relationships among the wild populations. Surprisingly, New York and Ohio populations showed more relatedness (51 %) than was found among the Ohio populations (44 %). The West Virginia populations were 43 % similar to one another and the same level of genetic relatedness was found within the Ohio to Ohio population comparisons. Yet the relatedness of West Virginia to both Ohio and New York was only 20 %. Perhaps the West Virginia populations have been geographically isolated and not been as subject to over-harvesting as some other areas. While this would lead to high degrees of similarity within an isolated population, it would also increase the genetic variation between that population and others.

Implications

Part of the aim of this study was to compare cultivated and wild populations and assess their degree of relatedness according to shared fragments. This is not possible based on the results; however, the implications of the results obtained separately for the wild and cultivated populations can be discussed.

The cultivated populations, with their high proportion of shared bands indicating genetic similarity, demonstrate that even when there are large geographic distances between populations, plants cultivated from root stock retain high degrees of similarity over time. It is possible that all of these plant populations originated from the same

geographic area and the genotype was distributed throughout the commercial community via root cuttings. However, the high levels of similarity are not the norm for clonal populations, according to Ellstrand and Roose (1987), who state that these types of populations typically show only intermediate levels of diversity.

The unexpectedly high degree of similarity found between the Garden in the Woods field and seed plants may indicate that there is a high amount of self fertilization occurring, which would have implications for wild populations as well. Lower levels of genetic variation can make a species susceptible to changes in climate, habitat, competition from other species, diseases and pests (Mayr 2001).

The lower proportion of shared DNA fragments among the wild populations can be seen as an indication of greater genetic variability within these groups, yet these results must be addressed with caution. If the amplification was affected by degradation of DNA in the leaf samples during shipping, then the results will be misleading. Further testing of freshly extracted DNA would yield more conclusive results. If these results are a true indication of the variability of the samples, the Ohio samples need to be investigated further as they show the most variability. Are these plants simply more successful at sexual reproduction, or are the populations so geographically removed from each other that they developed genetically in isolation, i.e. the founder effect.

From the results of this study *Hydrastis* appears to be a species with differing levels of genetic variation. The variations range from high similarity to low and the causes of those variations are complex. Variations may be due to geographic isolation, extensive propagation via root cutting (both reintroduction and cultivation), limited sexual reproduction and vegetative offshoots. The results supported original expectations that cultivated populations would be more similar than the wild grown populations. The few anomalies provide interesting questions and avenues to pursue with further study. *Hydrastis* is a good candidate for future study because the results show enough consistency to encourage a closer look at the variability of this interesting and useful plant.

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Tables

Table 1. Cultivated Populations.

Name of Population	Location	Date Sampled	Abbreviation
Garden in the Woods Field	Framingham, MA	6/01	GWS
Garden in the Woods Seed	Framingham, MA	6/01	GWF
Canada	Ontario, Canada	6/01	C1, C2
Mount Holyoke College Botanical Garden	South Hadley, MA	6/01	MHC

Table 2. Wild Populations.

Name of Population	Location	Date Sampled	Abbreviation
Joralamon Park	New York State	7/02	NY1
"	"	"	NY2
Alexandra Twp. Athens County	Ohio	9/01	#1
Waterford Twp. Washington County	"	10/01	#2
Morgan County	"	"	#3
Trimble Twp. Athens County	"	"	#4
"	"	"	#5
"	"	"	#6
Athens County Hopewood Farm	"	"	#7
Meigs County United Plant Savers	"	"	#8
Meigs County National Center for Preservertion of Medicinal Herbs	"	"	#9
Jackson's Mill	West Virginia	9/01	J
Murphy's Preserve	"	"	M
No Name Hollow	"	"	NH
Baker's Ridge	"	"	R
West VA Arboretum	"	"	W
Forks of Cheat	"	"	F
Zimmerman Tract	"	10/01	Z
Charlie Baer	"	"	C'
Briary Mountain	"	"	BM
Pull Off	"	"	P
Lycopersicon esculentum (Out-group 1)	South Hadley MA	4/01	L (T2)
Helleborus viridis (Out-group 2)	South Hadley MA	"	H

Table 3. F_6 values for cultivated populations, indicating percent of shared bands within each population.

Population	**F_6 Value**
Garden in the Woods Field	0.991
Garden in the Woods Seed	0.982
Canada	0.997
Mount Holyoke College	0.969

Table 4. F_6 values for wild populations, indicating percent of shared bands within each population.

Population	**F_6 Value**
New York	0.815
West Virginia	0.784
Ohio	0.657

Figures

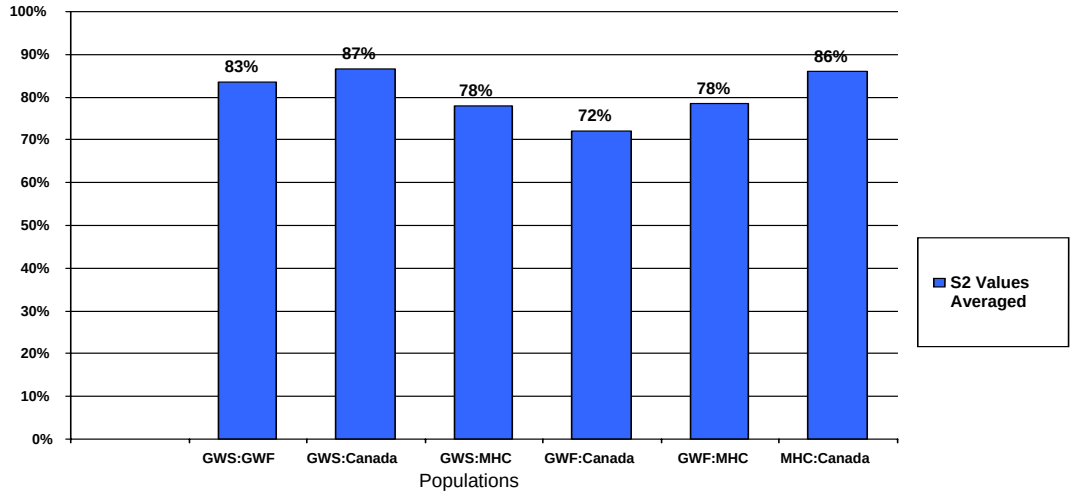

Fig. 1. Pair-wise comparison of shared bands between cultivated populations (S_2 values).

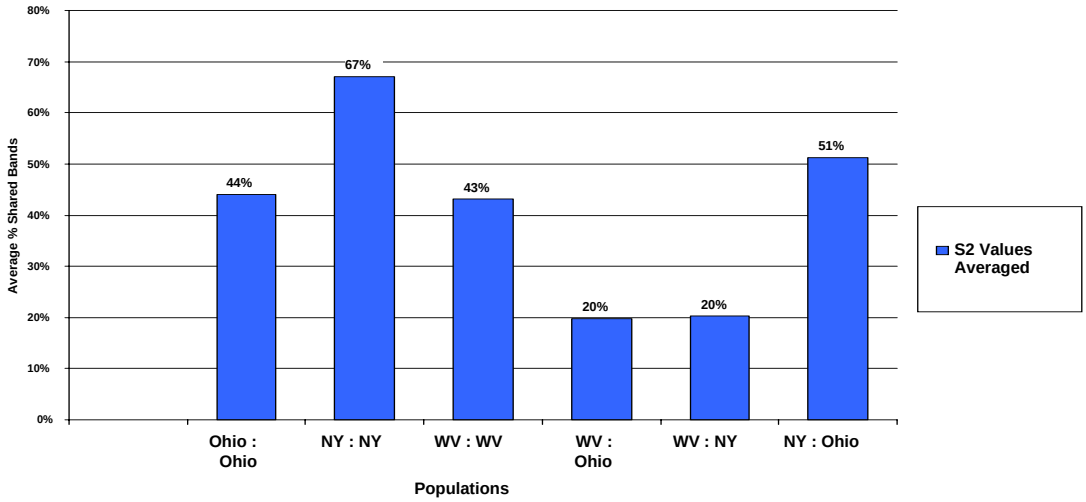

Fig. 2. Pair-wise comparison of shared bands between wild populations (S_2 values).