

**STUDIES OF THE REPRODUCTIVE METHOD  
AND THE INHERITANCE OF A LEAF  
CHLOROTIC TRAIT IN SELECTED  
*CYNODON* ACCESSIONS**

By

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## CHAPTER I

### INTRODUCTION AND LITERATURE REVIEW

#### Introduction

*Cynodon* is a genus of the family Gramineae (Poaceae) containing perennial, sod-forming, warm-season grasses distributed throughout the warmer parts of the world. Africa and Southeast Asia are the centers of *Cynodon* diversity where the genus is thought to have originated. *Cynodon* is not indigenous to North and South America, and is believed to have been introduced to North America in the early eighteenth century. Some *Cynodon* species are of high economic importance on account of their widespread use for livestock herbage, recreational turf and soil stabilization purposes (Taliaferro et al. 2004).

#### Taxonomy

A comprehensive biosystematic investigation of *Cynodon* was conducted at the Oklahoma State University during the 1960's (Harlan et al, 1970). These studies led to substantial revisions of earlier taxonomic classification of the genus by Hurcombe (1948) and Chippindall (1955). The revised classification (Harlan et al., 1970) differentiated the genus into nine species and ten varieties (Table 1). Robust East African, non-rhizomatous species of *C. aethiopicus*, *C. nlemfuensis*, and *C. plectostachyus* are commonly called

“stargrass” while the plants of the remaining six taxa are generally referred to as “bermudagrass”. Of the six *C. dactylon* varieties, *C. dactylon* var *dactylon* is cosmopolitan and is the most important for both turf and forage purposes. *C. dactylon* var. *dactylon* and *C. transvaalensis* Burtt-Davy are the taxa of predominant turf importance, with most of the economically important turf bermudagrasses originating from them. *C. arcuatus* J.S. Presl. Ex. C.B. Presl., *C. barberi* Rang. Et Tad and *C. dactylon* var. *polevansii* are of minor turf importance. Similarly, *C. dactylon* and the stargrass species are more preferred for livestock herbage use because of their higher biomass yield (Harlan, 1970).

### **Morphology**

Plants of different species of the genus are recognizable by virtue of their specific characteristic morphology. Cosmopolitan *C. dactylon* var *dactylon* has highly variable plant types ranging from small fine-textured plants used for turf to the large robust plants used for forage. Rhizomes are short and slender to stout and fleshy, while the stolons vary from very fine to coarse, and inflorescences bear two to several slender stiff racemes in one, or rarely two, whorls.

*C. arcuatus* is the most morphologically-distinct species of the genus, with large ovate-lanceolate leaves and long slender racemes in a single whorl (Harlan et al 1970; de Wet and Harlan, 1970). Distributed from Madagascar to Southeast Asia and Australia, it is devoid of rhizomes, has no winter hardiness and forms a loose open mat of inferior turf quality (Harlan et al, 1970).



*C. barberi* could be considered a miniature version of *C. arcuatus* except for the inflorescence differences. The racemes are shorter, more delicate and have rather widely spread spikelets. Native to south India, this species is also devoid of rhizomes and has no winter hardiness.

Plants of *C. transvaalensis* are mainly used as turfgrass due to their very fine-textured leaves and small inflorescences. The taxon is endemic to South Africa and is called African bermudagrass. *C. transvaalensis* plants are easily distinguished by their yellowish green color and erect narrow leaves. Rhizomes are short and fleshy, while stolons are slender and with short internodes. Plants of *C. transvaalensis* generally have levels of winter hardiness greater than needed in South Africa.

*C. X magennisii* is believed to have derived from a single clonal plant originated as a natural triploid hybrid from the cross between *C. dactylon* and *C. transvaalensis* (Harlan et al, 1970). Rhizomes are short and small, and racemes small and slender and in a single whorl in the inflorescence. This fine-textured, dark green, plant is a sterile triploid producing a good quality turf. It was released as the cultivar “Sunturf” in America(Huffine, 1957).

Three east African species, *C. aethiopicus*, *C. nlemfuensis* and *C. plectostachyus* are often confused with each other because of their similarity in appearance (Harlan et al, 1969). All three are devoid of rhizomes, have similar growth habits, and have little tolerance to freezing temperatures. The inflorescence of *C. aethiopicus* resembles that of *C. plectostachyus* with 2-3 whorls, but is smaller and dark red pigmented. In contrast, *C. nlemfuensis* inflorescences usually have only a single whorl of raceme.

## Reproductive Characteristics

Both sexual and asexual modes of reproduction are common in *Cynodon* species. Vegetative plant parts including rhizomes, stolons or crown buds facilitate asexual propagation, while sexual reproduction is through seeds produced by normal sexual processes. Flowers are perfect and contain a single pistil and three stamens which dehisce after extrusion from the flower. Plants usually flower in the early morning hours; however the time may differ depending upon location and climate. Pollen grains are mainly disseminated by wind currents. The ovules contain normal polygonium type embryo-sacs with an egg, two polar, two synergid and three antipodal nuclei (Burson and Tischler, 1980). Mature pollen grains respectively contain a generative and a vegetative nucleus. At pollination, the generative nucleus while passing through the pollen tube divides mitotically to produce two nuclei, which combine with the egg and the two fused polar nuclei to form the  $2n$  embryo and the  $3n$  endosperm respectively.

Significant variability exists among *Cynodon* species and plants for their seed set potential. Plants are usually cross-fertile and have relatively low self-fertility (Burton and Hart, 1967; Richardson et al., 1978, Taliaferro and Lamle, 1997). Strong out-crossing is enforced in *Cynodon* species by cross pollination and self- incompatibility (Burton, 1947; Burton and Hart, 1967, Taliaferro, 2003). Many forage and turf grass species, especially those with higher ploidy levels, reproduce through gametophytic apomixis (Hanna and Bashaw, 1987; Bashaw 1980; Bashaw and Funk, 1987). However, apomictic reproduction has not been documented in *Cynodon*.

## Hybridization Potential

The results from the extensive biosystematic studies of J.R. Harlan and colleagues in late 1960s have been the basis for inferences on the hybridization potential of different *Cynodon* species (Harlan et al, 1969 &1970, de Wet and Harlan, 1970). Accordingly, of the nine species, *C. arcuatus*, *C. barberi* and *C. plectostachyus* were reported to be completely isolated from each other and the rest of the genus. *C. aethiopicus* is also well isolated, with crosses made with other taxa only with great difficulty. The taxon *C. X magennisii* is a sterile triploid. The remaining species are fairly compatible with each other, but differ in degree of ease of hybridization. *C. transvaalensis* crosses easily with *C. dactylon* var *dactylon* and *C. nlemfuensis*. *C. nlemfuensis*, similarly, produces good seed set when crossed with *C. dactylon* var *dactylon*. Within *C. dactylon* though, low crossability has been observed among some of the different taxonomic varieties. Many crosses involving *C. dactylon* var *polevansii* have resulted in infertile seeds.

Controlled artificial crosses in bermudagrass are difficult due to the small spikelet size. Crosses can be attained through hand emasculation and pollination by painting pollen on the pistil, however the technique is tedious and time consuming (Richardson, 1958). Burton (1965), proposed the use of a fog chamber for ease in emasculation, whereby, the extruded anthers could be easily removed with the tweezers before dehiscing.

## **Apomixis as a Reproductive Mechanism**

Apomixis, in simple words, can be described as asexual reproduction through seeds, and referred to as agamospermy or gametophytic apomixis (Bashaw and Funk, 1987). It is a method of reproduction, specifically in polyploid plants, in which the normal meiotic gamete formation process is circumvented and seeds are produced from the asexually derived eggs, so that the resulting progeny plants are genetically the replica of the female parent.

Apomictic processes can be initiated at several points during gametophytic development. Depending upon the origin of the cells giving rise to apomictic embryo, two mechanisms are distinguished. In diplospory, the embryo-sac originates from the megaspore-mother cell either directly by mitosis or after arrested meiosis, without a reduction in chromosome number. As a result, an embryo-sac consisting of an unreduced egg cell is formed (Leblanc et al., 1995). In apospory, on the other hand, aposporous initial cells arise from nucellar cells of the ovules. They differentiate after three mitotic divisions into embryo-sacs containing a diploid egg cell that directly develops into an embryo without fertilization (Bashaw, 1962).

Two main forms of apomixis are recognized based on the extent of occurrence, facultative and obligate. Plants reproducing solely by apomixis are obligate apomicts, while the facultative ones employ both apomixis and sexual reproduction which could be within an individual plant or plant population (Hanna and Bashaw, 1987). As a consequence, variability can be observed in facultative apomicts in contrast to the exact clonal copies produced in the case of obligate apomicts.

Apomixis is not an uncommon mode of reproduction, particularly in polyploid grass species of family Poaceae (Hanna and Bashaw, 1987; Bashaw, 1975; Bashaw and Funk, 1987; Harlan and de Wet 1963; Taliaferro, 2004). It has been reported in It has been reported in over 300 species of at least 35 different plant families. Bashaw (1962), reported buffelgrass (*Pennisetum ciliare*) as an obligate apomict, and the mechanism of apomixis was apospory and pseudogamy. Similarly, eastern gamagrass (*Tripsacum dactyloides* L.) is characterized by diplosporous facultative apomixis (Leblanc et al., 1995, Burson et al., 1990), whereas *Poa* plants range from obligate to facultative to sexual with aposporous mechanism (Huff, 1992).

Cytological examination of megasporogenesis and embryo-sac development is required to identify the mechanism of apomixis. Lack of phenotypic variation among progeny plants is diagnostic of apomictic reproduction in species known to be allogamous.

Apospory is the most common mechanism of apomixis in higher plants. It is usually characterized by the presence of multiple embryo-sacs (Bashaw, 1980). Compared to apospory, diplospory is much more difficult to characterize on the basis of cytological evidence (Bashaw and Hanna, 1990). In mature ovules characterization is nearly impossible, as the embryo-sac may appear completely normal. Cytological evidence of diplospory requires examination of early megasporogenesis, when normal meiosis would be expected. Mitotic metaphase observed instead of meiosis in the megaspore mother cell indicates diplospory. Further evidence would be the absence of the linear tetrads of megaspores, absence

of the pre-megaspore dyad, and the position of the embryo-sac at the two- to four-nucleate stage (Bashaw and Hanna, 1990).

### **Molecular Markers in Genetic Studies**

A number of molecular techniques have been successfully used in *Cynodon* to study the genetic phylogeny of different species and cultivars. These include Random Amplified Polymorphic DNA (RAPD), Restriction Fragment Length Polymorphism (RFLP), Amplified Fragment Length Polymorphism (AFLP) and DNA Amplification Fingerprinting (DAF) techniques. Caetano-Anolles et al. (1995) used the DAF (DNA Amplification Fingerprinting) technique to study the genetic variation of 13 bermudagrass cultivars. DAF separated *C. transvaalensis* and *C. dactylon* into two distinct clusters, while the inter-specific hybrids clustered intermediate to the two species.

Ho et al. (1997) used DAF to identify the relationships among 18 Australian *Cynodon* cultivars using 20 random DAF primers. All the cultivars showed distinct polymorphism. Cluster analysis grouped the 18 cultivars into two distinct groups, one group included the bermudagrasses collected in Australia and the other group consisted of the hybrids of the *C. dactylon* and *C. transvaalensis* hybrid cultivars.

Caetano-Anolles et al. (1997) characterized the origin of bermudagrass off-types using DAF. They showed that off-types found in plantings of Tifway were distantly related to Tifway and probably resulted from mechanical contamination. They concluded that Tifway was not prone to somatic mutation and relatively genetically stable. Using DAF and Arbitrary Signatures from Amplification Profiles

(ASAP), Caetano-Anolles et al. (1997) showed that ‘Tifgreen’ and ‘Tifdwarf’ were closely related; and many of the offtypes found in plantings of the respective cultivars were also closely related to the two cultivars. They concluded that both Tifgreen and Tifdwarf are genetically unstable producing a relatively high frequency of somatic mutations (Caetano-Anolles et al., 1998)

DAF was also used by Asseffa et al. (1998) to assess the genetic relatedness among 62 accessions representing eight different species of *Cynodon*. The study showed *C. arcuatus* to be completely separated from other species by numerous monomorphic bands. The strongest species similarities were between *C. aethiopicus* and *C. arcuatus*; *C. transvaalensis* and *C. plectostachyus*; and *C. incompletus* and *C. nlemfuensis*. Intraspecific variation was the least for *C. aethiopicus*, *C. arcuatus* and *C. transvaalensis* and the greatest for *C. dactylon*.

Anderson et al. (2001) assessed authenticity of U-3 bermudagrass using the DAF technique. They found that bermudagrasses grown and sold in Oklahoma by several sod companies were distinctly different from putative true U-3.

The AFLP procedure has also been utilized for genetic studies of *Cynodon*. Zhang et al. (1999) differentiated a number of released and experimental bermudagrass genotypes using AFLP. Similarly Wu et al. (2004) used AFLP to study the genetic diversity within *C. transvaalensis* and its relatedness to *C. dactylon* accessions. The AFLP technique has better discrimination and greater repeatability as compared to the other techniques. However, it being more expensive in terms of reagents, time as well as labor, is preferred in more specific and extensive studies only.

## Objective of the study

The importance of breeding in producing plants with desirable characteristics is well established and is equally applicable for *Cynodon*. It is crucial to have proper knowledge of reproductive traits and characters of the plants to successfully breed them. Mutation in the normal plants can bring about certain diagnostic changes in the progenies, either in their morphology or other characteristics of economic importance, and would attract the attention of the scientists for further studies.

We have a mutated *Cynodon dactylon* var *dactylon* plant with alternate chlorotic strips in the shoot, and hence named Zebra. The Zebra trait is temperature sensitive, with increased phenotype intensity at higher temperatures. Zebra clonal plants are highly self-fertile in contrast to most *Cynodon dactylon* var *dactylon* plants. Information regarding the inheritance and genetic control of the zebra trait is lacking.

Several accessions of *C. arcuatus* maintained at Oklahoma State University produce morphologically uniform progeny. Lack of variation among the progeny is diagnostic either of inbreeding or of apomictic reproduction. No information is available on the reproductive mechanism of *C. arcuatus* other than the inability to cross plants of the taxon with the plants of the other species.

This research was therefore initiated with the following objectives:

- Elucidate the inheritance of the chlorotic stripe phenotype in the “Zebra” bermudagrass mutant line.
- Characterize the method of reproduction in *C. arcuatus* accession identified as potentially deviating in reproductive method relative to other *Cynodon* accessions.



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**Table 1: Taxonomic classification of the genus *Cynodon*<sup>1</sup>.**

<b>Epithet</b>	<b>Chromosome No.</b>	<b>Distribution</b>
<i>C. aethiopicus</i> Clayton et Harlan	18, 36	East African rift valleys
<i>C. arcuatus</i> J. S. Presl. Ex C. B. Presl.	36	Malagasy, and Southern India to Northern Australia
<i>C. barberi</i> Rang. et. Trad.	18	Southern India
<i>C. dactylon</i> (L.) Pers. var. <i>dactylon</i>	36	Cosmopolitan
var. <i>afghanicus</i> Harlan et de Wet	18, 36	Afghanistan steppes
var. <i>aridus</i> Harlan et de Wet	18	Southern Africa northward to Palestine, east to South India
var <i>coursii</i> (A. Camus) Harlan et Wet	36	Madagascar
var. <i>elegans</i> Rendle	36	Southern Africa, south of latitude 13° South
var. <i>polevansii</i> (Stent) de Wet et Harlan	36	Near Barberspan, South Africa
<i>C. incompletes</i> Nees var. <i>incompletes</i>	18	South Africa; Transvaal to Cape
var. <i>hirsutus</i> (Stent) de Wet et Harlan	18, 36	South Africa; Transvaal to Cape
<i>C. nlemfuensis</i> Vanderyst var. <i>nlemfuensis</i>	18, 36	East Africa
var. <i>robustus</i> Clayton et Harlan	18, 36	East Tropical Africa
<i>C. plectostachyus</i> (K. Schum.) Pilger	18	East Tropical Africa
<i>C. transvaalensis</i> Burt-Davy	18	South Africa
<i>C. magennisii</i> Hurcombe	27	South Africa

<sup>1</sup> Harlan et. al. 1970

## CHAPTER II

### Study of Reproductive Method in *Cynodon arcuatus*.

#### ABSTRACT

Plants of *Cynodon arcuatus* J.S. Presl & C.B. Presl Accession A10106 (PI 289613) are morphologically uniform indicative either of inbreeding or apomictic reproduction. No information is available on the method of reproduction in the species. Accordingly, this study was undertaken to determine the reproductive method in accession A10106. The study included observation of time of anther dehiscence, evaluation of the genetic relatedness of 30 full-sib progeny plants using DNA Amplification Fingerprinting (DAF) and examination of embryo-sacs. Observations indicated that anther dehiscence occurred in some cases, before the anthers were extruded from flowers, and in other cases, after anthers were extruded. The DAF study produced a total of 106 and 104 bands scored for four DAF and four MHP-DAF primers respectively. None of the DAF or MHP-DAF primers detected any polymorphism among the 30 progeny plants. Embryological examination of the ovules indicated only the presence of single embryo-sacs. Lack of multiple embryo-sacs ruled out the possibility of apospory. The lack of any DAF polymorphisms among the 30 full-sib progeny plants suggests apomictic reproduction, but does not rule out the possibility of strong inbreeding. Further study is required to elucidate the reproductive mechanism in *C. arcuatus* accession A10106.

## INTRODUCTION

*Cynodon* is a small genus of economically important grasses extensively used for turf and forage purposes. The genus is comprised of warm-season, perennial, sod-forming grasses adapted to a wide range of edaphic conditions. Usual asexual reproduction is through vegetative buds such as rhizomes, stolons and crown. However, almost all taxa reproduce by seeds assumed to be produced through normal sexual processes. The cosmopolitan species *Cynodon dactylon* var *dactylon* has been shown to be a strong outcrosser, as a result of cross-pollination and genetic self-incompatibility. The same is assumed true for the other *Cynodon* species exhibiting heterogeneity in plant populations (Burton, 1947; Harlan and de Wet, 1963; Taliaferro et al., 2004). Ploidy level within *Cynodon* varies from diploid to hexaploid. Most species are typically either diploid or tetraploid, with the exception being the triploid *C. magennesii* (Table 1).

*C. arcuatus* is easily recognizable from other species of the genus by virtue of its distinct morphology, comprising broad lanceolate leaves and long slender flexuous racemes arranged in a single whorl (Harlan et al., 1970, de Wet and Harlan, 1971). Pollination mechanisms have not been studied for *C. arcuatus*, however, it as well as the rest of the taxa of the genus is assumed to be cross-pollinating. The taxon is deemed to be genetically isolated, as evidenced by many failed sexual hybridization attempts in the past (Harlan et al., 1969). Furthermore, the genetic distance of *C. arcuatus* from other *Cynodon* species has been indicated at the molecular level (Assefa et al., 1999). Plants of *C. arcuatus* are highly fertile and prolific seed producers. Many *Cynodon arcuatus* accessions produce progeny that

exhibit less morphological variation than expected (Taliaferro, personal communication). Moreover, intra-specific variation among different accessions of the species has been minimal (Assefa et al., 1999). This lack of variation in progeny populations implies either strong inbreeding or gametophytic apomixis as possible modes of reproduction in *C. arcuatus*.

Gametophytic apomixis, or agamospermy, is defined as asexual reproduction via seeds formed in the absence of fertilization. The seeds are produced from the unreduced egg cells resulting from circumvented meiotic division, and hence generate progeny with the same genetic constitution as their female parent. Facultative apomicts reproduce through a combination of both sexual and apomictic reproduction within an individual plant or a plant population. Plants within such populations may vary from fully sexual to fully apomictic. Alternatively, plants within obligate apomictic species or species populations reproduce exclusively through apomixis (Bashaw, 1980).

The mechanism of apomixis, depending upon the cells giving rise to unreduced egg cells, is categorized into diplospory and apospory. In diplospory, meiotic cell division is short-circuited, and the megaspore mother cells, instead of producing a functional megaspore, divide mitotically to develop directly into an eight-nucleate embryo-sac (Leblanc et al., 1995; Bashaw, 1962). In apospory, embryo-sacs arise directly from nucellar cells of the ovule and unreduced nuclei in the aposporous embryo-sacs give rise to the embryo (Bashaw, 1980).

Apomixis is widespread in the plant kingdom and has been found in many polyploid forage and grasses (Bashaw, 1980, Bashaw and Funk, 1987; Hanna and



Bashaw, 1987). In these plants, a general trend is that the polyploid plants reproduce through apomixis while their diploid counterparts reproduce sexually. Bashaw (1962) reported obligate apomixis in buffelgrass with the mechanism of apospory and pseudogamy. Similarly, some plants of eastern gamagrass [*Tripsacum dactyloides* (L.)] plants have been shown to reproduce through facultative apomixis, along with pseudogamous diplospory (Leblanc et al., 1995, Burson, 1990). Burton and Forbes (1960) documented obligate apomixis in bahiagrass (*Paspalum notatum*), while Kentucky bluegrass (*Poa pratensis*) reproduced by aposporous pseudogamous facultative apomixis (Huff, 1992).

Use of DNA markers to estimate inherent genetic variability within and among the plant taxa has been ever increasing due to their power to discriminate based upon DNA sequence variation (Clegg, 1990; Patterson et al., 1991; Yang et al., 1996; Yerramsetty, 2004). Molecular fingerprinting systems like RFLP, AFLP, RAPD and DAF have been widely used in genetic studies of many plant species including bermudagrass. While all the techniques are relevant, each has its strength and weakness. The AFLP is very powerful in distinguishing between closely related genotypes; however, the cost of reagents, equipment and labor render it highly expensive. The RAPD procedure is simple and easy, but is less powerful in discriminating differences. In contrast, DAF is relatively simple, low in cost and has high resolution. Furthermore, the DAF technique is highly reproducible and can be easily accomplished with commonly available lab equipment. The DAF technique is based on PCR amplification of DNA fragments from genomic DNA using short 5-8 base arbitrary oligonucleotide primers (Caetano-Anolles, 1991). Amplification

products of a wide range of sizes are obtained, which are then separated through polyacrylamide gel electrophoresis and depicted to reveal bar code like patterns. This fragmentation pattern is unique and highly characteristic of the genomic DNA sequence of each individual tested.

Mini HairPin DAF (MHP-DAF) has been developed as an improvement over DAF with increased resolving power and higher specificity. The primers, containing palindromic sequences of 8-12 nucleotide basepairs, hybridize through intra-primer interactions creating a hairpin and a small looped priming structure (Caetano-Anolles and Gresshoff, 1994). The technique uses previously amplified DAF amplicons as templates to generate further banding pattern diversity. Caetano-Anolles et al. (1995) were able to reveal five times as many bermudagrass polymorphisms through MHP-DAF as conventional DAF primers. Thus, the procedure dramatically increases the resolving power of the DAF technique to separate even closely related species and accessions.

The DAF technique has been successfully used to study the phylogenetic relationship in a wide range of plants and animals. Several DAF studies of bermudagrass have been conducted including; 1) phylogenetic relationships among different *Cynodon* species (Assefa et al. 1999), 2) evaluation of off-types in bermudagrass cultivars (Caetano-Anolles, 1998), 3) determination of the fidelity of bermudagrass commercially sold as U-3 (Anderson et al., 2001) and 4) genetic relatedness of seeded bermudagrass cultivars (Yerramsetty et al., 2005). Ho et al. (1997) used the DAF technique to study the relatedness of 18 *Cynodon* cultivars available in Australia. Collectively, these studies demonstrated the ability of the

DAF procedure to provide information on the genetic relatedness of *Cynodon* plants or species to better assess their true identity or phylogeny.

Little information exists on the specific reproductive methods of *C. arcuatus* that would explain lack of morphological variation among plants in progeny populations. Accordingly, this study of *C. arcuatus* accession A10106 was initiated with the following objectives:

- Assess genetic variation among full-sib progeny using DAF DNA profiling,
- Evaluate the time of dehiscence of pollen from anthers i.e. whether dehiscence occurs before or after anthers are extruded from the flowers, and
- Examine serial sections to observe the number of mature embryo-sacs near or at the time of fertilization.

## **MATERIALS AND METHODS**

### **Plant Materials**

Around 250 seeds from *C. arcuatus* Accession No. 10106 were planted in the green house in a flat containing Metro Mix 250 growing medium (Scotts-Sierra Horticultural Products Co., Marysville, OH). The seeds originated from *C. arcuatus* plants growing in the greenhouse in proximity to plants of other *Cynodon* species, many of which flowered during the same period. Thirty vigorously growing plants were randomly selected and potted in 15 cm diameter pots under green house conditions. Plants were fertilized regularly with Peters Professional Peat-Lite (Scotts-Sierra, Marysville, OH) and Iron Chelate (Miller Chemical and Fertilizer Corp., Hanover, PA) to maintain good growth. The plants were treated with pesticides as needed.

### **DNA Isolation**

One gram of leaf tissue was harvested from each of the 30 pots, frozen in liquid nitrogen and ground to a fine powder using a mortar and pestle. Genomic DNA was isolated from 100 mg of powdered leaf tissue using the DNeasy plant mini-extraction kit (Qiagen Inc. Valencia, CA). DNA concentration was assessed spectrophotometrically at 260nm, and the quality was assessed by 260/280 ratio (Sambrook et al., 1989). All DNA samples were diluted to a final concentration of 20ng/  $\mu$ l in 0.5X TE. DNA quality was further assessed using TAE agarose gel electrophoresis. All samples showed good quality DNA with little or no DNA degradation.

### **PCR Amplification**

DAF fingerprints of the plants were resolved using four DAF and four MHP-DAF primers from Integrated DNA Technologies Inc., Corelville, IA (Table 3). The reactions were carried out in 200 $\mu$ l thin wall sterile tubes using 10X PCR buffer, 250 $\mu$ M dNTP, 9  $\mu$ M DAF primers, 50 $\mu$ M MgCl<sub>2</sub>, 1.5 U of Mango Taq polymerase (Bioline USA Inc., Randolph, MA) and 2 $\mu$ l of template DNA and added sterile water to make total reaction volume of 20 $\mu$ l. Positive control reaction mixes were used to ensure that specific bands were not due to random amplification of exogenous DNA. The amplification reactions were performed in a PTC-100 thermal cycler (MJ Research) with following temperature profiles: initial denaturation at 94°C for 60s, followed by 40 cycles each of 94°C for 30s, 30°C for 30s and 72°C for 60s, and a final extension of 72°C for 5 min. The reactions were hot started to increase the specificity of the initial PCR amplification reactions.

MHP-DAF analysis was conducted using the DAF PCR product that earlier showed maximum numbers of the bands in electrophoresis gels. All the procedures and reagents for MHP DAF were the same as with DAF except that 75  $\mu$ M MgCl<sub>2</sub> was used instead of 50  $\mu$ M and one  $\mu$ l of DAF product was used as the template.

### **Denaturing Polyacrylamide Gel Electrophoresis**

PCR products were run in 6% PolyAcrylamide Gel Electrophoresis (PAGE) gels in 1X TBE buffer using a BioRad Protein II apparatus (Bio Rad, Richmond, CA). The 20 cm long gel was made with Long Ranger Acrylamide (Cambrex Bio Science Inc., Rockland, ME), 1X TBE and 6M urea. Two gels were necessary because 34 wells were required to run all of the DNA samples, markers and controls. An Invitrogen (Carlsbad,

Ca) Low Mass DNA ladder was used as the marker. A total of 5  $\mu$ l of PCR product, with 2  $\mu$ l of formamide loading buffer containing the tracking dye bromophenol blue mixed, was loaded into the polyacrylamide gel and run at a voltage of 250V for about 3.5 hrs, until the bromophenol blue dye exited the gel. The gel was then stained with Syber Gold fluorescent stain (Molecular Probes Inc., Eugene, OR) in 1X TBE buffer for 20 minutes and images taken under UV light with the Gel Doc System (Bio Rad, Richmond, CA) according to manufacturers instructions.

### **Data Profiling and Analysis**

Each of the gel images was carefully examined for the presence of polymorphic bands, i.e. the bands present in at least one plant but absent in the others. Electrophoresis bands were visually scored, and the total number of bands present for each DAF and MHP-DAF primer was counted.

The whole DAF procedure, starting from DNA isolation, PCR reaction, gel electrophoresis, gel staining and bands scoring was repeated twice to ensure the reproducibility of the results.

### **Pollination Behavior**

In order to study the time-of-dehiscence of anthers, florets in pre-and post-anthesis stages were observed. Pre-anthesis florets were dissected under a stereoscope at 10X or 20X magnification using tweezers to reveal the anthers. The pre-anthesis florets were dissected as carefully as possible to negate or minimize anther dehiscence caused by the dissection. The pre-anthesis florets selected for dissection were judged to be nearing

the time for normal anther exertion. Post-anthesis florets were observed as close to the time of anther exertion as possible to determine if the anthers had dehisced prior to exertion. When necessary, these florets were placed under 10X and 20 X magnifications to confirm whether or not the anthers had dehisced. Florets were initially observed as close to the time of anther exertion as possible and repeated observations were made until dehiscence occurred.

### **Embryo-sac Analysis**

Inflorescences from the *C. arcuatus* progeny plants were collected at three different stages, from early maturity to 12 hrs after flowering, and fixed in formaldehyde acetic acid solution (FAA). Fixed ovaries were dehydrated with the tertiary butyl alcohol series, embedded in paraffin and sectioned at 12 thickness as outlined by Johansen (1940). Sectioned material was stained with saffranin- fast green dye combination. The slides were observed under phase contrast microscope to analyze the type of embryo-sac present.

## RESULTS AND DISCUSSIONS

### DNA Profiling

An average of 26.25 amplification products was obtained for the four DAF and four MHP-DAF primers. The total number of bands scored for the DAF and MHP-DAF primers were 106 and 104 respectively. Differences among the two runs (replications) were very small indicating high repeatability for the procedure. All the bands were monomorphic, and devoid of any polymorphism (Figs.1 and 2) even though the DNA profiles produced were clearly defined and repeatable. These results, along with the absence of any visual morphological variation, suggest genetic uniformity of the 30 progeny plants. The results are consistent with the studies by Asseffa et al. (1999) in which twelve DAF primers were unable to distinguish significant differences among the six accessions of *C arcuatus* under study.

DAF, along with MHP-DAF, have been demonstrated as a high resolution technique for assessing the genetic relatedness of *Cynodon* species and species cultivars (Caetano-Anolles et. al., 1995). Tifway and its irradiation mutant Tifway II were distinguished by using 81 DAF primers, through a single polymorphic band (Caetano-Anolles et. al., 1995). The high resolution capability of this technique suggests that even the most closely related plant lines are distinguishable, and conversely, if these techniques are unable to distinguish two or more plants then the plants must be very closely related or genetically identical. Furthermore, MHP-DAF primers are able to detect five times as many bermudagrass polymorphisms as conventional primers (Caetano-Anolles et. al., 1995). Even the MHP-DAF primers, in this case, failed to detect



any polymorphism. The failure of MHP-DAF to detect polymorphisms implies a high degree of genetic uniformity among the 30 plants. Such uniformity suggests either strong inbreeding or gametophytic apomixis as modes of reproduction.

### **Pollination Studies**

Observations indicated that the anthers dehiscence and pollen shed may occur either before or after the anthers are extruded from florets. Many of the florets observed showed the presence of dehisced pollens inside them (Fig. 3). However, there were other florets in which the anthers dehisced only after they were extruded from the florets. These observations suggest the occurrence of both self- and cross-pollination in the *C. arcuatus* plants.

### **Embryo-sac Analysis**

Examinations of 46 ovules were performed to look for evidence distinguishing the modes of inheritance at the embryological level. Ovules of *C. arcuatus* plants collected at different growth stages, from early maturity to 12 hrs after flowering, were section-sliced and fixed in the slides to examine the embryo-sac organization. There was no indication of multiple embryo-sacs in any of the ovules examined. However, none of the embryo-sacs examined had the organization definitive of the eight-nucleate polygonium type most characteristic of grasses. Nuclei were observed that were most likely either egg, polar or antipodal, though all of these nuclei types were not observed in any one sectioned ovule. Typical embryo-sacs observed in this study are depicted in Fig. 4.

The presence of the multiple embryo-sacs is indicative of apospory, the most common mechanism of apomixis in higher plants (Bashaw, 1980). The absence of multiple embryo-sacs is therefore, evidence against aposporous apomixis in *C. arcuatus*.

Hence the most likely reproductive modes that could account for absence of genetic variation among the 30 *C. arcuatus* progeny plants are inbreeding or diplosporous apomixis.

Diplospory is not a common method of apomixis in warm-season grasses. However, eastern gamagrass and members of weeping lovegrass (*Eragrostis curvula*) are two of a few warm-season grasses known to reproduce through diplosporous apomixis (Burson et al., 1990; Streetman, 1963 and Voigt and Bashaw, 1972, Burson et al., 1990). Determination of diplospory is much more difficult than apospory on the basis of cytological evidences (Bashaw and Hanna, 1990). The determination of diplosporous apomixis is nearly impossible by observation of mature ovules, as the embryo-sac may appear completely normal and identical to sexually derived embryo-sacs. The presence of meiosis, the resulting linear tetrad, and the functional megaspore as well as the deteriorating remains of non-functional megaspores confirm the presence of sexually reproducing ovules. In contrast, diplosporous apomixis is characterized by the absence of meiosis and resulting linear tetrad; and the presence of a long, tear-shaped megaspore mother cell (Burson et al., 1990). Similarly, sexual embryo-sacs originate from the chalazal megaspore and are hence located deeper within the ovule than the apomictic embryo-sacs developing from a megaspore mother cell near the micropyle. More comprehensive studies of megasporogenesis in *C. arcuatus* are necessary to determine the presence or absence of diplosporous apomixis.

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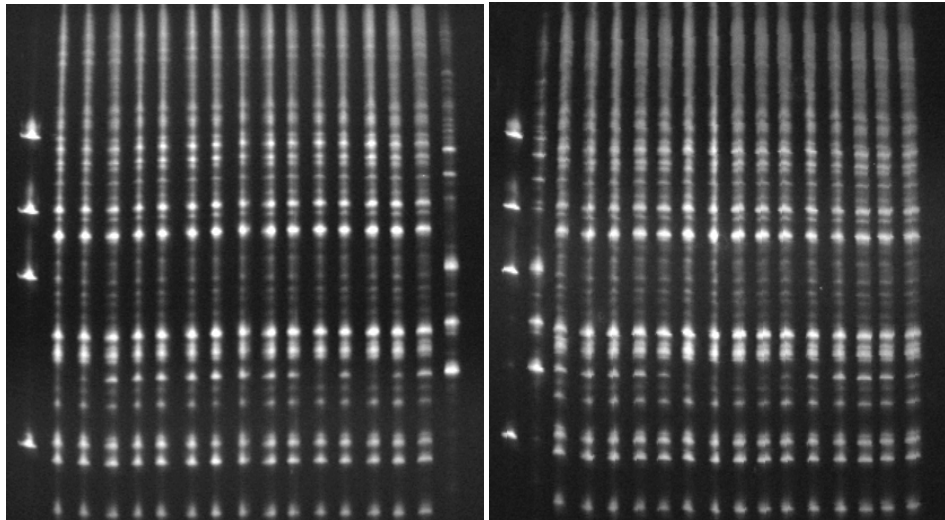
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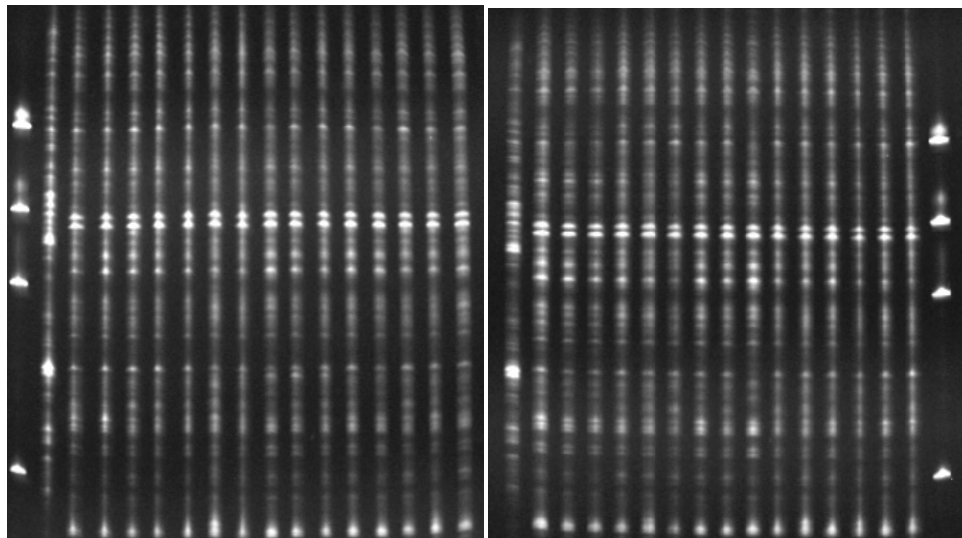
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**Table 1: Sequence of the DNA amplification Fingerprinting (DAF) and Mini Hair Pin-DAF (MHP-DAF) primers used in the study.**

<b>Primer Label</b>	<b>Primer Sequence</b>
DAF 9110	CAGAAACGCC
DAF9111	GAAACGCC
DAF9112	GTAACGCC
DAF9113	GTAACCCC
MHP-DAF 1	GCGAAGCGGA
MHP-DAF 2	GCGAAGCTACG
MHP-DAF 3	GCGAAGCCTA
MHP-DAF 4	GCGACAGCAGA



**Figure1.** Fingerprints of 30 progeny plants of *Cynodon arcuatus* accession A10106 using DAF Primer 9113.

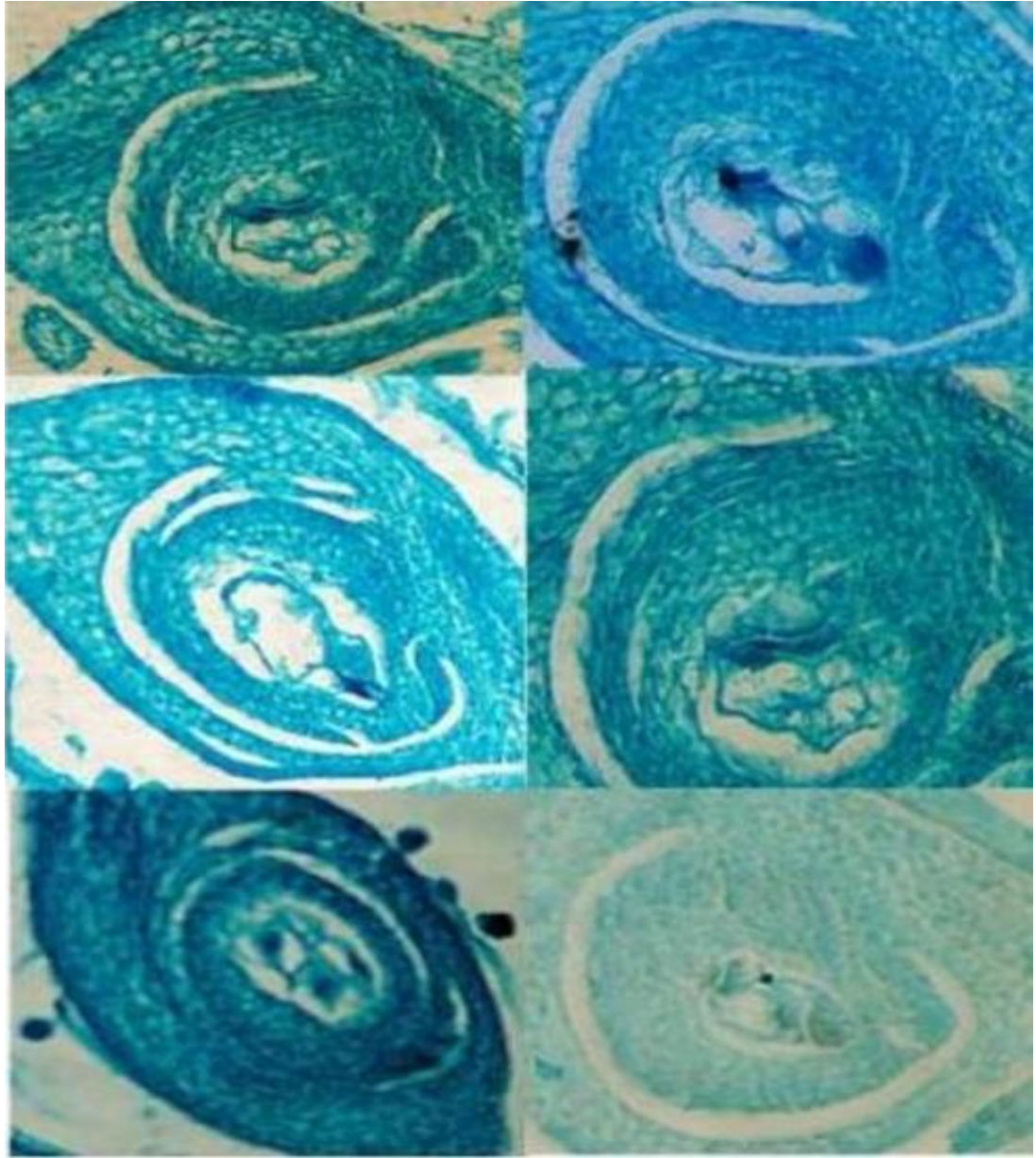


**Figure2.** Fingerprints of 30 progeny plants of *Cynodon arcuatus* accession A10106 using MHP-DAF Primer 1.





**Fig. 3** Excised anthers of *Cynodon arcuatus* accession A10106 in which dehiscence had occurred prior to extrusion from the flower.



**Fig. 4** Sections of ovules of *Cynodon arcuatus* accession A10106 showing mature single embryo-sacs.

### CHAPTER III

## Study of the Inheritance of a Leaf Chlorotic Trait in Bermudagrass

### ABSTRACT

The inheritance of a transverse chlorotic banding trait (zebra) on leaves of bermudagrass (*Cynodon dactylon* (L.) Pers.) was studied. The transverse chlorotic banding trait (zebra) was discovered on a single bermudagrass (*Cynodon dactylon* (L.) Pers) plant in an F<sub>1</sub> hybrid population in the 1970's. An F<sub>2</sub> progeny of the plant was clonally maintained because of its high self-and cross-fertility. Inheritance of the zebra trait has not been studied. Accordingly, this study was conducted to determine the inheritance of the zebra trait. Selfed progeny of the zebra plant and F<sub>1</sub> and F<sub>2</sub> progenies of crosses between Zebra and a bermudagrass plant with normal phenotype were used in the study. Selfed progeny (S<sub>1</sub>) of the Zebra clonal plant segregated into three phenotypic classes: albino, zebra, and normal green. Only 6 of 718 S<sub>1</sub> plants were normal green. S<sub>2</sub> progeny from each of four S<sub>1</sub> plants segregated only for albino and zebra phenotypes. Discounting the six normal green plants, the observed zebra: albino ratios were consistent with 9:7 ratio. Nine F<sub>1</sub> hybrids from Zebra x an unrelated normal green plant (Acc. 037) were initially of normal green phenotype, but after 9 months two and three plants developed intensive and faint chlorotic bands, respectively. F<sub>2</sub> and backcross (F<sub>1</sub> x Zebra male) progeny segregated for albino, zebra, and normal green phenotypes. Accurate

assessment of the zebra and normal green classes was not possible because of latent expression of the chlorotic bands in many plants. The data are not definitive of the inheritance of the zebra trait, but do provide evidence against maternal inheritance and implicate nuclear genes. Zebra trait expression in some plants is only after many months suggesting a strong environmental influence.

## INTRODUCTION

The genus *Cynodon* contains nine species and ten varieties, as taxonomically classified by Harlan et al. (1970) (Table 1). *C. dactylon* var *dactylon* is the most widely distributed taxon of the genus occurring worldwide distribution between latitudes of about 45°N and S (Harlan and de Wet, 1969). Plants of this taxon are commonly used for pasture, turf and soil conservation (Harlan, 1970; Burton and Hanna, 1995; Taliaferro, 1995).

Plants of *Cynodon* are warm-season (C-4), sod forming perennials. Studies on the reproductive behavior of *Cynodon dactylon* have demonstrated strong out-crossing resulting from cross-pollination and genetic self-incompatibility (Burton, 1947; Burton and Hart, 1967, Taliaferro et al., 2004). Sexual reproduction is through the normal sexually formed seeds, whereas individual plants are easily propagated from vegetative parts viz. rhizome, stolons or crown parts.

Although enormous genetic variation exists within some of the *Cynodon* taxa there is very little information documenting qualitative mutant phenotypes that might be useful in genetic studies. For instance, dominant markers would be especially useful in *Cynodon* as they have been in other plant species as indicators of hybridization and frequency thereof. Johnston and Taliaferro (1975) reported the discovery of a mutant bermudagrass plant with chlorotic stripes across the leaves but did not report its inheritance.

Chlorotic strips, or variegation patterns in general, have been reported in different plant species, including *Oryza sativa*, *Zea mays*, *Hordeum vulgare*, *Pennisetum glaucum*

and ornamental plants such as *Begonia*, *Dieffenbachia*, and *Dracaena*. In all of these plants, the shoots and especially the leaves have sectors of green and white in different patterns. Cells in the green sectors contain morphologically normal chloroplasts whereas the white sectors have plastids deficient in pigmentation and lamellar structure.

Different theories have been formulated regarding the development of chlorotic mutant phenotype. Mutation causing the chlorotic patterns could be in a nuclear gene governing chloroplast or mitochondrial phenotype, or else in extra-chromosomal genes present in chloroplast or mitochondrial genome, and therefore maternally inherited in consecutive progenies (Tilney-Bassett, 1975, Wu et al., 1999). There are also cases in which variegated mutants are produced as a result of insertion or excision of a transposable element in a gene locus (Nevers et al., 1986, Tilney-Bassett, 1991).

Some of the well-known examples of variegated phenotype induced by the recessive nuclear gene mutations include maize *iojap* and nonchromosomal stripe (NCS) mutations (Newton et al., 1990, Roussell et al. 1991, Han et al., 1992, Gu et al., 1993). The *iojap* phenotype is produced by the nuclear recessive gene *Ijij* segregating in a simple Mendelian 3:1 ratio (Jenkins, 1924), and giving rise to defective, maternally inherited plastids that undergo sorting out to form clonal sections containing non-pigmented cells.

Nonchromosomal stripe 5 (NCS5) mutations arise in maize from deletion of a portion of the cytochrome oxidase subunit 2 (Cox 2) gene in the mitochondrial genome. This mutant is also recessive. It produces permanently defective, maternally inherited mitochondria which secondarily affect chloroplast form and function in the form of white longitudinal stripes. A series of mitochondrial gene mutations named NCS2, NCS4,

NCS6 etc. have been documented with different mutation sites, all having different degrees of yellow strips on leaves, stunted growth and sectors of aborted kernels in ear.

Mutant nuclear genes in the other crops that produce transverse chlorotic stripes (hereafter called “Zebra”) include a single recessive gene in rice (Kusumi et al., 2000); a dominant gene in pearl millet (Werner & Burton, 1991); and at least two completely different and unrelated nuclear genes lying in different chromosome locations in barley: one a simple recessive mutant and the other an incompletely dominant (Tsuchiya, 1984).

Three different types of leaf mutant variegations in the model plant *Arabidopsis thaliana* have been studied (Sakamoto et al., 2003). 1) *chloroplast mutator (chm*; Redei, 1973; Martinez-Zapater, 1992; Sakamoto et al., 1996), 2) *immutans (im*; Redei, 1963, 1967; Wetzel et al., 1994; Wetzel and Rodermeil, 1998), and 3) *yellow variegated 1 and 2 (var1 and var 2)*(Martinez-Zapater, 1992). The *var1* and *var 2* mutations affect mitochondrial and chloroplast gene expression respectively. The *Immutans* mutant is similar to the Zebra mutant in our study in that it is also sensitive to temperature and light with elevated expressions at higher intensities. All the plants discussed above are diploids and involve single genes. The *Cynodon* Zebra plant in our study is, however, a tetraploid. Tetraploidy doubles the number of alleles in each gene locus, resulting in a wider range of possible phenotypes depending upon the number of mutant alleles. Another possibility could be the involvement of multiple interacting genes instead of a single gene with multiple alleles.

This study was initiated with an objective of determining the inheritance of the chlorotic trait in the zebra mutant bermudagrass plant.

## MATERIALS AND METHODS

The Zebra plant used in this study was a clonal progenitor of the same plant reported by Johnston and Taliaferro (1975). It was reported to be an F<sub>2</sub> plant from one original plant in a population of F<sub>1</sub> plants that exhibited the chlorotic stripping. The parentage of the F<sub>1</sub> plant was not reported and records of the cross have been lost. For this study clonal plants of Zebra were used to produce seeds under self-pollination and as parents in crosses with plants of normal phenotype. Initially, selfed seeds (S<sub>1</sub>) from the clonal “Zebra” plants were grown and the plants classified for phenotypic expressions including albino, zebra and normal. Two different plantings were made of selfed seed, the first in January 2004 and the second in June 2004. Plants displaying chlorotic stripping differed in the intensity of the chlorotic strips. Accordingly, these plants were assigned to arbitrary groups 1 to 4, with group 1 representing the most intense, and group 4 representing the least intense stripping.

Randomly selected plants from each group were selfed to obtain S<sub>2</sub> progenies. Small glycine pollinating bags placed over immature inflorescences maintained selfing and avoided any possibility of cross-pollination. When the seeds reached maturity, bags were collected and seeds were processed by hand rubbing and blowing with a Dakota seed processor. The F<sub>3</sub> generation plants were grown from the seeds and classified into albino, zebra and green phenotypic classes. The observed segregation ratios were compared with different expected genetic ratios using chi-square<sup>1</sup>.

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<sup>1</sup>Yates correction was added to the chi-square formula to increase the accuracy of results since there are only two classes and hence one degree of freedom

$$X^2 = \frac{\sum [(\text{observed} - \text{expected number}) - \frac{1}{2}]^2}{\text{expected number}}$$



Taking into consideration the possibility of maternal inheritance of the trait through chloroplast DNA, reciprocal controlled crosses were attempted between “Zebra” clonal plants and a randomly selected *C. dactylon* accession 037, of normal phenotype and the same ploidy with high fertility and seedset rate. Inflorescences of the pistillate parent were emasculated and then pollinated with pollens from pre-determined male plants. Fresh pollen was liberally painted on the stigmas of emasculated florets. Emasculation of florets was mainly accomplished by placing the female plants in a humidity chamber and removing the anthers in early morning hours after their exertion from the floret, but before they could dehisce. A few emasculations were also done by excising the anthers from florets as described by Richardson (1958). All unemasculated florets on inflorescences used in the crossing were excised. After crossing, the inflorescences were covered with the pollinating bags and harvested when the seeds were mature.

The F<sub>1</sub> plants from crosses between zebra and accession 037 were classified for phenotype (albino, zebra, normal green) and selected F<sub>1</sub> plants were selfed or backcrossed to female Zebra.

## RESULTS AND DISCUSSIONS

### Results

Selfed progeny of the Zebra plant segregated for albino, zebra and normal green (Table 2). That segregation of the selfed plants into the three phenotypic classes suggests that the zebra phenotype is not maternally inherited. Percentages of plants in the three phenotypic classes were similar for populations started in January and June of 2004. Plants with the zebra phenotype constituted the largest class with percentages of 57 and 63 for the January and June populations, respectively. Albino plants constituted the next largest class with percentages of 43 and 35 for the same respective populations. The albino seedlings died within about 14 days after emergence. The January population had one plant of normal green phenotype, while the June population had five normal green plants. Selfed progeny from single randomly selected plants in the respective four zebra classes segregated only for albino and zebra phenotypes (Table 2). The absence of the green phenotype in selfed progeny from zebra plants in groups 1 through 4 may have been due to the low number of plants evaluated. That the normal green phenotype occurs at very low frequency is indicated by the results from the two selfed populations from the clonally maintained Zebra plant. Although unlikely, there is a possibility that the normal green plants in the Zebra selfed progeny populations resulted from error; either through mechanical contamination during the seed cleaning process or genetic contamination via pollination of the Zebra plant with pollen from a normal green plant. While selfed seed were produced from clonal Zebra plants grown in greenhouse pots in isolation from other bermudagrass plants, it is possible that pollen was carried through wind currents even

from outside sources. Assuming that the normal green plants resulted from error, the ratios of zebra and albino progeny best fit a 9 zebra:7 albino ratio (Table 3). Only the June population of selfed plants from the clonal Zebra parent have a significant Chi-square (10.15,  $P < 0.01$ ) indicating lack of fit to a 9:7 ratio. Homogeneity Chi-square analysis indicated that the six populations (two  $S_1$  and four  $S_2$ ) could be pooled. The homogeneity Chi-square (4.95) was not significant indicating goodness-of-fit to the 9 zebra:7 albino ratio. Accordingly, these results alone suggest that the trait is conditioned by duplicate dominant genes. Under this hypothesis, the zebra phenotype results in any plant having at least one dominant allele at both loci, and the albino phenotype results in any plant that is homozygous recessive at one or both loci.

The nine  $F_1$  plants from the crossing of Zebra ♀ by Accession 037 were initially of normal green phenotype. However, after about 9 months, two of the nine plants developed prominent chlorotic strips and three other plants developed less prominent chlorotic strips.  $F_2$  progeny from three of the nine  $F_1$  plants segregated into albino, zebra, and normal green phenotypic classes (Table 4). The data in Table 4 are not accurate relative to numbers of plants with zebra and normal phenotypes, but are accurate for numbers of albino plants. The inaccuracies are due to delayed expression of the zebra phenotype in some of the  $F_2$  progeny. Many plants with initial normal green phenotype developed the chlorotic stripping as time progressed up to 6 months when classification was terminated. It is likely that additional plants will ultimately express the zebra phenotype. Albino plants constituted 60, 19, and 14 percent of the total  $F_2$  plants in populations P1, P6, and P8.

Backcrosses of three of the nine F<sub>1</sub> plants as female to the Zebra clonal plant produced progeny that also segregated into albino, zebra, and normal green phenotypic classes (Table 5). The numbers of plants with zebra and normal green phenotypes are inaccurate for the same reason as previously stated. The percentages of albino plants in backcross populations BCP1, BCP2 and BCP9 were 14, 61, and 46, respectively.

### **Discussion**

Prior to beginning the experiment, the zebra trait was hypothesized to be due to mutation of a chloroplast gene, and therefore the trait was anticipated to be maternally inherited. However, the finding that selfed and hybrid progeny involving the Zebra clonal plant segregated into three phenotypic classes is evidence against maternal inheritance and for involvement of one or more nuclear genes. The possibility of interaction of nuclear and cytoplasmic genes also exists.

Assuming the 9 zebra:7 albino ratio to be correct, and the Zebra clonal plant to be of genotype AaBb, then zebra plants with genotypes AABB (1/16), AABb (1/8), AaBB (1/8), and AaBb (1/4) should result. No segregation should result in selfed progeny of the AABB genotype, and about 75% of the selfed progeny from genotypes AABb and AaBB should be zebra phenotype and the remaining 25% should be albino. Much greater frequencies of albino progeny in some F<sub>2</sub> and backcross populations are evidence against this simplistic explanation of genetic control of the trait. Accurate assessment of the number of plants with zebra and normal green phenotypes is confounded by the latent expression of the zebra trait. Some plants express the trait only after many months. This points to a strong environmental influence on expression of the trait in many plants.

The results of the study are not definitive of the genetic inheritance of the zebra chlorotic banding trait, but do indicate that one or more nuclear genes are likely involved. Additional study involving segregation of selfed and hybrid progenies may provide information more definitive of the genetic control of the trait. Better defined environmental influences, particularly temperature and light, on expression of the trait would likely facilitate accurate classification of progeny.

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**Table 1: Taxonomic classification of the genus *Cynodon*<sup>1</sup>.**

<b>Epithet</b>	<b>Chromosome No.</b>	<b>Distribution</b>
<i>C. aethiopicus</i> Clayton et Harlan	18, 36	East African rift valleys
<i>C. arcuatus</i> J. S. Presl. Ex C. B. Presl.	36	Malagasy, and Southern India to Northern Australia
<i>C. barberi</i> Rang. et. Trad.	18	Southern India
<i>C. dactylon</i> (L.) Pers. var. <i>dactylon</i>	36	Cosmopolitan
var. <i>afghanicus</i> Harlan et de Wet	18, 36	Afghanistan steppes
var. <i>aridus</i> Harlan et de Wet	18	Southern Africa northward to Palestine, east to South India
var. <i>coursii</i> (A. Camus) Harlan et Wet	36	Madagascar
var. <i>elegans</i> Rendle	36	Southern Africa, south of latitude 13° South
var. <i>polevansii</i> (Stent) de Wet et Harlan	36	Near Barberspan, South Africa
<i>C. incompletes</i> Nees var. <i>incompletes</i>	18	South Africa; Transvaal to Cape
var. <i>hirsutus</i> (Stent) de Wet et Harlan	18, 36	South Africa; Transvaal to Cape
<i>C. nlemfuensis</i> Vanderyst var. <i>nlemfuensis</i>	18, 36	East Africa
var. <i>robustus</i> Clayton et Harlan	18, 36	East Tropical Africa
<i>C. plectostachyus</i> (K. Schum.) Pilger	18	East Tropical Africa
<i>C. transvaalensis</i> Burt-Davy	18	South Africa
<i>C. magennisii</i> Hurcombe	27	South Africa

<sup>1</sup> Harlan et. al. (1970).

**Table 2. Segregation of selfed progeny from the Zebra clonal plant and one plant from each of four classification groups . Group 1 consisted of selfed plants with the most intense stripping and group 4 the least intense stripping.**

Date Planted	Type of Seeds	No. of seeds	No. of seedlings germinated	Albino	Zebra	Normal
01/04	Zebra	1000	327	140(42.81%)	186(56.88%)	1(0.3%)
06/04	Zebra	750	391	138(35.29%)	248(63.42%)	5(1.3%)
07/04	Zebra Gr.1	100	36	13(36%)	23(64%)	0
”	Zebra Gr.2	150	58	19(32.75%)	39(67.25%)	0
”	ZebraGr.3	50	21	9(42.85%)	12(57.14%)	0
”	Zebra Gr.4	23	11	5(45.45%)	6(54.54%)	0

**Table 3. Chi-square values for goodness of fit to a 9:7 ratio of zebra: albino phenotypes based on segregation of selfed progeny.**

Zebra Plants Population	Number of Plants					X <sup>2</sup> value
	Total	Zebra		Albino		
		Observed	Expected	Observed	Expected	
01/04 zebra	326	186	183	140	143	0.078
06/04 zebra	386	248	217	138	169	10.15**
07/04 zebra Gr.1	36	23	20.25	13	15.75	0.92
” ” Gr.2	58	39	32.6	19	25.4	2.94
” ” Gr.3	21	12	11.8	9	9.2	0.061
” ” Gr.4	11	6	6.2	5	4.8	0.097
Homogeneity Chi-Square Value: 4.945 (P 0.50-0.30)						

**Table 4. Segregation data of F2 progeny plants from the three F1 plants from crosses of the Zebra clonal plant x accession 037.**

<b>S N</b>	<b>Albino plants</b>	<b>Total no. of plants</b>	<b>Zebbras</b>	<b>Normal plants<sup>1</sup></b>
P1	140	234	116	118
P6	21	112	41	71
P8	24	167	34	133

<sup>1</sup>Latent expression of the Zebra phenotype was still occurring when classification was discontinued approximately six months after the plants were started. Consequently, the numbers of zebra and normal plants are not accurate.

**Table 5. Segregation data of progeny plants from back-crosses of three F1 plants (Zebra x accession 037) as female to the Zebra clonal plant.**

<b>S N</b>	<b>Albino plants</b>	<b>Total no. of plants</b>	<b>Zebbras</b>	<b>Normal plants<sup>1</sup></b>
BCP1	21	154	81	73
BCP2	134	221	162	59
BCP9	42	92	66	26

<sup>1</sup>Latent expression of the Zebra phenotype was still occurring when classification was discontinued approximately six months after the plants were started. Consequently, the numbers of zebra and normal plants are not accurate.

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Thirty full-sib progeny plants of *Cynodon arcuatus* were evaluated using DAF. Lack of DAF polymorphisms among progeny plants, with eight DAF and MHP-DAF primers, suggests apomixis, but does not rule out the possibility of strong inbreeding. Histological studies indicating only single embryo-sacs suggested against aposporous, but not diplosporous apomictic reproduction.

The inheritance of a transverse chlorotic trait (zebra) on bermudagrass [*Cynodon dactylon* (L.) Pers] leaves was studied. F<sub>2</sub> progeny ("Zebra") of the F<sub>1</sub> plant with the trait was clonally maintained. Selfed progeny of the Zebra and F<sub>1</sub> and F<sub>2</sub> progenies of crosses between Zebra and a normal bermudagrass (accession 037) were used. Accurate assessment of plants segregating into zebra and normal phenotypes was not possible because of latent expression of chlorotic bands in many plants. The data are not definitive of inheritance of the zebra trait, but do provide evidence against maternal inheritance and implicate nuclear genes in its control.

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