INHERITANCE OF ALLOZYME MARKERS IN SWITCHGRASS (*Panicum virgatum* L.)

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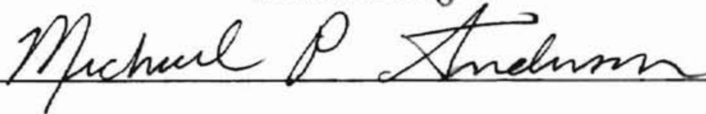

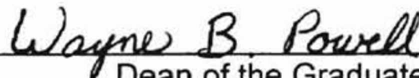

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CHAPTER I

INTRODUCTION

Switchgrass as a Crop Plant

Switchgrass (*Panicum virgatum* L.) is a perennial, cross-pollinated, warm-season grass that is found in all regions of the United States except the Northwest and California (Gould and Shaw, 1983). The species was classified into upland and lowland ecotypes based on morphology and habitat preference (Porter, 1966). Switchgrass is especially valuable in the Central and Southern Plains as a rangeland component and pasture, and has been recognized for its importance in soil conservation (Wolf and Fiske, 1996). Cooperative research on switchgrass breeding and management has been conducted between the U.S. Department of Agriculture and the University of Nebraska since the mid-1930's.

Switchgrass can be grown at low cost to give high stable yields while posing a minimum impact on the environment. As a non-food component of agricultural products, switchgrass offers a profitable alternative to conventional field crops owing to its durability and sustainability. The crop provides good animal forage when utilized in the earlier stages of growth, and mature plants may serve as biofuel feedstock for fuel to supplement traditional energy sources such as oil and gas (McLaughlin, 1993; Vogel and Masters, 1998). Switchgrass can be potentially used to generate chemical by-products for the manufacture of

fertilizers, solvents and plastics, and as substitute fiber for hardwood in the pulp and paper industry. Research programs have developed and released cultivars for commercial use and germplasms for further research and development (Alderson and Sharp, 1994; Hopkins et al., 1995; Vogel et al. 1996). Breeding projects are aimed at improving agronomic characters that are desired for specific purposes. High tillering and late maturing lines are being developed for maximum biomass production (Taliaferro and Das, 2000), while plants that produce fibers with good digestibility are bred for forage in ruminant animal production (Redfearn et al., 1999).

Switchgrass improvement can be achieved through conventional plant breeding methods, and understanding of basic genetic aspects of its genome will aid in its utilization. Information on switchgrass management, germplasm utilization, breeding and genetics has been accumulating (Moser and Vogel, 1995; Hopkins et al., 1996; Hultquist et al., 1996; Martinez-Reyna, 1998; Martinez-Reyna and Vogel, 1998; Taliaferro et al., 1999). As a species with varying ploidy levels and ecotypes, the following questions need to be further addressed: the origin of its polyploid nature (whether auto- or allopolyploidy), the mode of inheritance of characters (whether disomic or polysomic), the genetic variations associated with ploidy levels and ecotypes, and the relative number of genetic loci that are actually expressed at each ploidy level.

Chromosome Numbers and Ploidy Levels

Switchgrass has a basic chromosome number of nine and comprises several ploidy levels. Nielsen (1944) reported chromosome numbers of 18, 36, 54, 72, 90, and 108 among accessions representing a broad geographic range of the USA. A population of switchgrass can be a mixture of plants with varying ploidy levels and aneuploidy, which may contribute to poor seed set (Henry and Taylor, 1989). Aneuploid variants and multivalent pairing of chromosomes were reported at high ploidy levels (Barnett and Carver, 1967), although normal meiosis with bivalent pairing and regular segregation was usually observed in tetraploid and hexaploid accessions (Riley and Vogel, 1982).

Although chromosome numbers in the species have long been established, more detailed cytogenetic analyses were limited because of the small size of the chromosomes. Karyotypes that will characterize the gross morphology of individual chromosomes at lower ploidy levels are not available. Not much information is known about the homology that exists among copies of each chromosome, and if distinct sets of nuclear chromosomes are present. It is also not known whether bivalent meiotic pairing occurs randomly or through preferential pairing of chromosomes.

Cytological examination of switchgrass collected from Oklahoma indicated that lowland types were tetraploids whereas upland types consisted of octoploids and aneuploid variants of octoploids (Brunken and Estes, 1975). Hopkins et al. (1996) examined the nuclear DNA content and chromosome number of switchgrass populations representing an array of upland and lowland types by

means of laser flow cytometry and light microscopy. Based on both techniques, all lowland plants were found to be tetraploids (2n=4x=36) averaging 3.1 picograms DNA per nucleus, with the possible exception of *PMT-785* which contained an octoploid plant. Upland types were mainly octoploids (2n=8x=72) averaging 5.2 picograms DNA per nucleus, except for *Summer* which was tetraploid. The relationship between ploidy level and ecotype remains to be clarified.

Isozyme Markers in Genetic Studies

Isozymes or isoenzymes refer to alternative forms of an enzyme. They arise through amino acid changes that result in a shift in the net charge and electrophoretic migration of the original enzyme molecule, without affecting its catalytic function. These events are therefore classified as neutral mutations. Although a relatively old technique, isoenzyme electrophoresis coupled with histochemical staining (Hunter and Markert, 1957), remains a valuable tool for examining single gene variation in natural populations. The technique is particularly useful for plant species where genetic studies are generally limited by the scarcity of monogenic characters. Molecular data based on codominant allozyme markers, isozymic forms coded by the same genetic locus, facilitate determination of actual genotypes and genetic variation that can be associated to plant morphology, cytology, and ecological adaptation (Kephart, 1990). Isozymes have been useful for studying the origins and inheritance in polyploid plant species (Krebs and Hancock, 1989; Gutierrez et al., 1993; de Vicente and

Arus, 1996), for estimating genetic diversity in cultivated crops (Hamrick and Godt, 1997; Warnke et al., 1998), and for facilitating plant breeding works (Orton, 1983; Zamir, 1983). Tanksley and Rick (1980) noted the advantages of isozyme markers as having stable expression in a wide range of environments, while demonstrating no epistatic interrelationships thus permitting several genes to be surveyed simultaneously. In general, information based on molecular markers has provided substantial evidence on the mode of inheritance, parentage, formation, and immediate genetic consequences of polyploidy (Soltis and Soltis, 1995).

Objectives of the Study

The primary purpose of this study was to determine the mode of genetic segregation of selected allozyme markers in tetraploid switchgrass populations. Chromosome pairing in meiosis is mostly bivalent in tetraploid cultivars although multivalents are also observed (Riley and Vogel, 1982). Allozyme segregation was used to illustrate whether inheritance of traits is disomic as a result of specific bivalent chromosome pairing, or tetrasomic due to random bivalent pairing among four homologous chromosomes.

Isoenzyme polymorphism has not been characterized in switchgrass. As a consequence of the major objective, electrophoretic profiles of several enzyme systems were examined in the species, and specific isozyme loci were identified. Information obtained in this study should add to what is presently known about the genetics of switchgrass.

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CHAPTER II

SEGREGATION ANALYSIS OF ALLOZYME MARKERS IN TETRAPLOID SWITCHGRASS POPULATIONS

ABSTRACT

Switchgrass, *Panicum virgatum* (L.), has several levels (2x to 12x reported) of ploidy, with 2n=4x=36 and 2n=8x=72 being the most prevalent. However, there is no definitive information on whether the species is an autopolyploid or an allopolyploid, or whether it exhibits disomic or polysomic inheritance. The objectives of this study were to 1) identify polymorphic allozyme markers in tetraploid switchgrass, and 2) determine the pattern and mode of inheritance of the markers. Allozyme segregations at *Adh-1* and *Got-1* in 119 F_2 progenies of a tetraploid switchgrass cross conformed with expected disomic ratios ($p = 0.75–0.90$ and $p = 0.90–0.95$, respectively), and were significantly different from tetrasomic ratios ($p = 0.01–0.025$ and $p < 0.005$, respectively). These results suggest specific bivalent chromosome pairing in meiosis as opposed to random bivalent pairing among four copies of chromosomes. Analyses of PGM, LAP, and PGI in two progeny populations yielded nine data sets all conforming to the segregation of a simplex parental genotype with three doses of a null allele. Selfing of this genotype produces the same expected progeny ratio with disomic and tetrasomic inheritance. Null

alleles likely provide a means to downregulate expression of duplicate isozyme genes, and may present an opportunity for these genes to diversify. The following isozyme loci in switchgrass are proposed: *Adh-1, Got-1, Got-2, Pgm-1, Pgm-2, Pgm-3, Pgm-4, Lap-1, Lap-2, Lap-3, Lap-4, Pgi-1, Pgi-2, Pgi-3* and *Pgi-4*.

INTRODUCTION

One of the important populational aspects of polyploidy is the mode of inheritance which influences the resulting frequencies of genes and gene combinations in progenies of crosses. Studies of isozyme polymorphism and inheritance often provided clues about the effective number of genetic loci in polyploids, and how the genetic variation is distributed within and among loci (Gottlieb, 1982). Autopolyploids were shown to possess higher enzyme multiplicity and heterozygosity than their diploid progenitors as a direct result of polysomic inheritance.

Polysomic inheritance of isozyme markers was reported in a number of autopolyploid plant species, including alfalfa (Quiros, 1982), potato (Martinez-Zapater and Oliver, 1984; Quiros and McHale, 1985), blueberry (Krebs and Hancock, 1989), and the forage crop sainfoin (de Vicente and Arus, 1996). On the other hand, disomic inheritance of allozymes was reported in the tetraploid *Tragopogon* (Roose and Gottleib, 1976), cherry (Beaver and Iezzoni, 1993), and creeping bentgrass (Warnke et al., 1998), as well as in the hexaploid wheat (Hart, 1983) and in the octoploid strawberries (Arulsekar et al., 1981). In some instances, both polysomic and disomic inheritance were observed in the same species which may indicate segmental allopolyploidy (de Vicente and Arus, 1996).

Chromosome pairing in meiosis is not a conclusive method for discriminating between allo- and autopolyploids, as autotetraploid inheritance has been shown to occur through random bivalent pairing (Krebs and Hancock,

1989). Bivalents in polyploids may form through preferential pairing of chromosomes under the direction of pairing control genes, resulting in disomic inheritance. In wheat, the existence of pairing homoelogous (*Ph*) genes which restrict intergenomic chromosome pairing are well documented (Sears, 1976). Genetic divergence between component genomes of polyploids can be viewed in terms of pairing ability which may be due to one or a few genes (Jackson, 1982).

MATERIALS AND METHODS

Plant Materials

Parents and F_1s of intraploidy crosses from the Switchgrass Breeding Program at the Oklahoma State University, were used to identify polymorphic enzyme systems in the species. F_1 hybrids from tetraploid parents (2n=4x=36) were selfed to generate segregating F_2 progenies. Two crosses producing the highest numbers of F_2 plants were chosen for the inheritance study:

Cross 1: Female Parent: *Summer Rg2 R14 #5*, upland cultivar

Male Parent: *Alamo R1 #6*, lowland cultivar

F_1: *96-93B*; F_2 progenies were referred to as *96-93B* $\otimes$

Cross 2: Female Parent: *Alamo R7 #3*, lowland cultivar

Male Parent: *Kanlow R2 #3*, lowland cultivar

F_1: *96-144I*; F_2 progenies were referred to as *96-144I* $\otimes$

Starch Gel Electrophoresis of Isozymes

Horizontal starch gel electrophoresis was performed to visualize isoenzyme patterns initially for 15 commonly assayed enzyme systems. Electrophoresis and enzyme assays were according to the procedures of Glaszmann et al. (1988) and Manchenko (1994) with modifications. Suitable plant tissues to use (roots, young shoots, or green leaves) were identified, and electrophoresis conditions were optimized for each enzyme. For each plant, about 100 mg of plant tissue was ground in 200 ul solution containing 0.05 M

Tris-HCl buffer (pH 8.5), 0.05% 2-mercaptoethanol, 0.25% dithiothreitol, and 1% (w/v) polyvinyl-pyrrolidone using a mortar and a pestle. The crude protein extracts were absorbed in 1.0 X 0.7 cm-filter paper wicks (Whatman No. 3), which were then inserted into the slit of a 14% (w/v) starch gel to form a continuous arrangement. The starch gel held by an acrylic mold was mounted onto electrode trays inside the refrigerator, and a constant current of 65-75 milliamperes was applied to permit 3.5 hours of electrophoretic separation of proteins. The composition of gel and electrode buffers is listed in Appendix A. After electrophoresis, both anodal and cathodal parts of the gels were cut horizontally by passing a thin wire to produce several 1-mm slices. Gel slices were subjected to specific histochemical staining procedures (Appendix B) to visualize bands corresponding to zones of enzymatic activity. Bands were compared among plant samples for relative migration distances and intensities.

Allozyme Segregation Analysis

Five enzyme systems that displayed consistently well-resolved bands on zymograms were studied for allozyme segregation in the F_2 progenies. These enzymes were alcohol dehydrogenase (ADH), glutamate-oxaloacetate transaminase (GOT), phosphoglucomutase (PGM), leucine aminopeptidase (LAP), and phosphoglucose isomerase (PGI). ADH was assayed from young shoots, while zymograms for the four other enzymes were produced using green leaf tissues.

Allozyme segregation at each putative locus was analyzed in this manner:

1. Quaternary structure of the enzyme (monomeric or dimeric) and the number of isozyme loci present were established by examining the zymograms and by consulting the literature describing the general features of plant isozymes (Gottlieb, 1982; Weeden and Wendel, 1989).
2. Alleles at each locus were labeled consecutively with capital letters starting from the one nearest the origin of migration.
3. Genotypes of the parents were determined.
4. F_2 progeny ratios were predicted based on disomic and tetrasomic segregation assumming random chromosome segregation. Observed progeny ratios were compared with the expected disomic and tetrasomic ratios using the Chi-Square test ($p < 0.05$, significantly different ratios).

RESULTS

Fifteen isozyme loci belonging to five enzyme systems were identified in this study: *Adh-1, Got-1, Got-2, Pgm-1, Pgm-2, Pgm-3, Pgm-4, Lap-1, Lap-2, Lap-3, Lap-4, Pgi-1, Pg-2, Pgi-3* and *Pgi-4*. Two patterns of variation were observed for these genes. One pattern exhibited by ADH and GOT was characterized by loci wherein all four chromosomes carry alleles that produce active enzyme products. This indicated the tetraploid nature of the populations studied, and the existence of homologous or homeologous copies of chromosomes. Another pattern was displayed by PGM, LAP, and PGI, and was distinguished by the presence of duplicated loci, each locus apparently represented only by a single isomorph that has a corresponding null allele.

Alcohol Dehydrogenase, EC 1.1.1.1

(function: fermentation; common features in plants: dimeric, 1-3 cytosolic loci)

The single region of enzyme activity on the zymograms was identified as *Adh-1*, which displayed two alleles in the tetraploid plants examined (Fig. 1). The polypeptide products of these alleles form dimers, and heterozygous plants displayed the characteristic three-banded phenotypes. Five genotypes were distinguished from on the zymograms: two homozygotes (AAAA and BBBB), two single heterozygotes (AAAB and ABBB), and a double heterozygote (AABB).

Segregation in *96-93B* $\otimes$. Observed genotypic progeny classes and ratio were 4AAAA:32AAAB:45AABB:30ABBB:8BBBB. The genotype of the F_1 parent based on banding pattern was AABB. Assuming disomic inheritance

(AB/AB), the expected gametic ratio is 1AA:2AB:1BB for both chromosome pairs and the expected genotypic progeny ratio is 1AAAA:4AAAB:6AABB:4ABBB: 1BBBB. Comparison of this predicted disomic ratio with the observed ratio indicated goodness-of-fit ($p = 0.75–0.90$, Table 1.1). With tetrasomic inheritance (AABB⊗), the predicted gametic ratio was 1AA:4AB:1BB and the expected genotypic progeny ratio was 1AAAA:8AAAB:18AABB:8ABBB:1BBBB. The observed ratio in this case deviated significantly from tetrasomic segregation ($p = 0.01–0.025$, Table 1.2).

The disomic situation AA/BB, can be thought of where the two pairs of chromosomes each carry a different allele in two doses. This would be a better illustration of disomy suggesting the existence of two divergent diploid loci for the same gene which may not necessarily cosegregate as can be observed in allotetraploids. Here, selfing will only produce double heterozygous progenies similar to the F_1 parent, and heterozygosity is said to be fixed. This was not the case since five progeny classes were observed indicating that the two pairs of chromosomes were homologous and carried both alleles (AB/AB). Thus, *Adh-1* exemplifies typical genetic loci in autotetraploids that were simply duplicated as a result of doubling of the parental diploid genome.

Segregation in *96-144I*⊗. The F_1 genotype based on banding pattern was AAAB. Three genotypic progeny classes were observed with ratio 16AAAA:33AAAB:20AABB. Assuming disomic inheritance (AA/AB), the expected gametic ratios were 1AA and 1AA:2AB:1BB for the two chromosome pairs and the expected genotypic progeny ratio was 1AAAA:2AAAB:1AABB.

Comparison of this disomic ratio with the observed ratio indicated goodness-of-fit ($p = 0.50–0.75$, Table 1.3). With tetrasomic segregation (AAAB$\otimes$), the expected gametic ratio was 1AA:1AB and the expected genotypic progeny ratio was the same as that for disomic segregation. This situation does not distinguish between the two modes of inheritance. However, this result supported the proposed allelism at *Adh-1* since the observed ratio fit the expectation.

Glutamate-Oxaloacetate Transaminase, EC 2.6.1.1

(function: intermolecular transfer of amino groups; common features in plants: dimeric, up to 4 loci)

Two well-separated regions of GOT activity were observed, and were identified as *Got-1* and *Got-2* (Fig. 2). A faint zone of interlocus heterodimers was present between these two loci suggesting that their protein products are located within the same subcellular compartment. *Got-2* showed no scorable variability among the plants examined but appeared to comprise more than one band. The slower migrating region constitutes *Got-1* which revealed nine band positions corresponding to the homo- and heterodimer products of four alleles (A, B, C, and D). Dye reaction was less intense for bands further from the origin of migration and band intensity ratios may not have been displayed as expected under ideal conditions. Because of this, only the presence or absence of bands and not the allele dosage was considered when scoring some progeny genotypes.

Segregation in *96-93B*⊗. The F_1 genotype was ABCD based on the banding pattern. With tetrasomic inheritance (ABCD⊗), the expected gametic ratio was 1AB:1AC:1AD:1BC:1BD:1CD and there were 19 genotypic progeny classes expected at the following ratio: 1AABB:1AACC:1AADD:1BBCC:1BBDD: 1CCDD:2AABC:2ABBC:2ABCC:2AABD:2ABBD:2ABDD:2AACD:2ACCD: 2ACDD:2BBCD:2BCCD:2BCDD:6ABCD. Pooling together the genotypes that were difficult to distinguish from each other on the zymograms, the tetrasomic ratio was reduced to 11 classes: 1AABB:1AACC:1AADD:1BBCC:1BBDD: 1CCDD:6ABC:6ABD:6ACD:6BCD:6ABCD. A set of 9 specific progeny classes (out of the 19 enumerated above) was expected with disomic segregation, of which there were three possible cases based on the alleles carried by each pair of chromosomes, AB/CD, AC/BD, and AD/BC. Observed progeny classes and ratio were 9AACC: 10AADD:8BBCC:8BBDD:15ABC:11ABD:12ACD:13BCD: 33ABCD, which fit the disomic ratio resulting from allele combination AB/CD (p = 0.90–0.95, Table 2.2). The nine progeny types expected under the assumption of disomic segregation were 1AACC:1AADD:1BBCC:1BBDD:2ABCC:2ABDD: 2AACD:2BBCD:4ABCD (Fig. 2), which complement the observed classes. The two other cases of disomic segregation were not consistent with the observed ratio (p= 0.01–0.025 for AC/BD, p = 0.025–0.05 for AD/BC, Tables 2.3 and 2.4). Notably, genotypes AACC and BBDD, and genotypes AADD and BBCC, not expected with AC/BD and AD/BC combinations, respectively, were observed. In addition, genotypes AABB and CCDD expected in both cases were not carried by any progeny. The expected tetrasomic ratio was significantly different from

the observed ratio with $p < 0.005$ (Table 2.1).

Segregation in *96-144*/$\otimes$. The F_1 genotype was predicted to be ABCC and the expected tetrasomic progeny ratio was 1AABB:1CCCC:8ACC(4AACC+4ACCC):8BCC(4BBCC+4BCCC):18ABC(4AABC+4ABBC+10ABCC). Two possible allele combinations for disomic segregation are AB/CC yielding a progeny ratio of 1AACC:2ABCC:1BBCC, and AC/BC yielding a progeny ratio of 1AABB:1CCCC: 3ACC(1AACC+2ACCC):3BCC(1BBCC+2BCCC): 8ABC(2AABC+2ABBC+ 4ABCC). The observed progeny classes and ratio, 11AC:14BC:43ABC, fit tetrasomic segregation ($p = 0.10–0.25$, Table 2.5) as well as disomic segregation resulting from allele combination AB/CC ($p = 0.05–0.10$, Table 2.6), but were significantly different from disomic segregation resulting from AC/BC combination ($p = 0.01–0.025$, Table 2.7). However, the genotypes AABB and CCCC expected with tetrasomic segregation were not observed from the 68 progenies examined. If this remains true after analyzing a larger number of plants, the data will no longer fit tetrasomic expectation. The deviation of the observed ratio from disomic segregation AB/CC was nearly significant because of an apparent excess of genotype ABCC over AACC and BBCC (Table 2.6). The latter two genotypes, though, showed the expected 1:1 ratio, such that this deviation was not likely a result of random chance but due to an unknown factor that selects against these genotypes. This should also be resolved by examining a larger number of progenies. The genotype ABCO (O for null allele) was also tested in place of ABCC as the genotype of the F_1 parent but significant

results were obtained for tetrasomic and three cases of disomic segregation ($p < 0.005$ for all cases, Chi-Square tests not shown).

Overall, the data based on two populations supported the existence of the *Got-1* locus having at least four electrophoretically distinguishable alleles that are specifically paired and therefore segregate in a disomic fashion (AB/CD). Absence of an allele is likely often balanced by doubling the dosage of the other allele in the pair. The *Got-1* locus is a convenient system for this kind of analysis because several genotypic classes can be scored from zymograms, and some classes are not expected for certain modes of genetic segregation. This provides another basis for declaring goodness-of-fit in addition to the Chi-Square test. As in the case of the *Adh-1* allozymes, it can be noted that the four allozymes found at *Got-1* although segregating disomically were carried by homologous or homeologous pairs of chromosomes because they were shown to cosegregate as a single locus.

Phosphoglucomutase, EC 2.7.5.1

(function: converts starches to sugars; common features in plants: monomeric, at least 2 loci coding for enzymes localized in the cytosol and in the plastids)

The zymograms suggest a monomeric structure for the PGM enzyme (Fig. 3). Bands at four different positions were observed and were interpreted as alleles of separate loci, *Pgm-1*, *Pgm-2*, *Pgm-3* and *Pgm-4*. These bands were likely not allelic because they were not codominantly inherited, and their activities were generally varied. Each isomorph (designated by the letter A)

representing each PGM locus has a corresponding null allele (designated by the letter O) based on zymograms of two progeny populations (Fig. 3). *Pgm-3* appeared to be an ecotype-specific isomorph as it was present in all lowland parents, *Alamo R1 #6, Alamo R7 #3 and Kanlow R2 #3,* but not in the upland parent *Summer Rg2 R14 #5.* Dosage difference for a PGM allele was sometimes evident among plants but was difficult to score because the alternative allele by which comparison can be made was null. However, presence or absence of bands can be accurately determined. Additional bands near the identified PGM isozymes appeared much later on the zymograms, and may represent secondary or degradation products of primary bands.

Segregation in *96-93B*⊗. For *Pgm-1*, band 1A was either present or absent in the progenies. The observed progeny ratio was 89 A (at least one dose of the active allele was present) : 30 O (all four chromosomes carry null alleles), which fits the ratio 3 A (2AOOO+1AAOO) : 1 OOOO (p = 0.95–0.975, Table 3.1) expected with either disomic or tetrasomic segregation of parental genotype AOOO. The observed progeny ratio at *Pgm-2* was 96 A : 22 O which also fits the segregation of parental genotype AOOO (p = 0.10–0.25, Table 3.2). No segregation was scored for *Pgm-3* and *Pgm-4*. Allele 3A was not carried by the F_1 although present in the male parent while allele 4A was present in all progenies.

Segregation in *96-144I*⊗. Activity for *Pgm-1* and *Pgm-4* was absent in the parents and progenies of this population. The progenies segregated at *Pgm-2* as 53 A : 15 O indicating that the F_1 plant had genotype AOOO (p = 0.50–

0.75, Table 3.3). Allele 3A was present in all progenies at about the same intensity, so that the genotype of the F_1 must be AAAA or AAOO. Genotype AAOO was possible if segregation was disomic, AA/OO, which will only produce self progenies with similar genotype.

Leucine Aminopeptidase, EC 3.4.11.1

(function: protein catabolism, preferably splits leucine; common features in plants: monomeric often with null alleles, 2-3 cytosolic loci)

The zymograms suggest monomeric structure for LAP (Fig. 4). Four bands were observed and were interpreted as alleles of separate loci, *Lap-1*, *Lap-2*, *Lap-3* and *Lap-4*. *Lap- 3* and *Lap-4* often cosegregated in one set of progenies, suggesting tight linkage as a probable result of tandem duplication. Activity of *Lap-2* and *Lap-3* generally varied in different zymograms of the same individual plants, sometimes much lower compared to that of *Lap-1* and *Lap-4*. This may indicate changes in level of gene expression or differential stainability at different times of assays, and is consistent with the assumption of separate LAP loci. Because allele dosage was difficult to determine based on relative intensities of bands, only presence or absence of a band was scored.

Segregation in *96-93B*⊗ . *Lap-3* and *Lap-4* cosegregated in 112 of 117 progenies. Presence of alleles 3A and 4A was scored against a null allele. Observed ratios were 95 3A : 22 O and 96 4A : 21 O, both fitting the ratio 3 AOOO (1AAOO+2AOOO) : 1 OOOO (p = 0.10–0.25 and p = 0.05–0.10 respectively, Tables 4.1 and 4.2) expected from segregation of parental

genotype AOOO. Segregation at *Lap-2* was 86 2A : 31 O also fitting the same theoretical progeny ratio (p = 0.50–0.75, Table 4.3). Allele 1A was present in all progenies and no segregation data were scored for *Lap-1*.

Segregation in *96-144I*⊗. The progenies only varied at *Lap-4* where allele 4A was present in 52 out of 66 plants, fitting expected segregation of genotype AOOO (p = 0.25–0.50, Table 4.4). All plants showed enzyme activity at *Lap-1* and *Lap-2*, and absence of activity at *Lap 3*.

Phosphoglucose Isomerase, EC 5.3.1.9

(function: glycolysis, starch synthesis; common features in plants: dimeric, at least 2 loci coding for enzymes localized in the cytosol and in the plastids)

Two separate regions of PGI activity were present (Figure 5). The more anodal region represented by a single isomorph was identified as *Pgi-4,* and may correspond to the plastid enzyme. Five bands were observed in the more cathodal region and were interpreted as due to the activity of three loci, *Pgi-1*, *Pgi-2* and *Pgi-3*, each represented by a single isomorph. Interlocus heterodimers were observed between *Pgi-1* and *Pgi-2*, and between *Pgi-2* and *Pgi-3*. *Pgi-2* and *Pgi-3* migrated close to each other so that their products tend to smear on the zymograms. No heterodimers were apparent between *Pgi-1* and *Pgi-3*. Polypeptide products of different isozyme genes although they are present in the same cellular location may not be capable of forming intergenic heterodimers when the association is symmetrically unfavorable. Heterodimers were also not formed between *Pgi-4* and the other PGM loci, and this likely

indicates separate subcellular localizations. Only one set of progenies showed variation for a PGI locus.

Segregation in *96-93B*⊗. *Pgi-4* segregated as 93 A : 27 O, fitting the theoretical progeny ratio 3 AOOO (1AAOO+2AOOO) : 1 OOOO ($p = 0.50–0.75$, Table 5.1) expected from segregation of parental genotype AOOO. Segregation at *Pgi-1* was 89 A : 31 O which also fits the same expected ratio ($p = 0.75–0.90$, Table 5.2). All progenies showed activity for *Pgi-2* and *Pgi-3*. It was apparent from the zymograms that the homodimers of allele 1A were more intensely staining than those of allele 2A. Given that 1A was present at a single dose in the F_1 parent and 2A at more than one dose, the observed banding pattern does not support allelism between bands 1A and 2A.

DISCUSSION

Mode of Inheritance

Inheritance of allozyme markers was interpreted in this study based on the assumption of random chromosome segregation. The data provided no evidence of a double reduction event resulting from chromatid segregation. Low occurrence of chromatid segregation for isoenzyme loci has been attributed to localization on short chromosomes with low chiasma frequencies (Soltis and Soltis, 1989).

Allozyme data for *Adh-1* and *Got-1* in tetraploid switchgrass populations clearly indicated disomic inheritance, in agreement with cytological observations that diploid-like pairing of chromosomes can be observed in tetraploid plants (Riley and Vogel, 1982). Data for *Adh-1* based on first 50 randomly chosen F_2 progenies of *96-93B* conformed with disomic segregation ratio. This pattern did not change with each set of 10 plants added to the data although the p-values obtained for the two expected ratios differed more markedly. Likewise for *Got-1*, roughly 50 F_2 progenies of the same population were adequate to obtain good fit to the disomic ratio AB/CD. All nine progeny classes expected with this disomic ratio were observed after analyzing only about 20 plants. These results indicated that the set of F_2 progenies derived from *96-93B* was a truly random population suitable for predicting actual segregation ratios.

Disomic inheritance in polyploids results from specific bivalent pairing of chromosomes in meiosis. This requires pairing control mechanisms such as the

pairing homeologous (*Ph*) genes in wheat (Sears, 1984). Divergence among copies of chromosomes in polyploids may arise through chromosomal changes and rearrangements at the ultrastructural level (Jackson, 1982), and this may also affect pairing ability of homologous or homeologous chromosomes. Whether specific bivalent pairing in switchgrass is caused by *Ph*-like genes or due to structural differentiation between different pairs of chromosomes, presents a separate subject for investigation.

Genetic analyses of PGM, LAP and PGI enzymes did not distinguish between the two modes of inheritance because all nine sets of segregation data scored (Tables 3, 4 and 5) involved selfing a simplex parental genotype with three doses of a null allele (AOOO). This yields the same expected progeny ratios with disomic and tetrasomic segregation. Oddly, parental genotype AAOO was not encountered in the several duplicate loci of these enzyme systems. Selfing of genotype AAOO will produce a progeny ratio of 35 A (1AAAA:8AAAO:18AAOO: 8AOOO) : 1 OOOO with tetrasomic segregation, and 15 A (1AAAA:4AAAO: 6AAOO:4AOOOO) : 1 OOOO with disomic segregation (AO/AO). In instances where all progenies of either population appeared to show activity for the single isomorph of a PGM, LAP or PGI locus, not a single progeny that was null for the locus was recovered to suggest possible occurrence of parental genotype AAOO segregating as described. Missing these rare nulls was unlikely because the size of one of the populations (119 progenies) was large enough, and this population seemed to have unbiased representation of progenies based on the data obtained for ADH and

GOT. One possible explanation for this was that those plants were not recovered because they were completely null for all isozyme genes of the enzyme. This might be the case for *Pgm-4* and *Lap-1* in the progenies of *96-93B*, and for *Pgm-3* in the progenies of *96-144I* (Figs. 3 and 4). Another hypothesis would be that one pair of chromosomes in tetraploid switchgrass has been rendered permanently silenced for most of the duplicate loci of PGM, LAP and PGI. Only the other pair of chromosomes expresses the gene, which at the same time may carry a null allele. Thus, a plant may have the genotype AA/OO, AO/OO, or OO/OO. Genotypes AOOO and OOOO were directly demonstrated with the data presented in this study. Genotype AAOO must have been carried by the F_1 plants when all progenies showed activity for an isozyme locus, as selfing of this genotype under disomic segregation AA/OO yields progenies with similar genotype only. This hypothesis implies specific chromosome pairing, disomic inheritance, and possibly an undergoing process of differentiation between the two copies of genomes in the tetraploid switchgrass.

Further crosses and progeny analysis may be performed to demonstrate the existence of genotype AAOO segregating disomically as AA/OO. For example, the two F_1 plants utilized in this study can be crossed to determine their actual genotypes and modes of segregation at *Pgm-4* and *Pgi-3* (for *96-93B*), and at *Pgm-3* (for *96-144I*) (Figs. 3 and 5). This cross will essentially involve a plant with an "A" phenotype and one that is null for the locus. The expected genotypic and phenotypic progeny ratio in such cross for each of five combinations of possible genotypes and modes of segregation is given in

Table 6. As few as 20 F_1 progenies need to be examined in order to distinguish genotype AAOO segregating tetrasomically or disomically as AO/AO (cases 1 and 2) from the three other possible combinations of genotypes and modes of inheritance. The cross between genotypes AAOO and OOOO with tetrasomic and disomic segregation (AO/AO) will give phenotypic progeny ratios of 5 A : 1 O and 3 A : 1 O respectively, which are easier to illustrate than the 35 A : 1 O and 15 A : 1 O ratios expected when genotype AAOO segregating as such is selfed. If nulls are not recovered, the first two cases are ruled out. The three other genotypes described in Table 6 can then be distinguished from each other by crossing a few F_1 plants produced from the first step with a null for the loci being studied (*Pgm-3*, *Pgm-4* and *Pgi-3*) to generate another 20 hybrids per cross. This null must be selected such that no complete nulls for PGM and PGI would be produced in these crosses. Genotype AAAA (case 3) will produce all AAOO progenies in the first cross, and these progenies will in turn produce some nulls in the second cross. Genotype AAAO (case 4) will produce two progeny classes, AAOO and AOOO in the first cross, and progenies of class AAOO will produce some nulls in the second cross. Genotype AAOO segregating disomically as AA/OO (case 5) will not produce a null in both the first and second crosses.

Null Alleles in Duplicated Isozyme Genes

The majority of genes present in newly formed polyploids, especially in nonhybrid polyploids, are expected to be duplicated as a direct result of polyploidy (Hart, 1983). Duplicated genes found in polyploids are also generated by other mechanisms such as unequal crossing-over, and crossing-over in inversion and translocation heterozygotes, brought about by the instability of newly formed polyploids. These processes must have generated the duplicate genes of PGM, LAP and PGI that are found in switchgrass. These duplications may be present in the same chromosome such as in the case of *Lap-3* and *Lap-4*, or in non-homologous chromosomes.

The existence of multiple isozyme loci for an enzyme as observed in PGM, LAP and PGI denotes a polymorphic state, such that the individual loci need not be polymorphic. Thus, introduction of new allozymic forms seemed to occur favorably in certain loci but not in others, assuming that the rate of single DNA base changes is roughly the same for all genes. This must be particularly true for duplicate loci whose enzyme products, aside from sharing the same general function, do not exhibit much specificity for substrates. The duplicate loci of PGM, LAP and PGI were found close to each other and were not spread-out into separate regions of activity on the zymograms, suggesting that their protein products were highly similar in structure. Moreover as suggested in the zymograms, an individual plant only needs to carry a single active isomorph to produce a sufficient amount of the enzyme. Null alleles are likely a means to downregulate redundant expression of duplicate isozyme loci in polyploid

genomes, where gene duplications are further amplified by the presence of more than one set of genome. Economy of cellular resources is promoted by transcribing only one or a few copies of the gene. This illustrates well that the absolute number of genes in polyploids is not necessarily equal to the number of genetic loci being expressed. The drawback with null alleles being introduced in each duplicate locus, is that plants that are completely null for a given gene will be produced from some crosses. Deficiency for an enzyme that has a key role in basic metabolism may explain why some crosses have poor seed set and produce low number of viable progenies. The metabolic deficiency may exert its effect during seed development or in the seedling stage. In switchgrass, poor seed germination is attributed mainly to seed dormancy brought about by physical and physiological factors (Haynes et al., 1997). It is possible that other groups of duplicated genes in polyploid grasses exhibit similar phenomenon of downregulation by null alleles. Nevertheless, this situation should not pose a problem for species that reproduce prolifically both through sexual and vegetative means.

Null alleles result from deletions of gene sequences, untranscribed genes, inactive protein products, or from other irregularities at the level of gene expression. The occurrence of null alleles in this study was reproducible and must have been caused by a factor(s) that inhibits gene expression permanently. Gene silencing has been implicated as a major contributing factor to polyploid speciation (Garcia-Olmedo et al., 1978; Werth and Windham, 1990). Newly formed tetraploids appear to express most genes of the two component diploid

genomes (Roose and Gottlieb, 1976; Crawford, 1990), but over time, loci from one or the other genome may be turned off or will evolve new modes of regulation (Ford and Gottlieb, 1999). The loss of expression of duplicate genes may eventually lead to extensively diplodized genomes resulting to regulatory or functional divergence of duplicate genes (Soltis and Soltis, 1993). Current researches on this subject aim to describe at the molecular level the fate of duplicate genes during the formation and establishment of polyploid plant species (Gottlieb and Ford, 1997; Ford and Gottlieb, 1999). Duplicate genes with known functions are isolated, sequenced and compared to determine whether or not they maintain their original functions, or if new gene functions are acquired through neomorphic mutations.

Limitations of the Zymogram Technique

Despite its popularity, the zymogram technique has been characterized by irreproducibility and susceptibility to quantitative inaccuracy (Kephart, 1990), and that optimization of laboratory protocols is highly necessary. Many variables affect the preparation and analysis of gels for plants of various ploidal levels and phylogenetic histories (Carr and Johnson, 1980). The banding patterns for the five enzyme systems chosen for this experiment were reproducible. Many plants were scored repeatedly utilizing tissues harvested at different times and the same genotypes for each plant were obtained. As an added check, two to three samples of the appropriate tissues were harvested from each plant each time and pooled. Care was taken to equalize the concentration of protein

extracts among plants by measuring or weighing the tissues and adding a proportionate amount of extraction buffer. This improved the appearance of the zymograms and helped achieve parallel alignment of similar bands or alleles on the gel.

Other enzyme systems were initially examined in this study. Patterns for alanine aminopeptidase, arginine aminopeptidase, malic enzyme, shikimate dehydrogenase and proteinase were generally monomorphic even among plants of different ploidy levels. Multiple bands were obtained from zymograms of peroxidase, esterase, acid phosphatase, isocitrate dehydrogenase and malate dehydrogenase. Often the bands were clear enough for scoring although overlapping bands were sometimes observed.

Isozyme or allozyme markers correspond to whole gene sequences coding for proteins with known cellular functions, in contrast to DNA markers that are derived from short DNA sequences. For studying the mode of inheritance in polyploids, allozymes provide more significant information and are generally easier to interpret compared to alleles of random DNA markers such as Restriction Fragment Length Polymorphism and Randomly Amplified Polymorphic DNA. Isozyme markers are relatively few, which is the major limitation of the technique. Zymogram analysis was mainly designed for soluble proteins that function in glycolysis or the citric acid cycle. Moreover, some isozyme loci are typically monomorphic in a given population or species. Variations in DNA or amino acid sequences may not be detected in zymograms if they do not impart a significant shift in the net charge of the proteins. In

tetraploids, only duplex genotypes which possess balanced frequencies of alleles can distinguish between disomic and tetrasomic segregation and not the simplex genotypes. However, duplex genotypes are twice less frequent than simplex genotypes. It is thus necessary to employ different sets of progenies in order to determine the mode of segregation for several isozyme genes.

Notes on the Tetraploid Switchgrass Crosses

All allozyme data obtained in this study conformed with at least one known mode of Mendelian segregation which suggests absence of skewness. Also, no off-type genotypes were obtained dismissing the possibility of including plants produced through cross-pollination between the F_1 parents and nearby plants. Progeny genotypes were represented even if there were many classes being scored, such as in the case of the tetraallelic locus of *Got-1*. In a few instances, the Chi-Square tests did not provide very strong support for goodness-of-fit to a hypothesized progeny ratio (Tables 2.6, 3.2, 4.1 and 4.2). This may be due to sampling errors associated with population size, or to unknown factors that select against the underrepresented classes.

It was clearly suggested by the isozyme data that switchgrass crosses made with parents differing in ecotype generally possess higher polymorphism than when parents are of same ecotype. For duplicated enzyme systems with null alleles, the possibility of not finding certain isomorphs is high for crosses involving the same ecotype (Figs. 3, 4 and 5). Frequencies of alleles can be expected to vary greatly among populations occupying different ecological

habitats. This was illustrated by cluster analysis based on Randomly Amplified Polymorphic DNA markers which segregated switchgrass populations into two main groups consistent with the morphological differences between ecotypes (Gunter et al., 1996).

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Table 1.1

Chi-Square test comparing the observed and expected disomic segregation (AB/AB) ratios for the *Adh-1* locus in the self progenies of *96-93B*.

F1 genotype tested was AABB.

classes	expected ratio: disomic (AB/AB)	observed ratio	E	Chi-Square
AAAA	1	4	7.44	1.59
AAAB	4	32	29.75	0.17
AABB	6	45	44.63	0.00
ABBB	4	30	29.75	0.00
BBBB	1	8	7.44	0.04
total	16	119	119.00	1.81

p = 0.75-0.90, not signif.

Table 1.2

Chi-Square test comparing the observed and expected tetrasomic segregation ratios for the *Adh-1* locus in the self progenies of *96-93B*.

F1 genotype tested was AABB.

classes	expected ratio: tetrasomic	observed ratio	E	Chi-Square
AAAA	1	4	3.31	0.15
AAAB	8	32	26.44	1.17
AABB	18	45	59.50	3.53
ABBB	8	30	26.44	0.48
BBBB	1	8	3.31	6.67
total	36	119	119.00	11.99

p = 0.01-0.025, signif.

Table 1.3

Chi-Square test comparing the observed and expected tetrasomic=disomic segregation ratios for the *Adh-1* locus in the self progenies of *96-144I*.

F1 genotype tested was AAAB.

classes	expected ratio: tetrasomic=disomic	observed ratio	E	Chi-Square
AAAA	1	16	17.25	0.09
AAAB	2	33	34.50	0.07
AABB	1	20	17.25	0.44
total	4	69	69.00	0.59

p = 0.50-0.75, not signif.

Table 2.1

Chi-Square test comparing the observed and expected tetrasomic segregation ratios for the *Got-1* locus in the self progenies of *96-93B*.

F1 genotype tested was ABCD.

classes	expected ratio: tetrasomic	observed ratio	E	Chi-Square
AABB	1	0	3.31	3.31
AACC	1	9	3.31	9.81
AADD	1	10	3.31	13.56
BBCC	1	8	3.31	6.67
BBDD	1	8	3.31	6.67
CCDD	1	0	3.31	3.31
ABC	6	15	19.83	1.18
ABD	6	11	19.83	3.93
ACD	6	12	19.83	3.09
BCD	6	13	19.83	2.35
ABCD	6	33	19.83	8.74
total	36	119	119.00	62.61

p < 0.005, signif.

Table 2.2

Chi-Square test comparing the observed and expected disomic (AB/CD) segregation ratios for the *Got-1* locus in the self progenies of *96-93B*.

F1 genotype tested was ABCD.

classes	expected ratio: disomic (AB/CD)	observed ratio	E	Chi-Square
AACC	1	9	7.44	0.33
AADD	1	10	7.44	0.88
BBCC	1	8	7.44	0.04
BBDD	1	8	7.44	0.04
ABCC	2	15	14.88	0.00
ABDD	2	11	14.88	1.01
AACD	2	12	14.88	0.56
BBCD	2	13	14.88	0.24
ABCD	4	33	29.75	0.36
total	16	119	119.00	3.45

p = 0.90-0.95, not signif.

Table 2.3

Chi-Square test comparing the observed and expected disomic (AC/BD) segregation ratios for the *Got-1* locus in the self progenies of *96-93B*.

F1 genotype tested was ABCD.

classes	expected ratio: disomic (AC/BD)	observed ratio	E	Chi-Square
AABB	1	0	6.38	6.38
AADD	1	10	6.38	2.06
BBCC	1	8	6.38	0.41
CCDD	1	0	6.38	6.38
ABBC	2	15	12.75	0.40
AABD	2	11	12.75	0.24
ACDD	2	12	12.75	0.04
BCCD	2	13	12.75	0.00
ABCD	4	33	25.50	2.21
total	16	102	102.00	18.12

p = 0.01-0.025, signif.

Table 2.4

Chi-Square test comparing the observed and expected disomic (AD/BC) segregation ratios for the *Got-1* locus in the self progenies of *96-93B*.

F1 genotype tested was ABCD.

classes	expected ratio: disomic (AD/BC)	observed ratio	E	Chi-Square
AABB	1	0	6.31	6.31
AACC	1	9	6.31	1.14
BBDD	1	8	6.31	0.45
CCDD	1	0	6.31	6.31
AABC	2	15	12.63	0.45
ABBD	2	11	12.63	0.21
ACCD	2	12	12.63	0.03
BCDD	2	13	12.63	0.01
ABCD	4	33	25.25	2.38
total	16	101	101.00	17.30

p = 0.025-0.05, signif.

Table 2.5

Chi-Square test comparing the observed and expected tetrasomic segregation ratios for the *Got-1* locus in the self progenies of *96-144I*.

F1 genotype tested was ABCC.

classes	expected ratio: tetrasomic	observed ratio	E	Chi-Square
AABB	1	0	1.89	1.89
CCCC	1	0	1.89	1.89
ACC	8	11	15.11	1.12
BCC	8	14	15.11	0.08
ABC	18	43	34.00	2.38
total	36	68	68.00	7.36

p = 0.10-0.25, not signif.

Table 2.6

Chi-Square test comparing the observed and expected disomic (AB/CC) segregation ratios for the *Got-1* locus in the self progenies of *96-144I*.

F1 genotype tested was ABCC.

classes	expected ratio: disomic (AB/CC)	observed ratio	E	Chi-Square
AACC	1	11	17.00	2.12
ABCC	2	43	34.00	2.38
BBCC	1	14	17.00	0.53
total	4	68	68.00	5.03

p = 0.05-0.10, not signif.

Table 2.7

Chi-Square test comparing the observed and expected disomic (AC/BC) segregation ratios for the *Got-1* locus in the self progenies of *96-144I*.

F1 genotype tested was ABCC.

classes	expected ratio: disomic (AC/BC)	observed ratio	E	Chi-Square
AABB	1	0	4.25	4.25
CCCC	1	0	4.25	4.25
ACC	3	11	12.75	0.24
BCC	3	14	12.75	0.12
ABC	8	43	34.00	2.38
total	16	68	68.00	11.25

p = 0.01-0.025, signif.

Table 3.1

Chi-Square test comparing the observed and expected tetrasomic=disomic segregation ratios for the *Pgm-1* locus in the self progenies of *96-93B*.

F1 genotype tested was AOOO.

classes	expected ratio: tetrasomic=disomic	observed ratio	E	Chi-Square
A	3	89	89.25	0.00
O	1	30	29.75	0.00
total	4	119	119.00	0.00

p = 0.95-0.975, not signif.

Table 3.2

Chi-Square test comparing the observed and expected tetrasomic=disomic segregation ratios for the *Pgm-2* locus in the self progenies of *96-93B*.

F1 genotype tested was AOOO.

classes	expected ratio: tetrasomic=disomic	observed ratio	E	Chi-Square
A	3	96	88.50	0.64
O	1	22	29.50	1.91
total	4	118	118.00	2.54

p = 0.10-0.25, not signif.

Table 3.3

Chi-Square test comparing the observed and expected tetrasomic=disomic segregation ratios for the *Pgm-2* locus in the self progenies of *96-144I.*

F1 genotype tested was AOOO.

classes	expected ratio: tetrasomic=disomic	observed ratio	E	Chi-Square
A	3	53	51.00	0.08
O	1	15	17.00	0.24
total	4	68	68.00	0.31

p = 0.50-0.75, not signif.

Table 4.1

Chi-Square test comparing the observed and expected tetrasomic=disomic segregation ratios for the *Lap-3* locus in the self progenies of *96-93B.*

F1 genotype tested was AOOO.

classes	expected ratio: tetrasomic=disomic	observed ratio	E	Chi-Square
A	3	95	87.75	0.60
O	1	22	29.25	1.80
total	4	117	117.00	2.40

p = 0.10-0.25, not signif.

Table 4.2

Chi-Square test comparing the observed and expected tetrasomic=disomic segregation ratios for the *Lap-4* locus in the self progenies of *96-93B*.

F1 genotype tested was AOOO.

classes	expected ratio: tetrasomic=disomic	observed ratio	E	Chi-Square
A	3	96	87.75	0.78
O	1	21	29.25	2.33
total	4	117	117.00	3.10

p = 0.05-0.10, not signif.

Table 4.3

Chi-Square test comparing the observed and expected tetrasomic=disomic segregation ratios for the *Lap-2* locus in the self progenies of *96-93B*.

F1 genotype tested was AOOO.

classes	expected ratio: tetrasomic=disomic	observed ratio	E	Chi-Square
A	3	86	87.75	0.03
O	1	31	29.25	0.10
total	4	117	117.00	0.14

p = 0.50-0.75, not signif.

Table 4.4

Chi-Square test comparing the observed and expected tetrasomic=disomic segregation ratios for the *Lap-4* locus in the self progenies of *96-144I.*

F1 genotype tested was AOOO.

classes	expected ratio: tetrasomic=disomic	observed ratio	E	Chi-Square
A	3	52	49.50	0.13
O	1	14	16.50	0.38
total	4	66	66.00	0.51

p = 0.25-0.50, not signif.

Table 5.1

Chi-Square test comparing the observed and expected tetrasomic=disomic segregation ratios for the *Pgi-4* locus in the self progenies of *96-93B.*

F1 genotype tested was AOOO.

classes	expected ratio: tetrasomic=disomic	observed ratio	E	Chi-Square
A	3	93	90.00	0.10
O	1	27	30.00	0.30
total	4	120	120.00	0.40

p = 0.50-0.75, not signif.

Table 5.2

Chi-Square test comparing the observed and expected tetrasomic=disomic segregation ratios for the *Pgi-1* locus in the self progenies of *96-93B*.

F1 genotype tested was AOOO.

classes	expected ratio: disomic=tetrasomic	observed ratio	E	Chi-Square
A	3	89	90.00	0.01
O	1	31	30.00	0.03
total	4	120	120.00	0.04

p = 0.75-0.90, not signif.

Table 6. Possible genotypes and modes of genetic segregation of F_1 plants at PGM, LAP and PGI loci when the active isomorph (A) is expressed in all F_2 progenies. (O- null allele)

Genotype	Mode of Segregation	Expected Progeny Ratio When Selfed	Expected Progeny Ratio When Crossed With A Null (OOOO)
1. AAOO	disomic: AO / AO	1AAAA:8AAAO:18AAOO:8AOOO: 1OOOO; phenotypic ratio: 35 A :1 O (nulls may be missed in small population)	1AAOO:2AOOO:1OOOO phenotypic ratio: 3 A :1 O
2. AAOO	tetrasomic	1AAAA:4AAAO:6AAOO:4AOOO: 1OOOO; phenotypic ratio: 15A :1O (nulls may be missed in small population)	1AAOO:4AOOO:1OOOO phenotypic ratio: 5 A :1 O
3. AAAA	disomic = tetrasomic	all AAAA	all AAOO phenotype: all A
4. AAAO	disomic = tetrasomic	1AAAA:2AAAO:1AAOO phenotype: all A	1AAOO:1AOOO phenotype: all A
5. AAOO	disomic: AA / OO; gene is permanently silenced in one pair of chromosomes	all AAOO phenotype: all A	all AAOO phenotype: all A

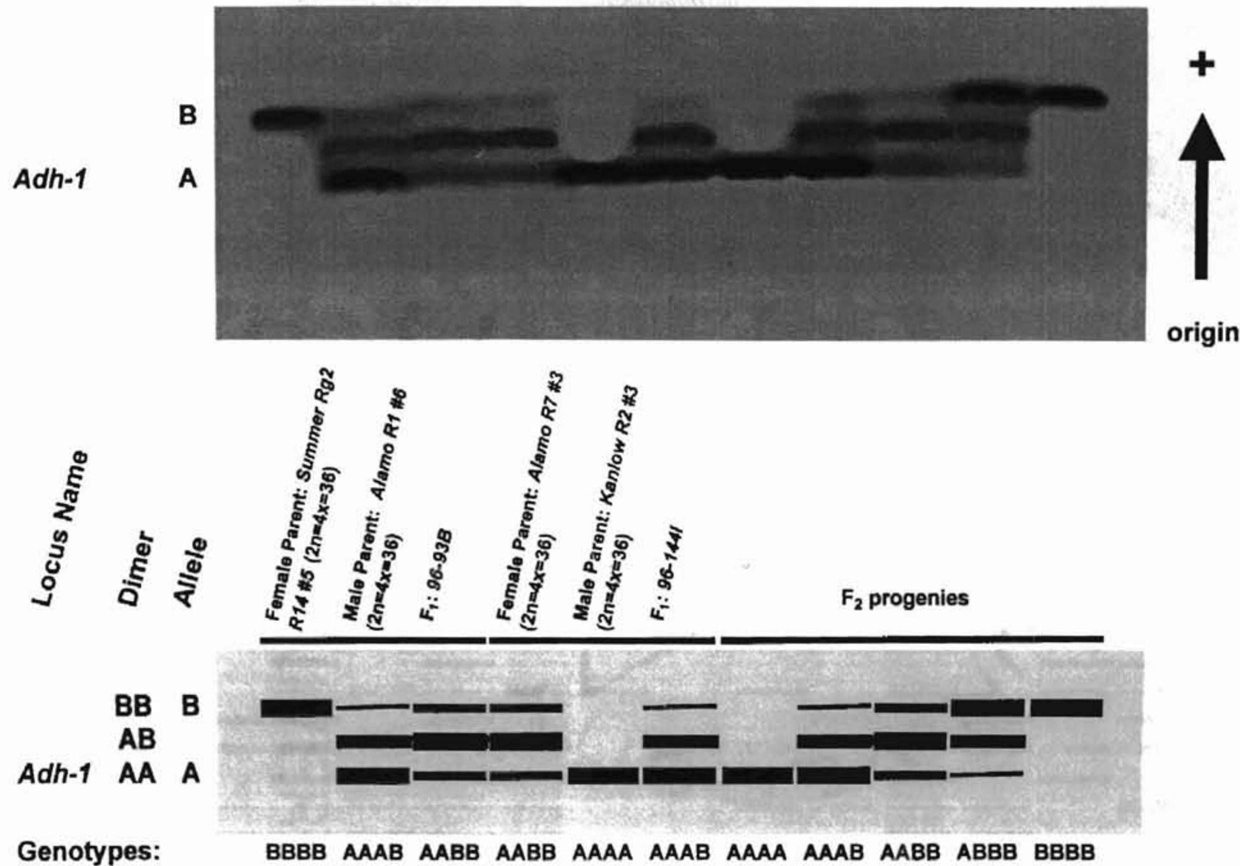
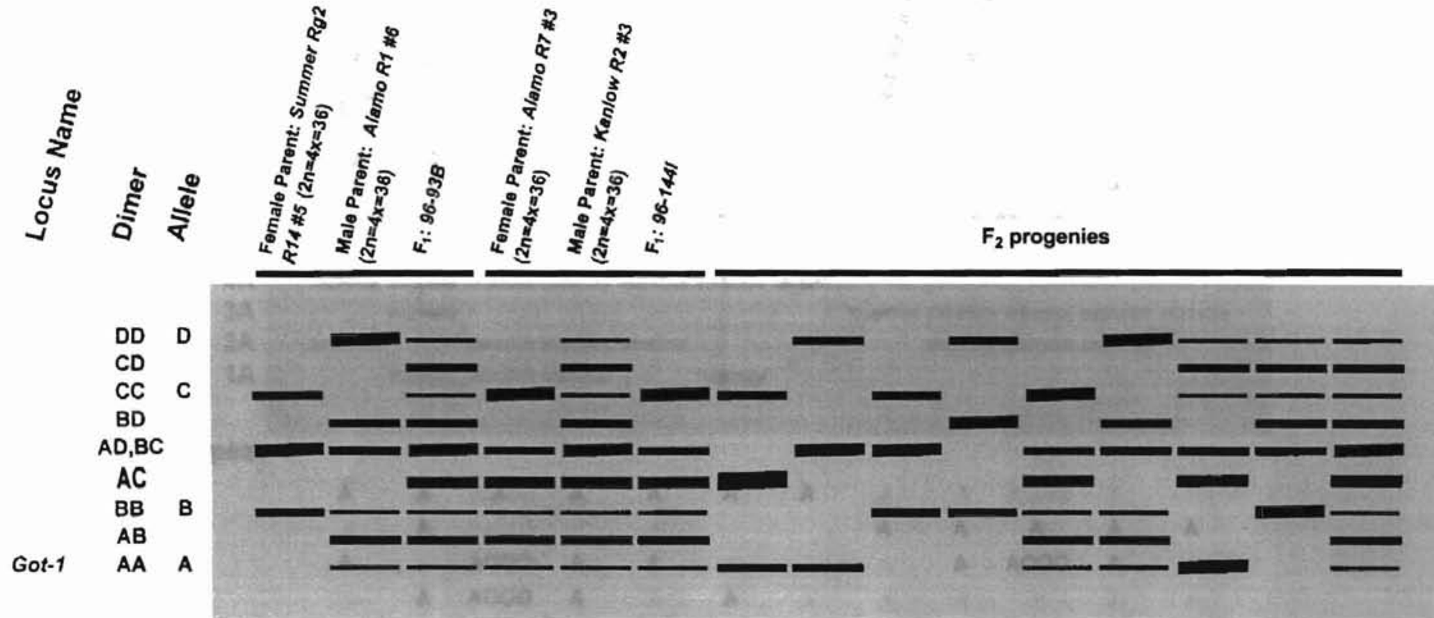


Figure 1. Zymogram and diagrammatic interpretation of ADH in the parents and F_2 progenies of 4x X 4x switchgrass crosses. Shown are five progeny classes possible with either disomic or tetrasomic inheritance when an F_1 parent in duplex state (AABB) is selfed. Theoretical band intensity ratio for genotype AABB is 1:2:1, and for genotypes AAAB and ABBB is 9:6:1.



Genotypes: BBCC ABDD ABCD ABCC ABCD ABCC AACC AADD BBCC BBDD ABCC ABDD AACD BBCD ABCD

Figure 2. Zymogram and diagrammatic interpretation of GOT in the parents and F_2 progenies of 4x X 4x switchgrass crosses. Shown are nine progeny classes that can be derived from selfing an F_1 parent in quadruplex state (ABCD) at *Got-1*, and when segregation is disomic (AB/CD). Theoritical band intensity ratio for each genotype is suggested in the diagram but may not be observed exactly on the zymogram.

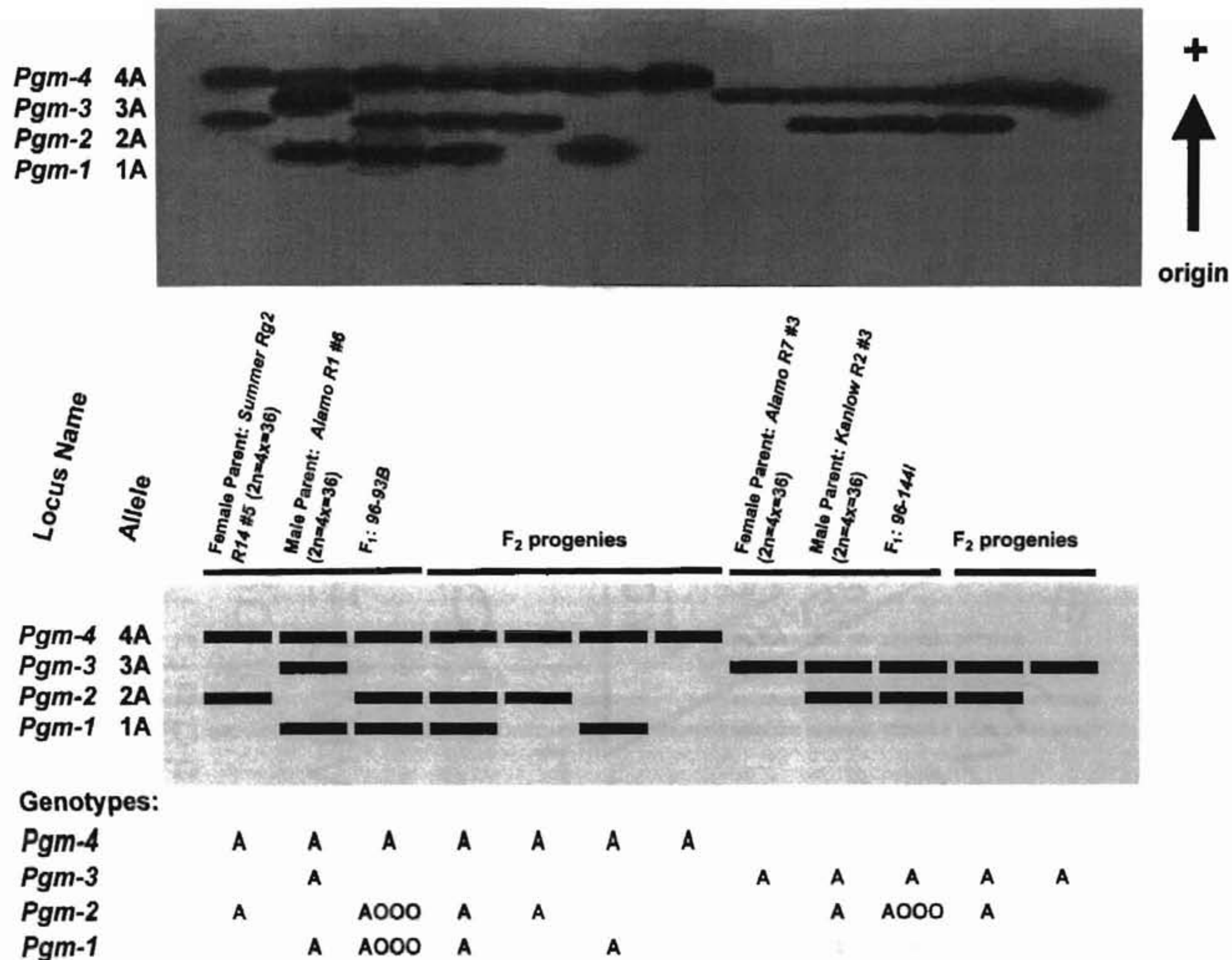


Figure 3. **Zymogram and diagrammatic interpretation of PGM in the parents and F_2 progenies of 4x X 4x switchgrass crosses.** Absence of a band indicates genotype OOOO (null), and a band indicates presence of at least one dose of the allele (A) that produces the functional enzyme. Actual genotypes of the F_1 parents are given when supported by segregation analysis.

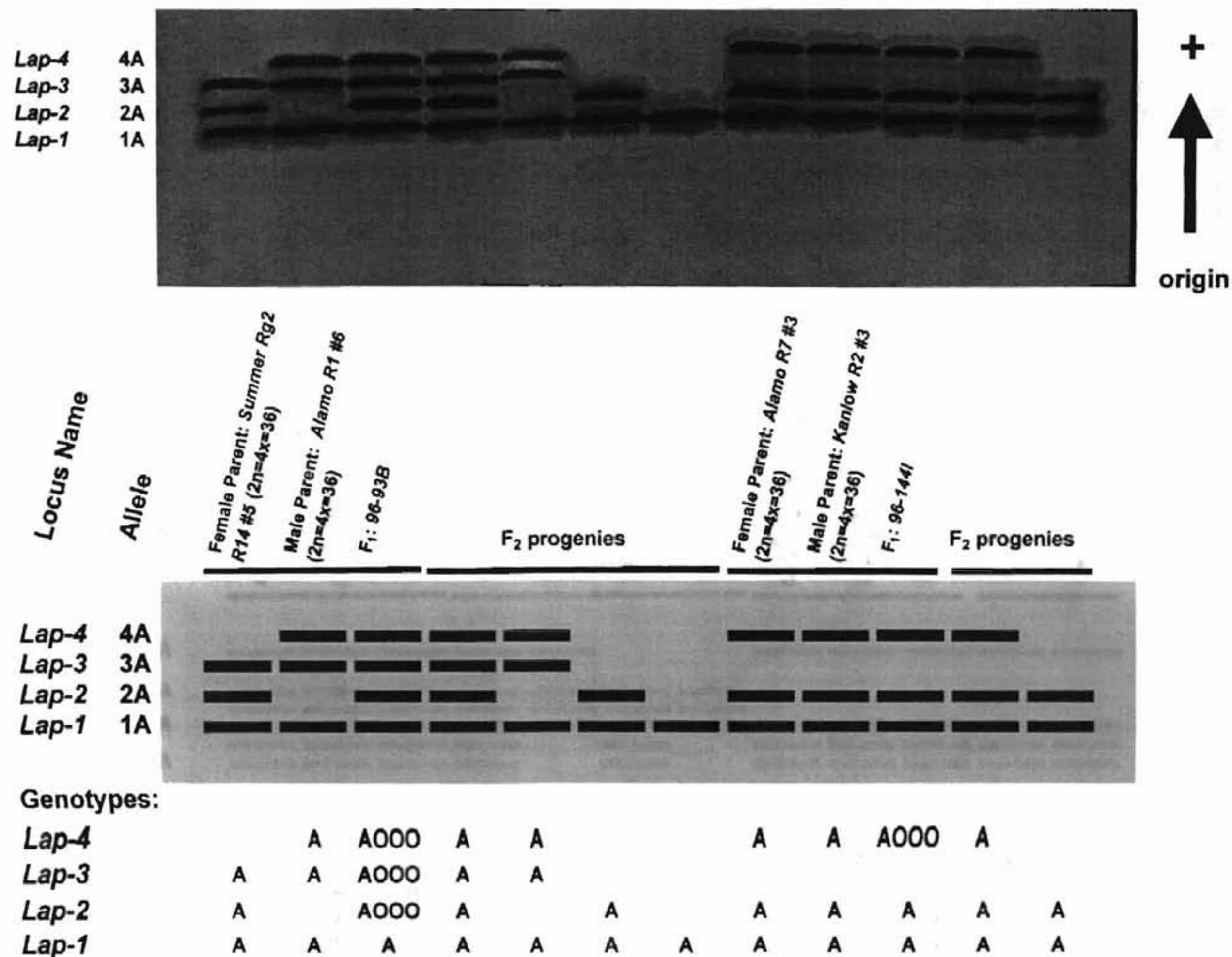


Figure 4. Zymogram and diagrammatic interpretation of LAP in the parents and F_2 progenies of 4x X 4x switchgrass crosses. Absence of a band indicates genotype OOOO (null), and a band indicates presence of at least one dose of the allele (A) that produces the functional enzyme. Actual genotypes of the F_1 parents are given when supported by segregation analysis.

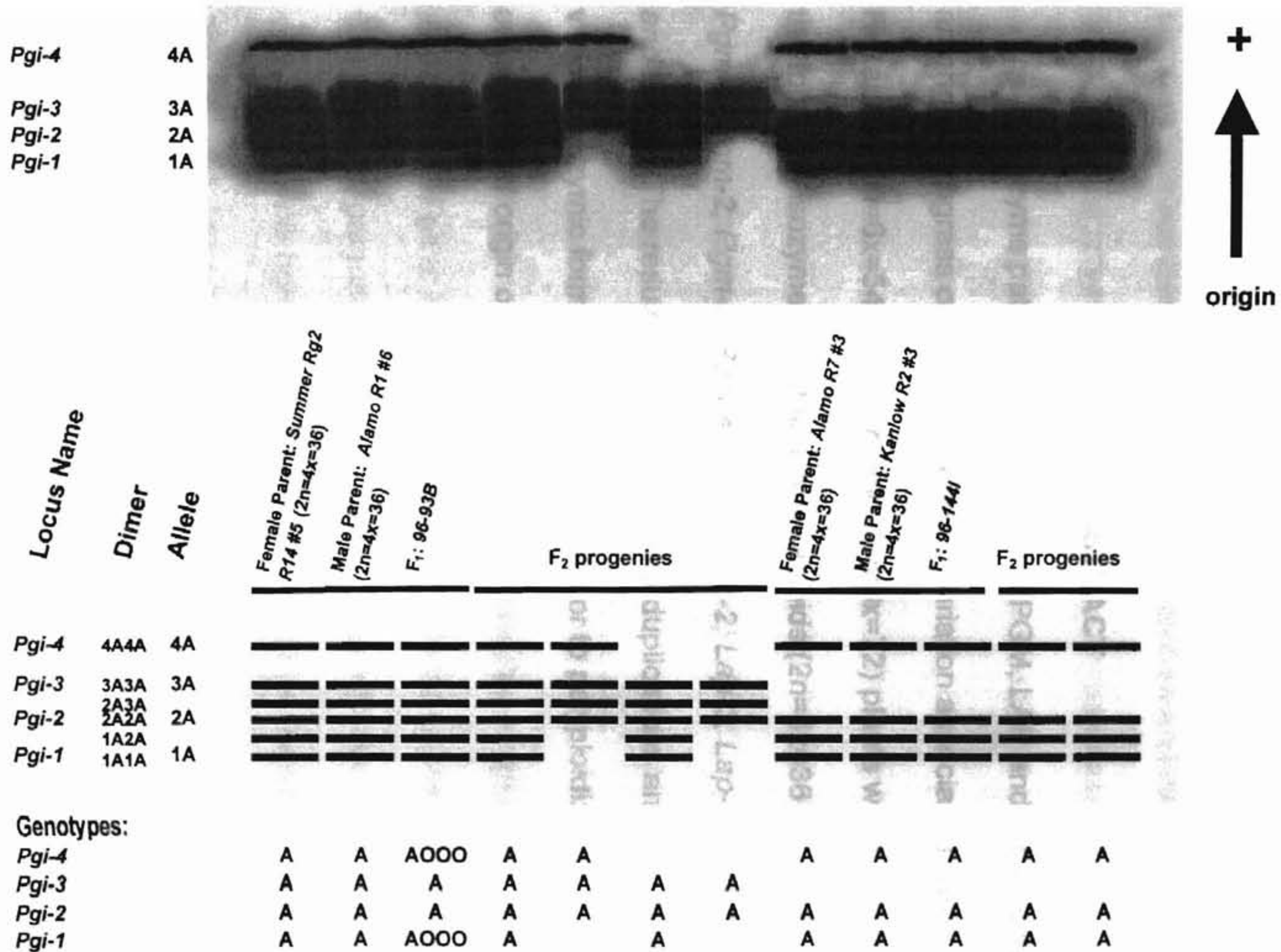


Figure 5. **Zymogram and diagrammatic interpretation of PGI in the parents and F_2 progenies of 4x X 4x switchgrass crosses.** Absence of a band indicates genotype OOOO (null), and a band indicates presence of at least one dose of the allele (A) that produces the functional enzyme. Actual genotypes of the F_1 parents are given when supported by segregation analysis.

CHAPTER III

ISOENZYME POLYMORPHISM AMONG SWITCHGRASS CULTIVARS

ABSTRACT

Isoenzyme patterns for ADH, GOT, PGM, LAP and PGI were compared among switchgrass cultivars to examine variation associated with ploidy level. Hexaploid (2n=6x=54) and octoploid (2n=8x=72) plants were found to possess the following isozymes identified in tetraploids (2n=4x=36): *Adh-1, Got-1, Got-2, Pgm-1, Pgm-2, Pgm-3, Pgm-4, Lap-1, Lap-2, Lap-3, Lap-4, Pgi-1, Pgi-2, Pgi-3 and Pgi-4*. The results indicate that gene duplications and divergence into various isozymic forms occurred mainly prior to polyploidization, and suggests an autopolyploid origin of switchgrass.

INTRODUCTION

The relationship between ploidy level and ecotype in switchgrass is not very clear although ecotypic differences appear to have more genetic bases than ploidy level differences. No consistent correlation was found between chromosome number and size of plants (McMillan and Weiler, 1959; Porter, 1966). Genetic diversity within the species has been assessed using Randomly Amplified Poymorphic DNA (RAPD) markers (Gunter et al., 1996), which clustered different populations into two distinct groups corresponding to ecotypic classification and regardless of ploidy level. This study indicated high genetic similarity within ecotypes, and found no significant differences in the number of RAPD bands between tetraploids and hexaploids. Chloroplast DNA restriction fragments indicated the presence of two distinct types of switchgrass which are directly associated with the lowland and upland ecotypes (Hultquist et al., 1996). A current phylogenetic analysis of switchgrass based on sequences of the plastid *ACC-1* gene indicates that cultivars are closely related to each other (Gornicki, P., personal communication). Less is known about the cytological evolution of switchgrass. Its genome composition, whether auto- or allopolyploid, has not been dealt with substantially in the literature. The diploid meiotic behavior of tetraploid cultivars was attributed to ancient autoploid or alloploid establishment events (Gunther, et al., 1996). Normal chromosome pairing in meiosis occurs in tetraploid hybrids of upland and lowland plants indicating that they have the same genome (Martinez-Reyna, 1998).

Polyploidy has been regarded as a major evolutionary force in higher plants, arising from nonreduction of gametic chromosomes, meristematic chromosome doubling, or zygotic chromosome doubling (de Wet, 1980). About 70% of the Gramineae, which includes many important crops and weeds, are polyploids. Most are probably allopolyploids but natural autopolyploids are frequent among perennial species (Muntzing, 1956). Isozyme electrophoresis has aided comparison of genotypes among populations of both diploid progenitors and polyploid derivatives, thereby facilitating reconstruction of evolutionary histories of intricate polyploid complexes such as the work done in the fern genera (Werth, 1989).

In this experiment, isoenzyme patterns of five enzyme systems were compared among tetraploid, hexaploid, and octoploid cultivars to examine variation associated with ploidy level. Isozyme loci of these enzyme systems were previously identified in tetraploid populations through allozyme inheritance study.

MATERIALS AND METHODS

Isoenzyme patterns for alcohol dehydrogenase (ADH), glutamate-oxaloacetate transaminase (GOT), phosphoglucomutase (PGM), leucine aminopeptidase (LAP) and phosphoglucose isomerase (PGI) were examined in the parents and F_1s of interploidy switchgrass crosses from the Switchgrass Breeding Program at the Oklahoma State University. The parents were cultivars or breeding lines which included plants known to be tetraploids (2n=4x=36), hexaploids (2n=6x=54), and octoploids (2n=8x=72). Two of these crosses based on tetraploid parents were used in an inheritance study of allozyme markers (Chapter II of this Thesis). The procedures for horizontal starch gel electrophoresis of isozymes were the same as described in Chapter II.

RESULTS

Zymograms showing isoenzyme patterns for ADH, GOT, PGM, LAP and PGI in 21 selected breeding lines are shown in Figs. 1-5. The tetraploid parents used in the inheritance study of allozyme markers were included to identify known loci and alleles of these five enzyme systems. Genotypes of the parents and F_1s of the interploidy crosses examined in this experiment were noted based on the bands observed in each plant, and were consistent with the allelism and locus assignments inferred from segregation analysis of tetraploid populations (data not shown).

For ADH (Fig. 1), two new isomorphs were observed at higher ploidy levels which either migrated slower or faster than the allozymes (A and B) of *Adh-1* identified in tetraploids (for example, see lanes 17 and 18). On the zymograms, all four ADH isomorphs thus identified appeared equidistant from each other. This may indicate that new isozymic forms were formed through successive mutations of one original allele decreasing or increasing the net charge of the enzyme product by roughly the same magnitude each time. Only a few amino acid changes can give rise to new isozymes, and they are those which do not alter the original function of the enzyme. The type and number of isomorphs present appeared to be correlated with ploidy level. The tetraploids only carried the two middle isomorphs (A and B) with the exception of *Alamo R1#11* (lane 2) which had three isomorphs. The hexaploids and octoploids always carried three isomorphs with the exception of *PMT785 R10 #1* (lane 16) which only carried two. *PMT785* was reported to include tetraploid plants. The

additional isomorphs observed at higher ploidy levels were likely alleles of *Adh-1* and not of separate ADH loci considering that this enzyme is polymorphic, and a single locus was found in tetraploid plants.

No additional GOT isomorphs were observed at higher ploidy levels (Fig. 2). An octoploid may only carry two allozymes of *Got-1*. Because null alleles were not found in the *Got-1* and *Adh-1* loci in the analysis of tetraploid plants, the eight copies of chromosomes in octoploids must each carry an active allele for these loci. Apparently, allozyme inheritance in *Got-1* at higher ploidy levels follows the disomic pattern observed in tetraploids. That is, allele A pairs with B, and C with D, so that at least one allele of each pair is present in an individual plant.

Null alleles also account for variation in PGM, LAP and PGI genes among plants at higher ploidy levels (Figs 3, 4 and 5). The same isozyme loci for PGM and LAP that were identified in tetraploid plants, were all that were found in hexaploids and octoploids (Figs. 3 and 4). Additional PGI isomorphs were observed (Fig. 5). These isomorphs likely represent separate PGI loci following the assumption of the existence of duplicate genes in this enzyme system. An isomorph migrating between *Pgi-3* and *Pgi-4* was observed in tetraploid, hexaploid and octoploid plants (lanes 3, 5, 10, 12, 16 and 17). This isomorph was not identified in the inheritance study because it was not observed in the tetraploid parents used (lanes 1, 6, 8 and 11). Another isomorph was found migrating below *Pgi-1* (lanes 14, 15 and 18) which was present only in octoploid plants. No heterodimers were formed between the products of *Pgi-4* and the

other PGI isomorphs as earlier observed in the analysis of tetraploid populations, suggesting that *Pgi-4* likely codes for the plastid enzyme.

DISCUSSION

Based on analysis of five enzyme systems, it was shown that hexaploid (2n=6x=54) and octoploid (2n=8x=72) plants possess the following isozyme genes identified in tetraploids (2n=4x=36): *Adh-1, Got-1, Got-2, Pgm-1, Pgm-2, Pgm-3, Pgm-4, Lap-1, Lap-2, Lap-3, Lap-4, Pgi-1, Pgi-2, Pgi-3 and Pgi-4*. This indicates that gene duplications and divergence into various isozymic forms occurred mainly prior to polyploidization, probably in the diploid ancestor. What followed was merely sequential doubling of the basic genome as well as a few occurrences of intraspecific hybridization among different ploidy levels. Hexaploid populations must have been derived from hybridization between tetraploid and octoploid progenitors following gametic reduction, although this event has not been demonstrated at present. The highly conserved isozyme profiles in the species may also indicate lack of introgression with divergent species and the absence of major genome reorganization at any ploidy level.

New isomorphs can be formed as ploidy level increases but not necessarily to match the number of duplicate copies of each chromosome. These new isozymic forms may be rarely found at lower ploidy indicating restricted gene flow across ploidal levels as this usually produces semi-fertile hybrids. This study did not include plant materials representing the breadth of genetic variability in switchgrass. Other isomorphs of ADH, GOT, PGM, LAP and PGI may be present in the species, that were not found in the plants examined in this experiment.

The occurrence and frequency of an isozyme in a given population must be determined by the amount of intercrossing that occurs among different populations or ecotypes. Restrictions to hybridization are posed not only by ploidy level differences but by physical separation due to habitat preference. Based on isoenzyme patterns, qualitative genetic variations in switchgrass can be explained both by ecotypic and ploidy level differences.

Most cross-pollinated grass species are believed to have an autopolyploid origin. Based on isoenzyme data presented here and other available information, it can be suggested that switchgrass has essentially one basic genome. Diploid-like pairing of chromosomes is a mechanism that has evolved to maintain fertility, and may indicate diploidization and differentiation that have taken place in the duplicated genome of this species.

The following support the hypothesis of an autopolyploid origin for switchgrass:

1. Several ploidy levels exist possibly including a diploid (which is not available in current collections), and these ploidy levels are in multiples of nine which suggests recurrent autopolyploidization: 2x=18, 4x=36, 6x=54, 8x=72, 10x=90, 12x=108.
2. Multivalent association of chromosomes can be observed in tetraploids, and more frequently at higher ploidy levels, indicating the presence of homologous or homeologous chromosomes.
3. The same isozyme genes can be found in tetraploid, hexaploid, and octoploid plants, suggesting genome homology.

4. Assessment of genetic diversity in switchgrass based on nuclear and organellar DNA markers indicated high genetic similarity within ecotypes. Diversity between ecotypes can be attributed mainly to low frequency of genetic exchanges.

Future studies utilizing a variety of approaches including cytogenetics, DNA marker analysis, and field experiments should verify the inferences made based on isozyme data about the relationships among switchgrass ploidy levels and ecotypes. These studies will find practical applications in the utilization of this crop and will contribute to the understanding of plant speciation with regards to polyploidy.

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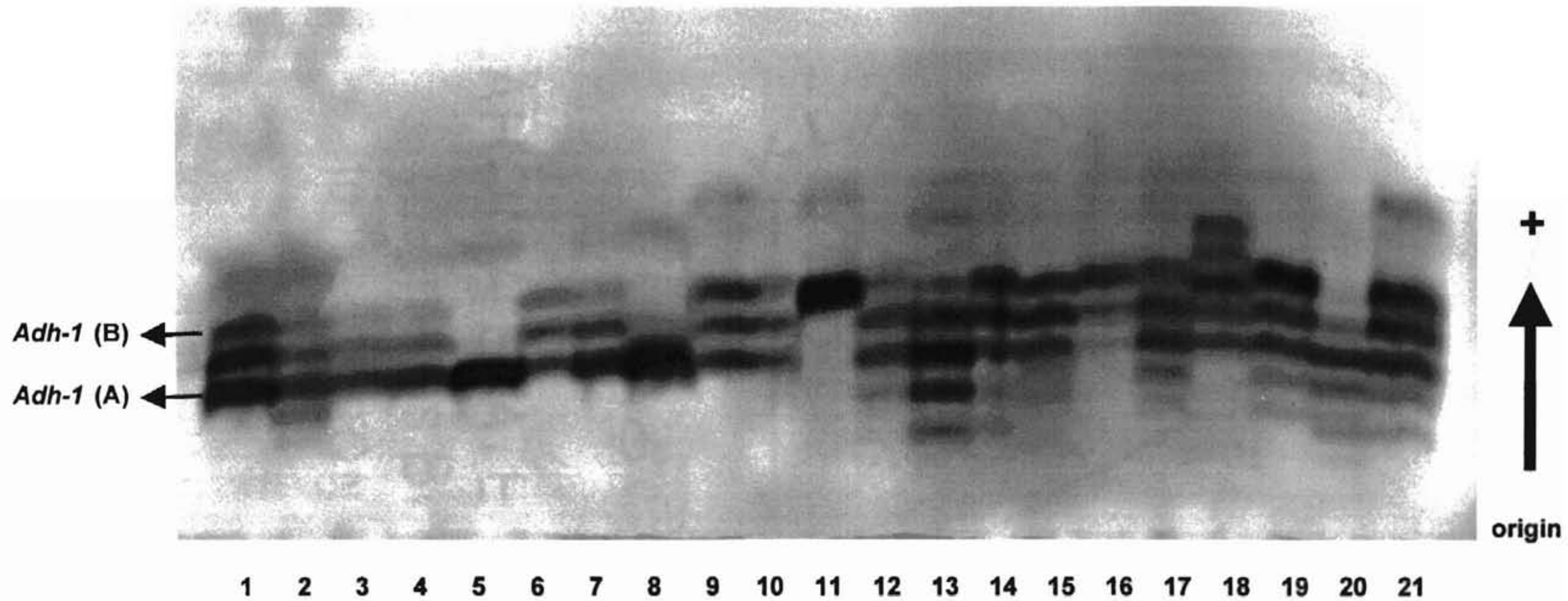

Figure 1. **Variation in ADH pattern among switchgrass breeding lines.**
Alleles of *Adh-1* as identified in the tetraploid parents used in the inheritance study (lanes 1, 6, 8, and 11) are indicated by the arrows. Lanes 1-11 (2n=4x=36): 1-*Alamo R1#6*, 2-*Alamo R1#11*, 3-*Alamo R4#1*, 4-*Alamo R7#1*, 5-*Alamo R7#2*, 6-*Alamo R7#3*, 7-*Kanlow R1#1*, 8-*Kanlow R2#3*, 9-*Kanlow R11#2*, 10-*Kanlow R11#4*, 11-*Summer R14#5*;
Lanes 12-13 (2n=6x=54): 12-*94-125A*, 13-*94-125B*;
Lanes 14-21 (2n=8x=72): 14-*Blackwell R20#7*, 15-*Caddo R4#5*, 16-*PMT785 R10#1*, 17-*SU94 R3#13*, 18-*SU94 R3#20*, 19-*SU94 R23#5*, 20-*SU94 R23#12*, 21-*SWG 068#3*

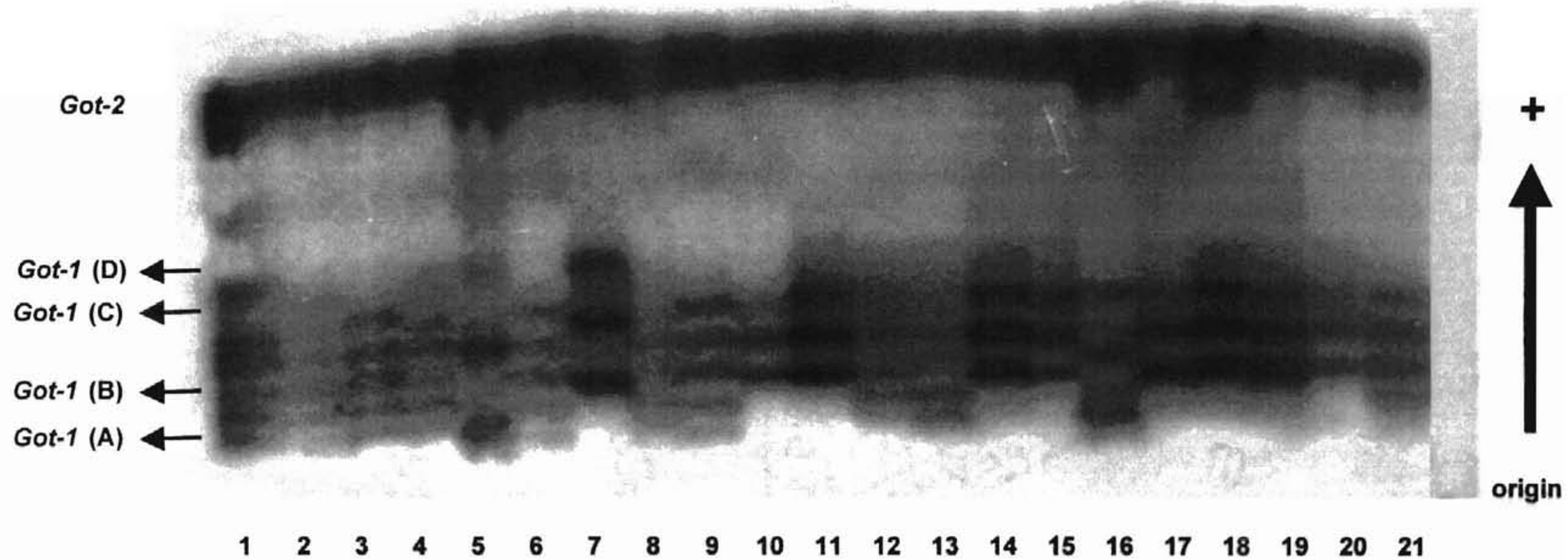

Figure 2. Variation in GOT pattern among switchgrass breeding lines.
Alleles of *Got-1* as identified in the tetraploid parents used in the inheritance study (lanes 1, 6, 8, and 11) are indicated by the arrows. Lanes 1-11 (2n=4x=36): 1-*Alamo R1#6*, 2-*Alamo R1#11*, 3-*Alamo R4#1*, 4-*Alamo R7#1*, 5-*Alamo R7#2*, 6-*Alamo R7#3*, 7-*Kanlow R1#1*, 8-*Kanlow R2#3*, 9-*Kanlow R11#2*, 10-*Kanlow R11#4*, 11-*Summer R14#5*;
Lanes 12-13 (2n=6x=54): 12-*94-125A*, 13-*94-125B*;
Lanes 14-21 (2n=8x=72): 14-*Blackwell R20#7*, 15-*Caddo R4#5*, 16-*PMT785 R10#1*, 17-*SU94 R3#13*, 18-*SU94 R3#20*, 19-*SU94 R23#5*, 20-*SU94 R23#12*, 21-*SWG 068#3*

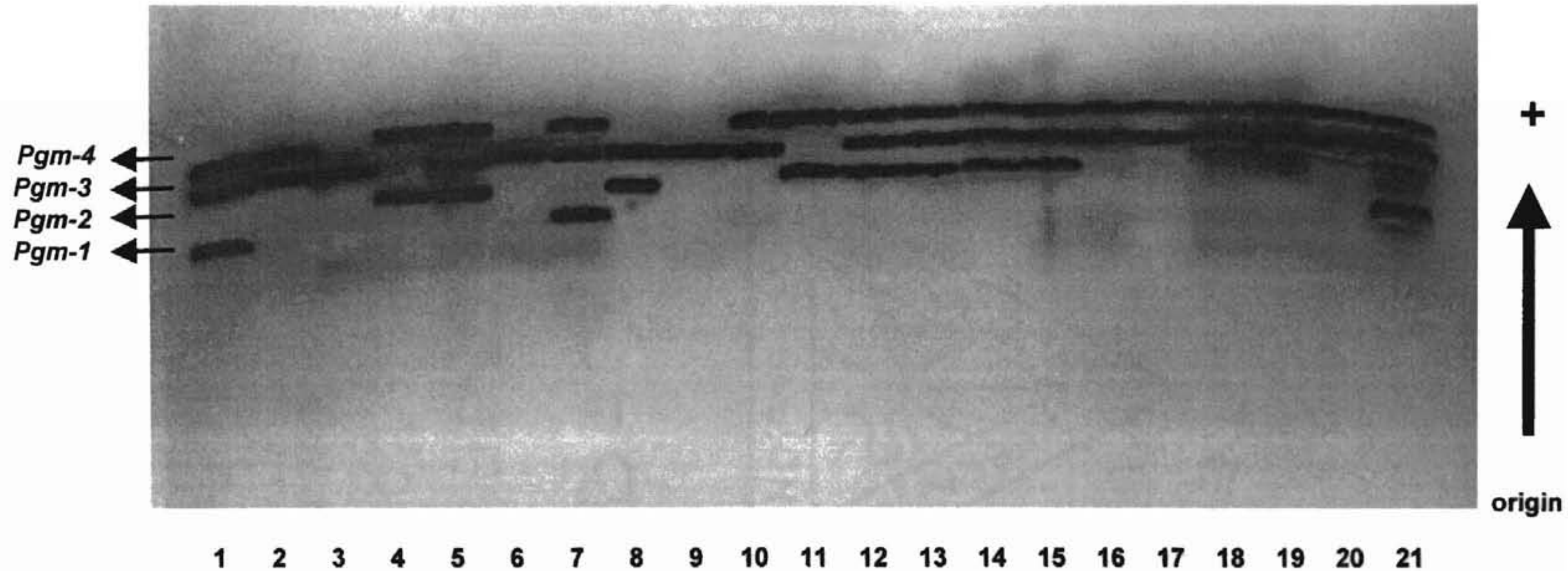

Figure 3. Variation in PGM pattern among switchgrass breeding lines.
The four PGM loci identified in the tetraploid parents used in the inheritance study (lanes 1, 6, 8, and 11) are indicated by the arrows. Lanes 1-11 (2n=4x=36): 1-*Alamo R1#6*, 2-*Alamo R1#11*, 3-*Alamo R4#1*, 4-*Alamo R7#1*, 5-*Alamo R7#2*, 6-*Alamo R7#3*, 7-*Kanlow R1#1*, 8-*Kanlow R2#3*, 9-*Kanlow R11#2*, 10-*Kanlow R11#4*, 11-*Summer R14#5*;
Lanes 12-13 (2n=6x=54): 12-*94-125A*, 13-*94-125B*;
Lanes 14-21 (2n=8x=72): 14-*Blackwell R20#7*, 15-*Caddo R4#5*, 16-*PMT785 R10#1*, 17-*SU94 R3#13*, 18-*SU94 R3#20*, 19-*SU94 R23#5*, 20-*SU94 R23#12*, 21-*SWG 068#3*

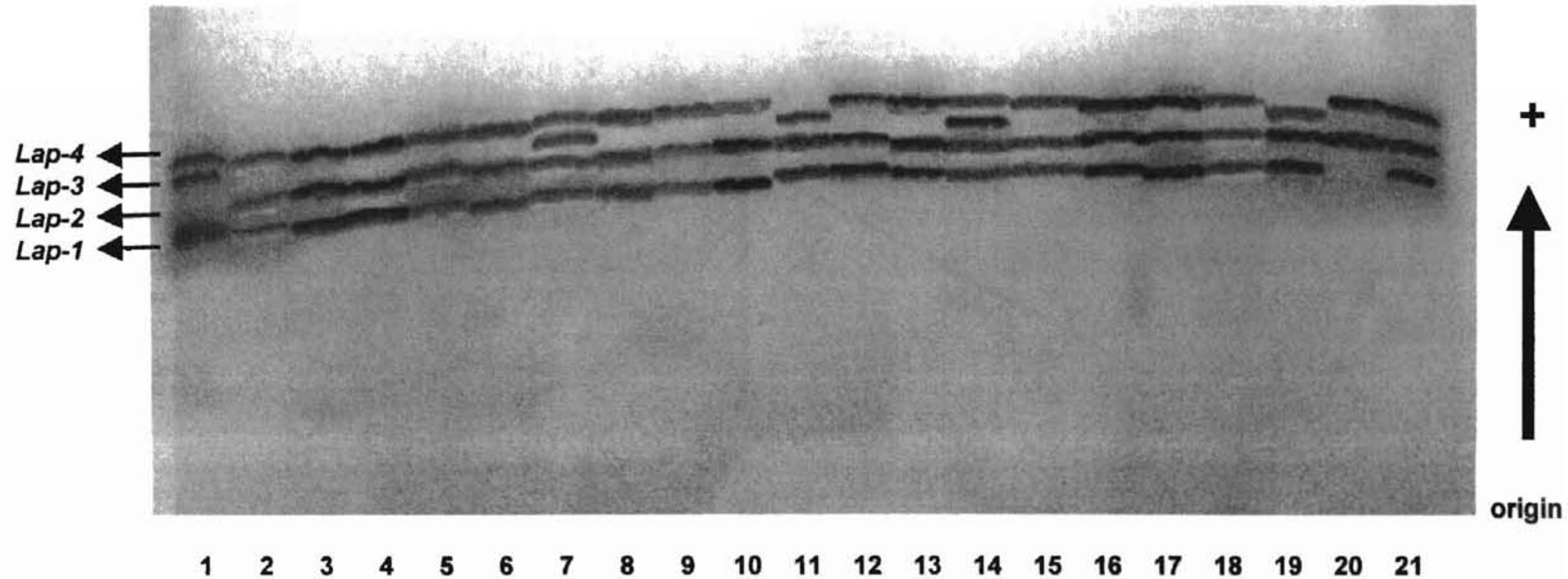

Figure 4. Variation in LAP pattern among switchgrass breeding lines.

The four LAP loci identified in the tetraploid parents used in the inheritance study (lanes 1, 6, 8, and 11) are indicated by the arrows. <u>Lanes 1-11 (2n=4x=36):</u> 1-*Alamo R1#6*, 2-*Alamo R1#11*, 3-*Alamo R4#1*, 4-*Alamo R7#1*, 5-*Alamo R7#2*, 6-*Alamo R7#3*, 7-*Kanlow R1#1*, 8-*Kanlow R2#3*, 9-*Kanlow R11#2*, 10-*Kanlow R11#4*, 11-*Summer R14#5*;
<u>Lanes 12-13 (2n=6x=54)</u>: 12-*94-125A*, 13-*94-125B*;
<u>Lanes 14-21 (2n=8x=72)</u>: 14-*Blackwell R20#7*, 15-*Caddo R4#5*, 16-*PMT785 R10#1*, 17-*SU94 R3#13*, 18-*SU94 R3#20*, 19-*SU94 R23#5*, 20-*SU94 R23#12*, 21-*SWG 068#3*

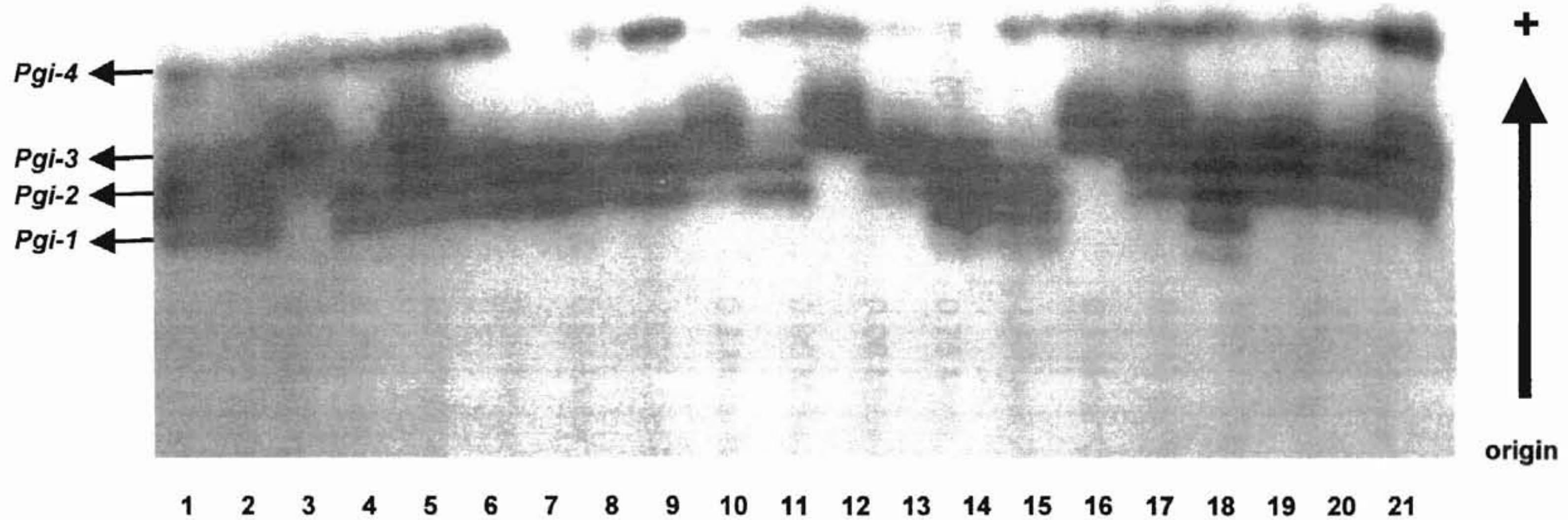

Figure 5. Variation in PGI pattern among switchgrass breeding lines.

The four PGI loci identified in the tetraploid parents used in the inheritance study (lanes 1, 6, 8, and 11) are indicated by the arrows. Lanes 1-11 (2n=4x=36): 1-*Alamo R1#6*, 2-*Alamo R1#11*, 3-*Alamo R4#1*, 4-*Alamo R7#1*, 5-*Alamo R7#2*, 6-*Alamo R7#3*, 7-*Kanlow R1#1*, 8-*Kanlow R2#3*, 9-*Kanlow R11#2*, 10-*Kanlow R11#4*, 11-*Summer R14#5*;
Lanes 12-13 (2n=6x=54): 12-*94-125A*, 13-*94-125B*;
Lanes 14-21 (2n=8x=72): 14-*Blackwell R20#7*, 15-*Caddo R4#5*, 16-*PMT785 R10#1*, 17-*SU94 R3#13*, 18-*SU94 R3#20*, 19-*SU94 R23#5*, 20-*SU94 R23#12*, 21-*SWG 068#3*

APPENDIX A

Electrophoresis Buffer Systems

Buffer System I (For assaying ADH)

Starch Gel Buffer (20x), pH 8.0:	0.18 Molar Trizma Base
	0.1 Molar L-Histidine Hydrochloride, Monohydrate
Electrode Buffer (1x), pH 8.0:	0.4 Molar Trizma Base
	0.1 Molar Citric Acid, Monohydrate

Buffer System II (For assaying LAP and PGI)

Starch Gel Buffer (10x), pH 8.8:	0.75 Molar Trizma Base
	0.06 Molar Citirc Acid, Monohydrate
Electrode Buffer (1x), pH 8.2:	0.06 M Sodium Hydroxide
	0.2 Molar Boric Acid, Anhydrous

Buffer System III (For assaying GOT and PGM)

Starch Gel Buffer (20x), pH 6.5:	0.04 Molar Trizma Base
	0.09 Molar L-Histidine Hydrochloride, Monohydrate
Electrode Buffer (1x), pH 6.5:	0.25 Molar Trizma Base
	0.1 Molar Citric Acid, Monohydrate

APPENDIX B

Staining Procedures for Enzyme Assays

ADH

Immerse gel in solution containing 35 ml water, 10 ml Tris-HCl (0.5 Molar, pH 8.5), 1 ml Absolute Ethanol, 1 ml β-Nicotinamide Adenine Dinucleotide (10 mg/ml), 2 ml Nitroblue Tetrazolium Salt (10 mg/ml), and 1 ml Phenazine Methosulfate (1 mg/ml). Incubate at 40°C for 45 minutes in the dark.

GOT

Immerse gel in solution containing 30 ml water, 20 ml Tris-HCl (0.5 Molar, pH 8.5), 0.1 g DL-Aspartic Acid, 0.05 g α-Ketoglutaric Acid, 0.002 g Pyridoxal-5-Phosphate, and 0.04 g Fast Blue BB Salt. Incubate at 40°C for 45 minutes in the dark.

PGM

Immerse gel in solution containing 25 ml water, 18 ml Tris-HCl (0.5 Molar, pH 8.5), 1 ml β-Nicotinamide Adenine Dinucleotide Phosphate (5 mg/ml), 2 ml Magnesium Chloride (0.1 Molar), 1 ml Glucose-1-Phosphate (50 mg/ml), 3.5 ul Glucose-6-Phosphate Dehydrogenase (10 units/ul), 2 ml Nitroblue Tetrazolium Salt (10 mg/ml), and 1 ml Phenazine Methosulfate (1 mg/ml). Incubate at 40°C for 1 hour in the dark, agitating occasionally. Bands diffuse out and must be scored at once.

LAP

Immerse gel in solution containing 20 ml Sodium Hydroxide (0.1 Molar), 25 ml Tris-Maleate Buffer (0.2 Molar, pH 3.3), 5 ml L-Leucyl-β-Naphthylamide (5 mg/ml), and 0.02 g Fast Black K Salt. Incubate at 40°C for 1 hour in the dark.

PGI

Immerse gel in solution containing 25 ml water, 18 ml Tris-HCl (0.5 Molar, pH 8.5), 1 ml β-Nicotinamide Adenine Dinucleotide Phosphate (5 mg/ml), 2 ml Magnesium Chloride (0.1 Molar), 1 ml Fructose-6-Phosphate (50 mg/ml), 3.5 ul Glucose-6-Phosphate Dehydrogenase (10 units/ul), 2 ml Nitroblue Tetrazolium Salt (10 mg/ml), and 1 ml Phenazine Methosulfate (1 mg/ml). Incubate at 40°C for 45 minutes in the dark. Bands diffuse out and must be scored at once.

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VITA

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Master of Science

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