Determination of microsatellite markers for prairie rose gentian, *Sabatia campestris*

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Introduction

With increasing frequency, tools of molecular biology are being used to answer questions in ecology, evolution, and conservation biology. Molecular tools such as microsatellite markers, tandem repeats of short nucleotide sequences (Queller et al., 1993), are especially useful when studying the genetics of threatened and endangered species. Using DNA techniques to better understand the biology of endangered species is significant for many reasons. First, only a small amount of tissue needs to be collected from individuals because DNA may be amplified using the polymerase chain reaction (PCR). Additionally, DNA markers such as microsatellites can detect greater amounts of genetic variation in populations which have little allozyme variation. This is especially important in the case of endangered species whose populations may have lost genetic variation due to genetic drift and/or population bottlenecks.

Prairie rose gentian (*Sabatia campestris*) is an annual plant species which is listed as state-endangered in Illinois (Herkert, 1991). Within Illinois, prairie rose gentian occurs within prairie remnants along railroad right-of-ways within three counties in the central portion of the state. Most of the seven populations known in Illinois are isolated from one another spatially, and often temporally, and thus, may have lost genetic variation. By better understanding the genetic structure of these populations, we may more effectively manage populations of prairie rose gentian and use this species as a model for better conserving other state-endangered annual plants. Data gathered using molecular tools may allow managers to improve conservation efforts of endangered species by identifying populations with relatively high genetic diversity which is a potential indicator for the ability to withstanding environmental change.

The main objective for this study was to develop microsatellite markers for prairie rose gentian. Microsatellite markers are highly variable within the genome and thus, are expected to contain many polymorphic loci. Once the markers are determined, they can be used to examine the population genetic structure of prairie rose gentian statewide as well as along the range of the species which extends as far south as Texas (Bolick, 1991). Additionally, the markers will be used to quantify the occurrence of gene flow between two populations which occur on Twelve Mile Prairie in Marion County.

Methods and Results

The development of microsatellite markers is a step-by-step process in which one procedure leads to another. This discussion of methods and results will follow in the same manner. First, a partial DNA library must be created, followed by screening of the library for

microsatellites within the genome. The procedures which I have followed are based upon Travis Glenn's procedures for developing microsatellites (unpublished manuscript).

Development of a partial DNA library:

A genomic library is constructed by inserting recombinant plasmids into bacteria. The recombinant plasmids contain DNA fragments from the genome of the study organism. The bacteria are cultured, forming colonies which, when taken collectively, contain the entire genome of the organism. To determine microsatellite markers, I constructed a partial library for prairie rose gentian by using DNA which was between 300 and 900 base pairs in length rather than incorporating DNA from the entire genome.

Extraction of DNA

The first step in the process of developing a library is to extract DNA from prairie rose gentian. I extracted DNA from 1 gram of prairie rose gentian tissue which originated from a single plant which was collected from Texas. The quality of the DNA was checked by running an aliquot of the sample on an 0.5% agarose gel. Additionally, I quantified the amount of DNA extracted using a spectrophotometer.

From the extraction, high molecular weight DNA was obtained which was relatively clean of RNA and proteins. Besides determining the quality of the DNA, I also quantified the amount of DNA in this sample. From a 1 gram sample of plant tissue, eleven micrograms of DNA were extracted, which is enough to develop a library.

Preparation of insert DNA: Cut and Size-select prairie rose gentian DNA

The prairie rose gentian DNA was digested using the Sau3A I restriction enzyme. This enzyme was chosen as it is complementary with another restriction enzyme, BamH I, which was used to digest the plasmid vector. The restriction enzymes act by cutting the DNA at specific recognition sites. For BamH I and Sau3A I, the recognition site is at GATC. Following digestion of the DNA, the sample was run on an agarose gel and size-selected for fragments ranging from 300 to 600 base pairs and from 600 to 900 base pairs. These fragments were excised from the gel and purified using the Qiagen Gel Extraction Kit. Thus, I have size-selected fragments containing 300-600 and 600-900 base pairs and have cleaned the DNA from the agarose gel.

Preparation of the plasmid vector: Cut and Dephosphorylate

The bacterial plasmid vector used was pcDNA II. The plasmid was cut using the restriction enzyme, BamH I and dephosphorylated with SAP (Shrimp Alkaline Phosphatase).

The plasmid was run on a 0.5% agarose gel, excised from the gel, and purified using the Qiagen Gel Extraction Kit. Additionally, the amount of purified plasmid DNA was determined.

Electroporation of ligated recombinant DNA into bacteria

I combined the prairie rose gentian size-selected DNA with the pcDNA II plasmid using a 1:1 mixture of insert (plant) DNA to vector DNA. The enzyme, ligase, was added to make recombinant DNA plasmids such that plant DNA was incorporated into bacterial plasmids. The recombinant plasmids were inserted into competent bacterial cells using electroporation. This technique involves using an electric current to insert the recombinant plasmids into bacterial cells so that many copies of the recombinant DNA can be made by growing bacterial colonies. This facilitates the screening of microsatellites in the plant genome.

Blue/White Colony Selection

The electroporated bacterial cells were plated onto petri dishes to quantify the amount of successful recombinant DNA colonies which were made. The number of recombinant colonies was titered by plating 10 ul and 100 ul of bacteria on separate LB petri dishes and incubating them for twelve hours.

The growth of white colonies indicates the incorporation of prairie rose gentian DNA within the plasmid. In contrast, blue colonies do not contain recombinant plasmids. I obtained only ten colonies from the plates with the small aliquot, of these 40% were white colonies. On the plate with 100 ul, numerous colonies formed with at least 45% of the colonies containing recombinant DNA.

After titering the bacterial cells, the colonies with recombinant DNA plasmids were plated onto petri dishes using LB mixed with tetracycline and ampicillin. Approximately, 12,000 colonies were plated on three large petri dishes and incubated for 12 hours at 37°C.

At the completion of this step, a partial DNA library has been constructed. Next, the library was screened for microsatellite DNA.

<u>Screening for microsatellite DNA:</u>

Lifting colonies onto filters

Using nylon membranes, two lifts were performed on each of the three plates. Thus, two copies of the colonies on the plates were transferred to the membranes. Two lifts were necessary to serve as a control when probing the membranes for microsatellites. The membranes were processed to denature the DNA and convert it to single-strands using Boehringer Mannheim's DIG system for filter hybridization (van Miltenburg et al., 1995). DNA from the colonies was secured to the membranes by ultraviolet crosslinking.

Preparation of Probe

Oligonucleotide probes are used to screen the library. The probes are labeled to allow for detection of the probes as they hybridize to a complementary microsatellite sequence on the nylon membranes. I used a non-radioactive probe to label oligonucleotides using the DIG oligonucleotide 3'-end labeling kit by Boehringer Mannheim. To date, I have labeled the following DNA oligonucleotides: AC-10 (indicates that AC is repeated 10 times), AG-10, AGC-8, AGG-8, and ACC-8 (note that the letters indicate DNA nucleotides). Each probe was quantified to ensure successful labeling.

Hybridization of probes to colonies on filters

Hybridization is the adherence of a probe to a complementary sequence of DNA which is linked to the nylon membrane. Hybridizations were conducted following Boehringer Mannheim's protocol for hybridization (van Miltenburg et al., 1995). The AGG-8, ACC-8, AGC-8 oligonucleotide probes did not hybridize to the membranes at a temperature of 70°C. The hybridization temperature was lowered to 60°C. At this temperature, many hybridizations occurred indicating that false positives were present. Currently, I am in the process of obtaining positive controls for the hybridizations. By using DNA which contains known microsatellites, I can confirm if true hybridization of the oligonucleotide probe to the complementary DNA sequence occurred.

I hypothesize that when I probed the membranes at 70°C, no hybridization occurred because none of the tri-nucleotide microsatellites which I probed were found in the genome. To test this hypothesis, I need to use positive controls. For our next screening, I plan to use the di-nucleotides, AC-10 and AG-10, as they are found more often in the genome of plants than tri-nucleotides.

Discussion

Significant progress has been made in determining microsatellite markers for prairie rose gentian. I have developed a partial DNA library for prairie rose gentian. Additionally, I have been screening the library for microsatellites and will begin using positive controls to more carefully scan for markers. Following screening, I will sequence the inserts which appear to contain microsatellites and design oligonucleotide primer pairs for use in PCR. Once PCR conditions are optimized, I can investigate which microsatellites are polymorphic in prairie rose gentian.

Ultimately, the determination of these genetic markers is important because it will enable us to better understand the population genetics of prairie rose gentian. These microsatellites will provide sufficient variation to allow for detailed study of population structure and gene

flow within the typically, small and isolated populations of this plant species in Illinois. Additionally, it will more easily provide for long-term monitoring of genetic variation of Illinois populations. Finally, the data obtained will assist conservation biologists in long-term management of this species and other species with similar life histories.

Summary

The development of a genomic library and screening for microsatellite loci is a long and complex process. I have developed a partial DNA library and have begun screening for markers. Following the screening process, I will sequence the inserts, develop primers, optimize PCR conditions and look for variation in the microsatellites. Determination of these molecular markers sets the stage for many opportunities to study prairie rose gentian. They will be powerful tools for use in identifying population genetic structure and gene flow, as well as for use in the long-term management of this state-endangered species.

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