Detection of Polymorphic Minisatellite Regions by PCR Amplification in a Segregating Population of *Tripsacum dactyloides* Parental, First, and Second Filial Generations

An Honors Thesis (HONRS 499)

by

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Abstract

The goal of this study concerned the localization and comparison of variable number tandem repeat sequences (VNTRs) within the diploid genome of *Tripsacum dactyloides* (Eastern gamagrass), a wild relative of modern maize. Genomic DNA was isolated from harvested plant tissue. The sequences of VNTR minisatelitite segments were then amplified by polymerase chain reaction (PCR). The amplified DNA was visualized by neutral agarose gel electrophoresis and ethidium bromide staining. This process allowed for photographic recording and identification of polymorphisms within a segregating parental, first, and second filial generations of *Tripsacum*. The data obtained revealed that primer FVIIex8 is appropriate for detecting novel polymorphisms segregating at the PCR amplification level. Scored data was analyzed using MAPMAKER (Lander et al. 1987) computer software to determine linkage of the minisattelite sequence containing the FVIIex8 primer core sequence to markers on the genetic map of *Tripsacum*. 
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Abstract

The goal of this study concerned the localization and comparison of variable number tandem repeat sequences (VNTRs) within the diploid genome of *Tripsacum dactyloides* (Eastern gamagrass), a wild relative of modern maize. Genomic DNA was isolated from harvested plant tissue. The sequences of VNTR minisattelite segments were then amplified by polymerase chain reaction (PCR). The amplified DNA was visualized by neutral agarose gel electrophoresis and ethidium bromide staining. This process allowed for photographic recording and identification of polymorphisms within a segregating parental, first, and second filial generations of *Tripsacum*. The data obtained revealed that primer FVIIex8 is appropriate for detecting novel polymorphisms segregating at the PCR amplification level. Scored data was analyzed using MAPMAKER (Lander et al. 1987) computer software to determine linkage of the minisattelite sequence containing the FVIIex8 primer core sequence to markers on the genetic map of *Tripsacum*.

Introduction

*Tripsacum dactyloides* is the most closely related species to *Zea mays* (modern maize) outside of the *Zea* genus (de Wet et al. 1982; Doeby 1983). Galinat et al. found in 1974 that 10 out of the 18 chromosomes of *Tripsacum* have alleles corresponding to homologous alleles in *Zea mays*. Other useful tools, such as a RFLP (restriction fragment length polymorphism) linkage map of the genes of *Tripsacum*, constructed by Blakey (1993), aid in further comparison of the genetic relatedness between *Tripsacum* and *Zea mays*. Because of genetic similarities between *Tripsacum* and *Zea mays*, many investigations are underway at a number of sites (Ball State University, USDA-ARS, and industry) to use modern molecular techniques to move genes from *Tripsacum* to maize and vice versa. The more information that can be obtained regarding the structure of the *Tripsacum* genome, the more successful these investigations will be.

One area of genomic structure that is often ignored for comparison analysis is repetitive DNA, which includes two classes: tandemly repeated (VNTR) and interspersed repetitive DNA. VNTR regions of DNA are short, repeated segments of DNA that are connected tandemly along the genome. A form of extremely hypervariable VNTR sequence, known as a minisatellite, has been used as a tool to differentiate between plant species within a genus (Zhou, et al, unpublished). Minisatellite regions usually contain anywhere
from 5-50 repeats of a 14-100 base pair sequence (Lewin 1997). This type of VNTR can be scattered throughout the genome and are often located within close proximity upstream or downstream of functional genes and some within intron (sequences of DNA that do not code for a protein) sequences (Krontiris 1995). These sequences are also highly polymorphic across individuals and are inherited in a Mendelian fashion (Zhou et al., unpublished). Minisatellites can also have an influence on phenotypic expression. In humans, instabilities in these sequences can lead to a variety diseases such as many forms of cancers (Krontiris, 1995). Due to their close proximity to genes, minisatellite sequences can be used as genetic markers to follow genes as they segregate through generations of organisms and are helpful in genetic mapping (Nakamura et al. 1987).

PCR (polymerase chain reaction) is a method used to amplify a specific region of the genome. A short sequence DNA primer (10-20 bases) is used to target this particular region. This segment is then replicated thousands of times to create the copious amounts of DNA necessary for comparison analysis (Lewin 1997). By using random primers, the random amplification of polymorphic DNA (RAPD) can be used, along with gel electrophoresis, to detect genetic polymorphisms among individuals (Zhou et al., unpublished). The RAPD products can be size-separated on an agarose electrophoresis gel to reveal an ethidium-stained bands, a sort of genetic fingerprint, used to discern individuals. In the DAMD process, the core sequences of minisatellites are used as the primer for PCR to produce RAPD like data to identify polymorphisms in minisatellite regions among individuals (Heath et al. 1993).

This process was used by our group to amplify minisatellite regions in diploid *Tripsacum dactyloides* utilizing the primer FVIIex8. Gel electrophoresis was used to follow genomic polymorphisms in a segregating, diploid F₂ population based on band patterns revealed in the parental and F₁ samples. Data was used to determine a linkage map location of the satellite sequence in the *Tripsacum* genome. Results and discussion of this data is presented herein.
Materials and Methods

Plant Material

All *Tripsacum* plants used in this study were grown at the USDA-ARS Southern Plains Range Research Station, Woodward, Oklahoma. Samples were freeze dried and stored in a -20°C until ground on a Tecator Cyclotec sample mill (Fisher Scientific, cat# TC 1093-002). Ground tissue was then stored in 10 ml scintillation vials in a -20°C freezer until used for DNA extraction.

DNA Isolation

Genomic DNA was isolated and purified at the University of Toledo and Ball State University using a modified procedure of Saghai-Maroof et al. (1984). DNA quantification and qualification was accomplished using UV spectrophotometry at 260 and 280 nm wavelength. The final purified DNA was aliquoted for PCR to a concentration of 10 ng/µl in TE pH 8.0 (10 mM Tris-HCl, pH 8.0 +1.0 mM EDTA, pH 8.0). Remaining genomic DNA was precipitated using 100% ethyl alcohol (2.5ml/ml of sample volume) and 5M NaCl (50 µl/ml of sample volume) and stored in this solution at -20°C for long term storage.

DNA Amplification

The primers used were based on the following minisatellite core sequences: 1) primer 14C2 (GGCAGGATTGAGGC) (Vergnaud 1989); 2) a derivative of 33.6 (GGAGGTGGGCA) (Jeffreys et al. 1985); 3) FVIIex8 (ATGCACACACACAGG) (Murray et al. 1988); and FVIIex8-C (TACGTGTGTTGTGC) (Murray et al. 1988). PCR reactions were performed in a Perkin Elmer GeneAmp® PCR System 2400 Thermal Cycler. The amplification was programmed as: denaturation for 4 min. @ 94°C, followed by 35 cycles of: 1 min. @ 94°C, 1 min. @ 55°C, 1 min. 30 sec. @ 72°C, and a final extension for 10 min. @ 72°C. Each reaction was carried out in a volume of 20 µl containing 100 ng of genomic DNA, 10X thermal buffer (Promega), 10X dNTPs (2.5 mM each dNTP), 2.5 mM MgCl₂, 10µM primer, 1 unit of Taq DNA Polymerase (Promega). The PCR products were electrophoresed in 3% (w/v) agarose, and 1X TAE running buffer (50 mM Tris pH 8.0 base + 0.41 g/l NaOAc, anhydrous, +
0.292 g/l Na₂EDTA; pH 8.0 with glacial acetic acid), at 100V for 8 hours. The loading dye used was 1X SGB (50mM Tris pH 8.0 + 50% glycerol + 5 mM EDTA pH 8.0 + 0.5% SDS + 1.5 mg/ml bromophenol blue, sodium salt, + 1.5 mg/ml Xylene cyanole). The molecular weight markers used was a wide range DNA marker (50bp-10,000 bp, Sigma Chemical Company) and a lambda DNA EcoR I/Hind III digest (Sigma Chemical Company). Amplified DNA fragments were visualized by staining with ethidium bromide [10 mg/ml] (50 µl/500 ml of distilled water for 30 min. followed by destaining with deionized distilled water for 30 min. The gels were then photographed using a Fotodyne UV illuminator, Gel Print 2000i BioPhotonics® Corp. camera system, Mitsubishi P90 Printer, and a Javelin monitor. Linkage analysis was performed using MAPMAKER computer package (Lander et al. 1987).

Results

Four primers (primers 14C2; a derivative of 33.6, FVIIex8, and FVIIex8-C) were originally tested with the male and female parental samples (WW1218, and WW1582) and the first filial (F1) sample (WWI748) for polymorphisms at the PCR level. Multiple-band patterns (5-7 bands) were detected in each sample analyzed in ethidium-stained gels. Two primers, the derivative of 33.6 and 14C2, displayed no polymorphic bands and therefore were not used in subsequent PCR runs. A second gel was run using primers FVIIex8 and FVIIex8-C (photo. 1). Primer FVIIex8 was then chosen for subsequent runs because it displayed two polymorphic bands, one approximately at 1500 bp, present in WW1582 and the F1 sample and a second band at approximately 550 bp (present in parent WW1218 and the F1 sample). The other primer, FVIIex8-C, only displayed one polymorphic band (present in parental WW1218 and the F1 sample) at approximately 800 bp. FVIIex8 was then used in PCR reactions with the second filial generation samples. Segregation was found to be maintained through this generation (photo. 2, 3, 4, and 5 with wide range molecular marker (Sigma Chemical Co.) and in duplicate using a lambda DNA-ECOR I/Hind III double digest as a molecular weight marker). The lower band (550 bp) proved very difficult to amplify and was dropped from analysis. The upper band (1500 bp) was used for segregation analysis. Bands were identified as present (+) or absent (-) and were found to segregate in the following pattern:
This data was entered into the computer program MAPMAKER (ver. 2.0, Lander et al., 1987) along with current *Tripsacum* RFLP map (Blakey, 1993) data to determine if the minisatellite core sequence was linked (maximum recombination of 40%) to any mapped *Tripsacum* loci. Bands that were present were scored as a C, bands that were absent were scored with an A, and samples which did not amplify were scored with a dash (-). MAPMAKER performed pairwise comparisons and two-point linkage analysis between the minisatellite data and existing map data. Primer sequence FVIIex8 was not found to be linked to any of the presently mapped gene loci in the *Tripsacum* genome database.

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+ = band present  
- = band absent  
NA = sample did not amplify
Discussion

The band of interest (Band a, photo #1 ~ 1500 bp) was seen to segregate in a Mendelian fashion through the parental and F1 samples into the F2 generation, therefore the minisatellite sequence corresponding to primer FVIIex8 was stably maintained through the generations.

Analysis with MAPMAKER suggested that the minisatellite sequence was not linked (maximum recombination of 40%) to any of the known mapped loci of the mapped Tripsacum genome. This leads to a number of possible conclusions: 1) The minisatellite amplified by primer FVIIex8 is not linked to any loci in the Tripsacum genome. 2) The minisatellite is linked but the number of population samples analyzed (42) was insufficient to give a representative sample for this locus. 3) The minisatellite is linked, but the loci that it is linked with has not currently been mapped.

Due to time constraints our sample set was small (42). It is very possible that this small F2 sample set is not representative of the true segregation pattern of this minisatellite locus. There was also the other band at approximately 550 bp that proved very difficult to amplify to quantities sufficient for gel electrophoresis analysis, this band may have supplied sufficient data to suggest linkage of this sequence. A possible solution to this would be to adjust PCR conditions to provide sufficient DNA for gel analysis of this band.

The genetic linkage map of the entire Tripsacum genome has not yet been completed. It is possible that this minisatellite sequence is linked to a loci in the Tripsacum genome that is not currently in the genetic map data base. Until the entire genome of Tripsacum dactyloides has been mapped, it will be impossible to determine whether this minisatellite is linked to any single locus or linkage group.

Due to the genetic similarity of maize genes to Tripsacum and vice versa, it is important to examine each of these genomes as completely as possible. Locating minisatellite sequences that are linked to genes in either genome and can be used as genetic markers will be extremely useful.

Possible areas of continued research to be explored includes analysis with additional primers, investigations with a larger population cohort to ensure statistical significance, and variations of PCR conditions that could possibly provide an increased number of polymorphic bands for analysis. Continued mapping of the Tripsacum and maize genomes will also provide helpful reference points.
References


Photo Legend

photo 1) from left to right: lane 1 - empty; lane 2 - WW1748 (F1); lane 3 - WW1582 (parental 2);
  lane 4 - WW1218 (parental 1); lane 5 - empty; lane 6 - empty; lane 7 - WW1748;
lane 8 - WW1582; lane 9 - WW1218; lane 10 - empty. Samples on the left are with primer
FVIIex8-C, Samples on the right are with primer FVIIex8. Polymorphic bands were denoted
with an a or b, band scored for linkage analysis was band a.

photo 2) lanes from left to right: WW1218, WW1582, WW1748, molecular weight marker, samples 13,
  14, 15, 16, 17, 18, 20, 21. All samples were produced using primer FVIIex8.

photo 3) lanes from left to right: samples 22, 23, 24, 25, 101, 104, 2, 3, 4, 5, 6, 8. All samples were
  produced using primer FVIIex8.

photo 4) lanes from left to right: molecular weight marker, samples 9, 106, 107, 108, 111, 112, 113,
  115, 10, 11, 12. All samples were produced using primer FVIIex8.

photo 5) lanes from left to right: samples 19, 26, 28, 30, 31, 32, 33, 34, 35, 36, 114, molecular weight
  marker. All samples were produced using primer FVIIex8.
Photo 3
Acknowledgments

At this time I would like to thank Dr. C. Ann Blakey for all of her help and guidance through my college research career and senior thesis process, without her this thesis would not have evolved. I would also like to thank Dr. Robert Hammersmith for his professional advice and mentorship. I would like to thank all of the professors of the Biology Department of Ball State who have influenced and molded me as a scientist throughout my college career. Lastly, I would like to thank my parents for providing love and support that I have received all my life.