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POPULATION DYNAMICS OF THE GYNODIOECIOUS *BOUTELOUA CHONDROSIOIDES* (POACEAE)

by

Laura M Zahn

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As members of the Final Examination Committee, we certify that we have read the dissertation prepared by Laura M Zahn

entitled Population dynamics of the gynodioecious Bouteloua chondrosioides (Poaceae)

and recommend that it be accepted as fulfilling the dissertation requirement for the Degree of Doctor of Philosophy

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Final approval and acceptance of this dissertation is contingent upon the candidate's submission of the final copy of the dissertation to the Graduate College.

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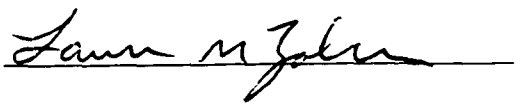
SIGNED: 

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DEDICATION

This dissertation is for those people who gave me the support and love I needed to make it through. Thank you Alex, Amy, Dan, Jeff, Karen, Linda and Mark. I love you all.

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ABSTRACT

This dissertation investigated the evolution and ecology of male sterility in the gynodioecious *Bouteloua chondrosioides* (Poaceae) by studying the distribution, inheritance, phenotypes of male sterility, mitochondrial DNA polymorphisms, and the distribution and effect polyploidy has on sex type expression in *B. chondrosioides*. *B. chondrosioides* has two male sterile types, one of which is described for the first time in this dissertation. Field studies determined that the proportion of male sterility was highly variable among populations and non-randomly distributed within populations. Investigations of the progeny of individuals of known sex type rejected models of simple nuclear recessive and dominant inheritance of male sterility. Examination of characters that may affect reproduction demonstrated that there were few significant reproductive differences explaining the maintenance of the two male sterile forms. In order to investigate if male sterility is due to cytoplasmic factors, mitochondrial DNA restriction fragment length polymorphisms were examined to determine if there were correlations between unique restriction fragment patterns and male sterile forms. These studies demonstrated that some, but not all, male sterile individuals do have unique mitochondrial restriction fragments. In addition to these investigations, the distribution of polyploidy was characterized and investigations performed to determine if there are correlations between sex type and ploidy level and if polyploidy has evolved once or multiple times in *B. chondrosioides*. Flow cytometry resulted in data that demonstrated

no correlation between male sterility and ploidy level, and that while most populations are either only diploid or tetraploid, some populations had both diploid and polyploid individuals. The examination of the relationships of cpDNA sequences from individuals of known ploidy level demonstrated that polyploidy appears to have originated and established more than once in the history of *B. chondrosioides*. The results from these three studies exhibit patterns that are in accord with the hypothesis that male sterility in *B. chondrosioides* is due to cytoplasmic male sterility.

INTRODUCTION TO THE STUDY OF MALE STERILITY IN THE GYNODIOECIOUS *BOUTELOUA CHONDROSIOIDES* (POACEAE)

Chapter 1

Rationale for the dissertation: The reproductive system of an angiosperm is the mechanism by which genes are passed from one generation to the next by producing pollen and ovules for fertilization, fruiting, and dispersing seeds, which leads to seedling germination and establishment (Barrett and Eckert, 1990). The basic means by which angiosperms accomplish reproduction is through a mating system composed of perfect, self-compatible, hermaphroditic flowers with both male and female reproductive function. The mating system of a plant directly affects the evolutionary potential of a species because variation in the reproduction mechanisms is under stringent selection (Bradshaw *et. al.*, 1995). Any modification in the floral phenotype is of great interest because changes in the plant mating system will directly impact patterns of genetic diversity, speciation and evolution.

Many studies of male sterility have been performed on grass crop species, but studies of male sterility within natural populations are of major importance for understanding the evolution of gynodioecy (Belhassen *et al.*, 1991). Because modifications in the floral phenotype have the greatest impact on the fitness of an individual, the study of mating strategies of a species should be a high priority in scientific investigations of plants (Barrett, 1998). Hypotheses concerning the evolution of polymorphisms in mating systems have been proposed but are problematic to test as dimorphism in plant reproduction is relatively rare (Barrett, 1992). The main source of intraspecific floral dimorphism is found in diclinous plants, those species that separate male and female function either within the individual or among individuals within a population. One such species is the gynodioecious grass *Bouteloua chondrosioides*, which is of interest to

studies of plant mating systems because it has intra-specific floral dimorphism. *B. chondrosioides* is gynodioecious, with a mating system in which populations are composed of both hermaphroditic and male sterile individuals. This dissertation examined the genetic basis of male sterility in *B. chondrosioides*. In addition, investigations of *B. chondrosioides* were performed to determine the occurrence and distribution of male sterile phenotypes and an investigation of phenotypic characters affecting reproduction among the sexual phenotypes, if mitochondrial polymorphisms are correlated with male sterility, and the effects and evolution of polyploidy.

Theoretical and empirical studies of gynodioecy: Mutations causing male sterility are common in plants, but the maintenance of male sterile individuals within populations as a gynodioecious mating system is fairly rare, occurring in 4-8% of all angiosperms (Kaul, 1988; Richards, 1997). Male sterile plants are at a reproductive disadvantage, relative to hermaphrodites, because of their loss of male function. Male sterile individuals can only reproduce through their ovules, causing a reproductive deficiency in the number of genes they are able to pass to offspring in the next generation (Lewis, 1941; Lloyd, 1975; Agren and Willson, 1991; Manicacci, *et al.*, 1997). Theory dictates that plants with genetically determined male sterility should not be maintained within hermaphroditic populations without a significant reproductive advantage in their offspring (Lewis, 1941; Lloyd, 1975; Ross, 1978; Charlesworth and Charlesworth, 1978; Pannell, 1997). The major focus of the study of gynodioecy is the ecological and evolutionary significance of how gynodioecious species are able to maintain male sterile individuals within populations.

In order to address how gynodioecy is maintained, many studies have examined if adaptations are found that increase the reproductive potential of male sterile individuals, relative to hermaphrodites. The degree of the advantage required in sexual reproduction depends on the mode of inheritance (Ross, 1978). Male sterile individuals require only a slight advantage over hermaphrodites if male sterility is cytoplasmically inherited (Charlesworth and Charlesworth, 1978; Agren and Willson, 1991). However, for nuclear inheritance, male sterile individuals must be at least twice as fit as hermaphrodites (Lloyd, 1975; Stevens and Van Damme, 1988; Frank, 1989). Significant differences between male sterile individuals and hermaphrodites, in gynodioecious species, have

been observed in characters relating to fitness. These characters include: an increase in flower or inflorescence number (Eckhart, 1999; Delph and Carroll, 2001), resource allocation to the sex organs (Connor, 1973; Ashman, 1992; Eckhart and Chapin, 1997), fruit number (Connor, 1973; Van Damme and Van Delden, 1984; Delph and Carroll, 2001), seed set (Connor, 1973; reviewed in Gouyon and Couvet, 1987; Tarayre and Thompson, 1997), seed mass (Krohne *et al.*, 1980), seed quality (Couvet *et al.*, 1986), germination frequencies (Couvet *et al.*, 1986), survival in variable or harsh environments (Krohne *et al.*, 1980; Van Damme and Van Delden, 1984) and adult survival and life span (Gouyon and Couvet, 1987; Tarayre and Thompson, 1997).

The maintenance of male sterility within a species requires several stages to become established. Initially a single genetic element, either nuclear or cytoplasmic, must arise within a population (Ross, 1978; Bawa, 1980; Richards, 1997). This is an inherently unstable condition and the population will vary in levels of male sterility and can fluctuate between gynodioecy and hermaphroditism in a process driven by genetic variability and natural selection (Ross, 1978; Bawa, 1980; Richards, 1997). If a second unlinked genetic element, typically a nuclear gene, arises within the population, then stable gynodioecy can be established (Richards, 1997). Once gynodioecy has been established in a population or species, the frequency of male sterile individuals is determined and maintained by the relative degree of inbreeding depression, the frequency of seeds that each sex type produces, and the proportion of survival of offspring of each sex type (Richards, 1997; Charlesworth and Laporte, 1998). If female functions are selected for in male sterile individuals, there may also be concurrent selection towards

maleness in hermaphroditic individuals (Lloyd, 1975). This selection for male and female function may eventually lead to the full separation of the sexes and a dioecious mating system (Richards, 1997).

Because of the advantages male sterile individuals have due to selection against inbreeding, relative to hermaphrodites in a population with a mixed mating system, the gynodioecious mating system effectively acts the same as other mating systems that enforce outbreeding such as dioecy and self incompatibility (Kaul, 1988). Theoretically, gynodioecy fulfills the same function in reducing self pollination (Delph, 1990) within populations and may be an alternate reproductive strategy and be established in place of dioecy (Richards, 1997). Support for this theory comes from the fact that few floras have both gynodioecy and full dioecy (Richards, 1997). In floras that typically have high levels of dioecy, in the absence of dioecy, especially islands, gynodioecy tends to be found in lieu of dioecy (Richards, 1997). Typically, gynodioecy and dioecy are found in different ecological zones as gynodioecy is far more common in temperate zones and dioecy more common in tropical zones (Richards, 1997).

Other support for dioecy and gynodioecy being similar mating systems is that gynodioecy and dioecy are the only plant mating systems that theoretically can be maintained within a species as an evolutionary stable system (ESS) (Richards, 1997). An ESS is an evolutionary strategy which dominates an ecological strategy, in this case a plant mating system, such that once established it cannot be replaced by any other strategy under natural selection (Smith and Price, 1973; Smith, 1982). As an ESS, gynodioecy can be established such that it is maintained at equilibrium and is not in

transition between hermaphroditism and dioecy. Gynodioecy is the only plant mating system theorized to have the ability to achieve evolutionary stability through the establishment of a single sex in a hermaphroditic population (Bawa, 1980). If gynodioecy is an evolutionary stable mating system, male sterility must be determined by two unlinked genetic factors, either two unlinked nuclear genes or an interaction between cytoplasmic and nuclear genes (Richards, 1997).

However, besides establishment as an ESS or as an alternative to dioecy, gynodioecy can also be a transitory stage between hermaphroditism and dioecy (Ross, 1978). Theoretical studies have demonstrated that selection for the separation of sexual function can arise when selection against inbreeding rates result in the physical separation of the anthers and stigmas (Doust and Laporte, 1991). When a single gene determines sex expression it is an inherently unstable condition (Richards, 1997). Therefore, in cases of unstable gynodioecy it is expected that either the population will revert to hermaphroditism or that natural selection will maintain male sterile individuals until a 2^{nd} genetic element can be established within the population to produce stable gynodioecy or selection that acts to increase maleness in hermaphrodites and femaleness in male sterile individuals and the species becomes fully dioecious (Richards, 1997).

Two major hypothesis have been proposed to explain the evolution of dioecy via gynodioecy: The selection against inbreeding hypothesis and the resource allocation hypothesis. The selection against inbreeding hypothesis states that for dicliny to evolve, the mating system must have some degree of selfing which results in inbreeding depression (Ross, 1978; Bawa, 1980; Freeman *et al.*, 1997). Plant species with both

selfing and outcrossing tend to have less fit offspring that result from self-pollination and more fit offspring that result from outcrossing (Charlesworth and Charlesworth, 1978; Holsinger, 2000). Because male steriles cannot self-pollinate, they avoid inbreeding and are more fit due to a combination of inbreeding avoidance and out-crossing advantage (Lloyd, 1975; Thomson and Barrett, 1981; Gouyon and Couvet, 1987; Freeman *et al.*, 1997). Because most diclinous mating systems prevent selfing, the selection against inbreeding hypothesis states that male sterile plants will have an immediate advantage over hermaphrodites and will quickly establish within populations that have some degree of inbreeding (Ross, 1978; VanDamme and VanDelden, 1982). Selection against inbreeding, however, cannot result in dioecy, the total separation of male and female function, without concurrent selective adaptations towards the separation of the sexes (Freeman *et al.*, 1997).

The resource allocation hypothesis states that selection towards optimal sexual characters results in the evolution of sexually differentiated individuals (Freeman, *et al.*, 1997). Sexual selection towards differential resource allocation and beneficial pleiotropic effects in genes coding for male sterility can eventually result in dioecy (Lloyd, 1975; Bawa, 1980; Givinish, 1980). This selection can work in concert with selection against inbreeding (Charlesworth and Charlesworth, 1978). This hypothesis is strongly supported in theoretical models and is supported by the occurrence of dicliny in self-incompatible species (Bawa, 1980).

Despite theory, an advantage in male sterile individuals is not always required to establish gynodioecy because the need for a sexual fitness advantage can be negated by

alternative reproductive strategies. Asexual reproduction maintains male sterility in the grass genera *Cortaderia* and *Poa*, where several species in each genus are composed exclusively of male sterile individuals which are fully asexual and reproduce only by apomixis (Connor, 1983; Anton and Connor, 1995). In addition, in the cytoplasmically male sterile *Plantago lanceolata*, male sterility is maintained through a combination of effects due to maternal inheritance of male sterility caused by founder effects and an increase in vegetative establishment in male sterile individuals (VanDamme and VanDelden, 1984; Stevens and VanDamme, 1988; Maurice *et al.*, 1994).

Genetic and environmental determination of gynodioecy: The underlying genetic causes of gynodioecy in plants are difficult to identify because sex determining systems are often very complex and can be complicated by environmental effects (Gouyon and Couvet, 1987; Connor and Charlesworth, 1989; Charlesworth and Laporte, 1998). Male sterility in plants is caused by the environment (Heslop-Harrison, 1959; Kaul, 1988; Van Damme and Van Delden, 1984; Richards, 1997), the incomplete penetrance of genes coding for dioecy (Richards, 1997), dominant or recessive nuclear genes (Kaul, 1988), or cytoplasmic male sterility (CMS) (Kaul, 1988; Richards, 1997). CMS has been documented in several species within distantly related families (Richards, 1997) and it has been well established that CMS is the most common cause of gynodioecy (Frank, 1989; Richards, 1997). No natural gynodioecious populations that have been intensively studied have had male sterility attributed solely to nuclear factors (Budar and Pelletier, 2001), although complex nuclear sex determination has not been ruled out (or fully supported) in several gynodioecious species (Connor and Charlesworth, 1989; Charlesworth and Laporte, 1998). In addition, both nuclear and cytoplasmic factors conferring male sterility have been identified within *Plantago lanceolata* and male sterility is conferred by more than one genetic factor in many mutants found within crop species (Van Damme, 1983; Kaul, 1988; Charlesworth and Laporte, 1998).

Because of the value of male sterility to crop breeding, male sterility has been extensively documented in crops and domestic plant species (Kaul, 1998). The most common form of male sterility in crop plants is CMS (Kaul, 1988). Many domestic crop species have also had numerous spontaneous mutations identified that cause male sterility

by nuclear recessive inheritance (Charlesworth and Charlesworth, 1978; Kaul, 1988). In crop plants, representing most major lineages of the Poaceae, CMS has arisen spontaneously or been created through intra- or inter- specific or generic crosses (Kaul, 1988). This suggests that the underlying nuclear-cytoplasmic system causing male sterility is widespread within the grass family. However, the scarcity of gynodioecy in the grasses suggests that male sterility caused by CMS is typically deleterious in the field which prevents its establishment as a mating system in most instances. Less commonly, male sterility can be determined by dominant and multi-genic nuclear inheritance (Kaul, 1988; Connor and Charlesworth, 1989). In the gynodioecious grass *Cortaderia sellanoa*, male sterility is most likely due to a multi-genic recessive system, although CMS has not yet been fully excluded as the underlying source of male sterility (Connor and Charlesworth, 1989).

In addition to genes that cause male sterility, approximately 10% of plants express diclinous mating systems as a response to environmental signals (Lloyd and Bawa, 1984). Plant sex expression can be affected by density, temperature, light intensity, soil moisture and fertility of the soil (Lloyd and Bawa, 1984; Kaul, 1988). In some Poaceae, photoperiod and temperature can influence anther development (Heslop-Harrison, 1959; Moss and Heslop-Harrison, 1968). In the cases of environmentally determined sex expression, the sex ratio is often skewed within a population (Lloyd and Bawa, 1984). In environmentally induced sex expression, the distribution of male sterile individuals and male individuals have demonstrated a tendency to segregate where hermaphrodites and males tend to occupy more marginal habitats (Lloyd and Bawa, 1984; Quinn, 1991).

Overview of cytoplasmic male sterility: CMS results from rearrangements in the mitochondria that produce chimeric genes composed of the sequences of several genes and open reading frames (Hanson, 1991). These chimeric genes are either translated into new, hybrid, proteins that are expressed in the developing anthers, disrupting male fertility or are co-transcribed and affect wildtype mitochondrial genes by a currently unknown mechanism (Dohmen, *et al.*, 1994; Saumitou-Laprade *et al.*, 1994; Schnable and Wise, 1998). Alternative forms of mitochondrial rearrangements (cytotypes) can give rise to different male sterile phenotypes within a single species as seen in *Plantago lanceolata* (Van Damme, 1982), *Daucus carota* (Van Damme, 1983) and *Silene vulgaris* (Charlesworth and Laporte, 1998). The converse is also true where multiple male sterile cytotypes can produce a single male sterile phenotype (de Haan *et al.*, 1997a). Plants with CMS that have been characterized molecularly support this as most populations with male sterility have multiple cytotypes associated with male sterility within relatively small geographical distributions (Belhassen *et al.*, 1991; Saumitou-Laprade, *et al.*, 1993; Ronfort *et al.*, 1995; de Haan, *et al.*, 1997a; Hidalgo-Fernandez *et al.*, 1999, Budar and Pelletier, 2001).

Most male sterile cytoplasms have a unique nuclear restorer system that re-establishes male fertility (Hanson, 1991; Schnable and Wise, 1998; Budar and Pelletier, 2001). Nuclear restorers can affect the genes causing male sterility at all stages from transcription to modification of the mature protein (Schnable and Wise, 1998). The most clearly understood genetic fertility restorers are mRNA processing enzymes that splice chimeric mRNA transcripts which prevents the formation and accumulation of chimeric

proteins in the mitochondria (Hanson, 1991; Schnable and Wise, 1998; Srivastava. 2000: Budar and Pelletier, 2001).

Plant species with CMS often have a non-random and clustered distribution of male sterile individuals within the population (Manicacci *et al.*, 1997; McCauley, 1997; McCauley and Taylor, 1997; Thompson *et al.*, 1998; Taylor *et al.*, 1999). In addition, the sex ratio of gynodioecious species with CMS vary enormously, even among proximal populations, as is exemplified by *Thymus vulgaris* where populations contain from 0-95% male sterility (Tarayre and Thompson, 1997). This variation within and among populations in the distribution of male sterility has been explained as a function of the maternal inheritance of male sterility and its effect on the underlying genetic structure as well as population structure and drift (Couvet *et al.*, 1998). The distribution of male sterility can be affected by differential dispersal of seed and pollen, and differences in survival or expression of sex types due to the physiology, phenology and environment of the plant (Freeman *et al.*, 1997; Taylor *et al.*, 1999). The age of the population and position in the equilibrium cycle also affect the amount and distribution of male sterility in populations with CMS (Couvet *et al.*, 1986; Frank, 1989; McCauley and Taylor, 1997; Manicacci *et al.*, 1998; Delph and Carroll, 2001).

Recent advances in plant genetics have led to the ability to examine all the genes expressed within an individual plant at a specific developmental stage (Finkelstein *et* al., 2002; Roberts, 2002). Due to the high sequence conservation of mitochondrial genes (Laroche et al., 1997), it soon should be possible to use the entire mitochondrial genome of maize or rice as a template and examine which genes are differentially expressed

between individuals of each type of male sterile and hermaphrodites. In addition, once the rice genome is fully sequenced, we can test if there are differences in nuclear gene expression in both types of male sterile individuals and hermaphrodites using conserved rice genes as probes. Overall coding regions of genes within plants, even from distant families, show some degree of conservation (Aharoni and Vorst, 2002). These experiments may indicate which genes are acting to influence the expression of male sterility in *B. chondrosioides*.

Grass mating systems: Mating systems in the Poaceae are most likely influenced by the fact that grass pollen is only fertile a short time (Richards, 1997) and is relatively heavy (Connor, 1979), resulting in effective population sizes in sexual individuals of less than a kilometer (Connor, 1979). As a result of these constraints, the grasses have evolved anthers that tend to dehisce (release pollen) when there is wind to maximize cross pollination (Connor, 1979). The dehiscence of the anthers is also often coincidental with high temperature and humidity, conditions that facilitate successful pollination (Connor, 1979).

Individuals with only male or female sexual function are relatively uncommon in the grasses (Connor, 1979; 1981), although monoecy and its variants are common mating systems in the grasses (Connor, 1979). Gynodioecy is the rarest mating system found in the grasses, occurring only in three genera, *Bouteloua* (Reeder and Reeder, 1966), *Cortaderia* (Connor, 1979; 1981) and *Poa* species (Connor, 1979; 1981; Anton and Connor, 1995). Dioecy, where individual plants have only male or female function, has been described in 15 genera including *Bouteloua*, *Cortaderia* and *Poa* (Connor, 1979; Columbus, 1999). Androdioecy, where plants are either male or hermaphroditic, has not been documented in the Poaceae.

In species of both *Cortaderia* and *Poa*, apomixis, asexual seed formation, has replaced sexual reproduction by gynodioecy and has resulted in populations composed solely of male sterile individuals (Connor, 1979; 1983). However, apomixis is not associated with the separation of the sexes in the grasses as it is also found in hermaphroditic, and monoecious grass species (Connor, 1979). Several grass species are

known to be solely maintained by asexual reproduction (Chapman, 1992. Anton and Connor, 1995) while others are facilitative apomicts in response to day length (Connor. 1979).

Polyploidy, especially counts of 6X and higher, is often associated with apomixis in the grasses (Gould, 1951; Connor, 1979; Holsinger, 2000). Although rare, some diploid grasses are able to set asexual seed but most apomictic individuals are polyploid (Chapman, 1992). In the genus *Dichanthium*, diploid species or populations are fully sexual, those that are tetraploid are facilitative apomicts and those that are hexaploid are fully asexual (Connor, 1979). Similar observations on ploidy and asexuality have been made in *Bouteloua curtipendula* where individuals with chromosome counts higher than 52 set only apomictic seed (Gould, 1951).

The genus *Bouteloua:* Grasses of the genus *Bouteloua*, in the sub-family Chloridoideae. are tropical and sub-tropical grasses that are widespread and common in North and South America (Gould, 1979, Clayton and Renvoize, 1992). *Bouteloua* species are widely distributed and many species are the dominant vegetation of the short grass prairies, desert grasslands, and xeric desert shrub (Gould, 1979; Clayton and Renvoize, 1992). The hypothesized origin of this genus is central Mexico, the current center of Chloridoid diversity (Columbus *et al.*, 1998; Felger, 2000).

Recent additions to the genus *Bouteloua* form a monophyletic clade with 45 hermaphroditic species, eight primarily dioecious species with six of these exhibiting occasional monoecy, three species that are both monoecious and andromonoecious, and one gynodioecious species (Columbus, 1999). High diversity in mating systems are uncommon in the grasses and are found only in *Bouteloua* and *Poa*. Selection towards a reduction in self pollination may be driving the evolution of dicliny in *Bouteloua*, as self-pollination is known to occur in several genera in the Chloridoideae (Connor, 1981).

The mode of inheritance of sex in the dioecious *Bouteloua dactyloides* (formerly *Buchloë dactyloides*) has been well characterized as a genetic system, affected by plant density, inherited through the nucleus (Yin and Quinn, 1995). The pathway towards dioecy in *B. dactyloides* and other Chloridoid grasses is hypothesized to have evolved from a hermaphroditic, self-compatible, ancestor through a monoecious form that then evolved into a dioecious breeding system (Connor, 1981). Other diclinous species in *Bouteloua*, especially those that are monoecious, may also have sex determining systems like those demonstrated in other plant families (Yin and Quinn, 1995; Ainsworth, *et al.*,

1998). However, as all documented gynodioecious species are believed to have evolved directly from a hermaphroditic ancestor (Lloyd, 1975), dicliny in *Bouteloua* may have arisen through both monoecy and gynodioecy.

Chromosome counts have been performed on the majority of species within the genus *Bouteloua* (*sensu* Columbus, 1999). Most *Bouteloua* species, *sensu* Columbus (1999), are polyploid or have multiple ploidy levels. Of 28 *Bouteloua* species, where X=10 (Jacobs, 1986), 16 have one ploidy level that is either diploid or tetraploid (2X=20, 4X=40), two have two ploidy levels and are either diploid and tetraploid (2X=20, 4X=40) or are tetraploid and hexaploid (4X=40, 6X=60), and 10 have more than three documented ploidy states including aneuploid counts where particular chromosomes are present in extra or fewer copies instead of having chromosome numbers that are a direct multiple of the base chromosome number (Gould, 1979; J Reeder personal communication).

In many polyploid plants there are characters used to identify polyploids from diploids including, asexuality, larger size of the plant or floral organs, and adaptations to novel habitat (Stebbins, 1950, Keeler, 1998). Upon inspection, however, these characters vary from species to species and cannot be used for sweeping generalizations about concurrent changes that occur with polyploidy (Keeler, 1998). No defining characters are known to exist in most polyploid *Bouteloua* (J. Reeder, personal communication) although, in *B. curtipendula* pollen size and fertility have been correlated with polyploidy (Harlan, 1949; S. Smith, personal communication).

Bouteloua chondrosioides: *B. chondrosioides* (H.B.K.) Bentham ex S. Watson (Poaceae), commonly known as sprucetop grama, is considered one of the better forage grasses adapted to the arid grasslands of North America and dry tropical forests of Central America, making it an economically important species (US Department of Agriculture, 1937; Gould, 1951; 1979). *B. chondrosioides* is a bunchgrass that is often prevalent in arid grasslands on dry open south facing slopes and grassy plateaus (Gould, 1979; Bock and Bock, 2000) at elevations of 750-2000m in the Big Bend region of Texas and southern Arizona in the United States. In Arizona it is found on lithic torriothents and lithic haplustolls soil types (University of Arizona AgNIC, Ecological Site Guides at: <http://great-sandy.arid.arizona.edu/scripts/esrimap.dll?name=siteguides&cmd=Map>). The range of *B. chondrosioides* extends from the USA southwards into the interior of Mexico where it is distributed in the foothills of the Sierra Madre and throughout the central plateau (Gould, 1979). The distribution of *B. chondrosioides* shifts in more southern latitudes from the arid grasslands to the dry tropical forests of southern Mexico and extends through Central America to its southernmost locations in Costa Rica (Gould, 1979). In the dry tropical forests, *B. chondrosioides* is found at lower elevations of 50-150m (Columbus, personal communication). Besides experiencing a habitat shift, some Mexican populations have been described as a stoloniferious lawn (US Department of Agriculture, 1937; L Zahn, personal observation).

Male sterility in *B. chondrosioides*: The inflorescence of *B. chondrosioides* is characterized by 9-11 densely packed branches with 8-12 spikelets per branch from 10 to 100cm high. The spikelets are enclosed within a pair of glumes composed of a single sterile floret (grass flower) and a single fertile floret (Gould, 1979). The fertile hermaphroditic florets of *B. chondrosioides* are typical of the grasses: a pistil composed of a single ovary with two stigmas and styles and three stamens, composed of an anther borne on a filament (Gould, 1951). No studies have been performed on if apomixis occurs in *Bouteloua chondrosioides*, but it is known that some vegetative reproduction via tillering does occur (US Department of Agriculture, 1937; L Zahn, personal observation). Chromosome counts in *B. chondrosioides* have identified two ploidy levels: 2X=20; 4X=40 and one aneuploid count 2X=22 (Gould, 1979; Reeder and Reeder, 1966).

Bouteloua chondrosioides was initially thought to be hermaphroditic but in the 1960's male sterile plants were noted in Mexican populations (Reeder and Reeder, 1966). These MA (for Malformed Anthers) male sterile individuals have, instead of anthers borne on filaments, filaments that end with saggitate anther remnants that make no pollen and are aborted early in development that can only be seen with magnification (Reeder and Reeder, 1966). I have observed that these male sterile individuals have significantly larger pistils than hermaphrodites (data presented in Chapter 2), an observation that was diagramed but not discussed in Reeder and Reeder's paper.

I also have discovered a second, independently inherited, form of male sterility that co-exists in most populations with the MA male sterile form. This form has highly

reduced anthers when compared to fertile anthers. These MS (for Male Sterile) individuals have stunted anthers visible to the naked eye, always lack pollen grains, never exert from the floret, and are pale yellow in color. This trait is morphologically distinct from the MA male sterile form described by Reeder and Reeder as well as from fertile anthers from hermaphroditic individuals (data presented in Chapter 2).

Bouteloua chondrosioides is a good species in which to investigate male sterility as it is the only gynodioecious species in the genus *Bouteloua*, *sensu* Columbus (1999), and is closely related to species with a range of mating systems including hermaphroditism, dioecy and monoecy (Gould, 1979; Columbus, 1999). By investigating the natural distribution, inheritance, means of maintenance, mitochondrial polymorphisms, and effects of polyploidy on male sterility in *B. chondrosioides*, I will add to the body of knowledge about gynodioecy, and how male sterility is maintained in the grasses. These investigations are the basis for the study of the evolution of gynodioecy, as they allow us to directly examine natural selection at work and will provide information for future studies testing models and investigating how polymorphisms in plant mating systems evolve.

THE DISTRIBUTION, INHERITANCE, AND MAINTENANCE OF MALE STERILITY IN *BOUTELOUA CHONDROSIOIDES* (POACEAE)

Chapter 2

Summary of chapter 2: Experiments were performed on *Bouteloua chondrosioides*, a gynodioecious grass with two male sterile types, in order to investigate the distribution of male sterility in the field, the inheritance of the two male sterile types, and to document phenotypic differences between individuals that are hermaphrodites, individuals that are MA male sterile, and individuals that are MS male sterile. Data collected in the field were used to calculate the frequency and distribution of male sterile types in natural populations, seeds were collected in the field and grown in a greenhouse to test if the distribution of sex types among the offspring of parents of known sex type fit a model of either simple recessive or dominant genetic inheritance, and measurements of flowers of each sex type were performed to determine if floral characters and reproductive traits in individuals of known sex and their offspring were significantly different among the three sex types. The results of analyses of the data collected in field studies demonstrated that there are significant differences in the proportion of male sterile individuals among and within populations in Arizona, Mexico, and Texas. The distribution sex types among the progeny of parents of known sex type led to the rejection of the hypothesis that male sterility is due to either simple recessive or dominant nuclear inheritance for both the MA and MS male sterile types. The examination of floral characters established that there are significant floral morphological differences between MA male sterile individuals, MS male sterile individuals and hermaphrodites. The results from these studies indicate that male sterility in *B. chondrosioides* may be due to cytoplasmic male sterility.

Introduction to chapter 2: Gynodioecy is a plant mating system consisting of individuals that are either hermaphroditic, with full male and female reproductive function, or are male sterile and have only female reproductive function. Depending on the species, gynodioecy can be an evolutionarily stable system, maintained at a constant equilibrium within a species, or it can be unstable and a point in the transition between hermaphroditism and dioecy, the full separation of male and female function in plants (Ross, 1978; Richards, 1997). In either case, the maintenance of male sterility is dependent on an increase in fitness to counter the reproductive disadvantage due to the loss of male function (Lewis, 1941; Lloyd, 1975; Agren and Wilson, 1991; Manicacci, *et. al.*, 1997). Studies of gynodioecy are important to investigations of the ecological and evolutionary forces affecting floral reproduction. Studies of gynodioecious plants allow for the examination of the transition between hermaphroditism and dioecy, the differences between male and female reproductive function, how plants cope with the loss of male function, and how genetic and evolutionary factors influence the evolution of mating system polymorphisms (Ross 1978; Richards, 1997; Thompson *et al.*, 1998).

Male sterility in plants may be caused by the environment (Heslop- Harrison, 1959; Kaul, 1988; Van Damme and Van Delden, 1984; Richards, 1997), the incomplete penetrance of genes coding for dioecy (Richards, 1997), dominant or recessive nuclear genes (Kaul, 1988), or cytoplasmic male sterility (CMS) (Kaul, 1988; Richards, 1997). Studies have confirmed that in most non-domestic plants with gynodioecy, male sterility results from CMS (Frank, 1989; Manicacci *et al.*, 1997). In addition, CMS is known to

occur naturally in 70 different plant species, spanning many plant families (Frank, 1989). In addition, intra- or inter-specific hybridization may also result in CMS (Charlesworth, 1981; Kaul, 1988; Frank, 1989; Maurice *et. al.*, 1994; Charlesworth and Laporte, 1998). Determining the cause of gynodioecy is often difficult, as complex sex-determining systems are not easily identified (Gouyon and Couvet, 1987; Connor and Charlesworth, 1989; Charlesworth and Laporte, 1998). In cytoplasmic male sterile species, determining the inheritance of male sterility can be problematical due to nuclear genes that restore male fertility (Frank, 1989; Connor and Charlesworth, 1989). Nuclear fertility restorers vary greatly within a species as they can be cytoplasmic specific, have multiple genes that restore fertility, can be recessive, dominant or multigenic and can differ depending on the male sterile cytoplasm and population (de Haan *et al.*, 1997a). This is further complicated by the fact that both nuclear and cytoplasmic male sterility can be maintained within a single species (Van Damme, 1983; Kaul, 1988; Charlesworth and Laporte, 1998).

If the inheritance of male sterility within a species is due to multiple unlinked nuclear factors, the frequency of male sterility tends to be low, with a constant and even distribution of male sterile individuals within and among populations (Manicacci *et al.*, 1997). In contrast, plants with CMS and nuclear restorers often have populations with high frequencies of male sterility, high variation in the level of male sterility among populations, and populations that have non-random distribution of individuals of different sex types (Belhassen et al., 1989; Sun and Ganders, 1986; Frank, 1989; Gouyon *et al.*, 1991; Manicacci *et al.*, 1997; McCauley and Taylor, 1997).

No matter the sex determination system, an increase in fitness is required for male sterility to be maintained (Lewis, 1941). The degree of advantage needed for the maintenance of male sterility in a gynodioecious population depends on the mode of inheritance and life history of the species (Pannell, 1997). Theoretical studies have demonstrated that male sterile individuals need only a slight reproductive advantage if male sterility is due to CMS (Charlesworth and Charlesworth, 1978; Agren and Wilson, 1991). However, for nuclear inheritance, theory dictates that male sterile individuals must be at least twice as fit as hermaphrodites (Lloyd, 1976; Stevens and Van Damme, 1988; Frank, 1989; Pannell, 1997). Differences in characters relating to fitness between male sterile individuals and hermaphrodites have been observed in flower or inflorescence number (Eckhart, 1999; Delph and Carroll, 2001), fruit number (Connor, 1973; Van Damme and Van Delden, 1984; Delph and Carroll, 2001), seed set (Connor, 1973), seed mass (Krohne *et al.*, 1980), survival in variable or harsh environments (Krohne *et al.*, 1980; Van Damme and Van Delden, 1984) and resource allocation to the sex organs (Connor, 1973; Ashman, 1994; Eckhart and Chapin, 1997). In the case of two independent phenotypes, even if both are caused by cytoplasmic factors, selection is expected to differ for each (Gouyon *et al.*, 1991).

However, despite theoretical predictions, the maintenance of male sterility does not always appear to be due to an increase in sexual reproduction. In *Thymus vulgaris*, the high frequencies of male sterility detected in some populations are due to spatial dynamics between nuclear and cytoplasmic sex-determining genes (Thompson *et al.*, 1998). Furthermore, male sterile individuals of *Plantago lanceolata* and *Geranium*

sylvaticum persist despite no identifiable advantage in sexual reproduction (Van Damme. 1984; Agren and Willson, 1991). Male sterile individuals in *Plantago*, a species with CMS, are maintained through founder effect and maternal inheritance during colonization. In addition, an increase in vegetative establishment has been observed in male sterile individuals of *Plantago*, relative to hermaphroditic individuals (Van Damme and Van Delden, 1984; Stevens and Van Damme, 1988; Maurice *et. al.*, 1994).

Bouteloua chondrosioides (H.B.K.) Benth., or sprucetop grama, is a perennial, gynodioecious grass found in the desert grasslands of Arizona, Texas and north and central Mexico and the dry tropical forests of southern Mexico to Costa Rica at elevations of 50-2000 meters (Gould, 1951; 1979). Chromosome counts have demonstrated that *B. chondrosioides* can be either diploid (2n=20) or tetraploid (2n=40) (Reeder and Reeder, 1966; Gould, 1979). Ploidy has not been linked to sex type expression in other grasses (Anton and Connor, 1994) and preliminarily investigations of sex type in *B. chondrosioides* indicate that sex type is not correlated with ploidy levels (Chapter 4). No study has been performed to determine if apomixis occurs in *B. chondrosioides*, as documented in other polyploid *Bouteloua* species (Connor, 1979). *B. chondrosioides* does reproduce vegetatively via tillering (US Department of Agriculture, 1937; Zahn, personal observation). The extent of vegetative reproduction within populations of *B. chondrosioides* is currently unknown.

Reeder and Reeder (1966) described a male sterile type of *B. chondrosioides* with malformed anthers, referred to in this dissertation as the MA male sterile type. MA male sterile plants have florets with enlarged ovaries and stigmas, and instead of anthers borne on filaments, filaments end with sterile, colorless, saggitate anther remnants that make no

pollen, are aborted early in development, never exert and are invisible to the naked eye (Reeder and Reeder, 1966; Zahn, this study). Reeder and Reeder's study planted seeds. of unknown parental sex, collected in Aguascalientes, Guanajuato and Jalisco, Mexico in a greenhouse in New Haven, CT. From these seeds, they recovered 45-74% MA male sterile progeny (Reeder and Reeder, 1966). In addition, MA male sterility was identified on herbarium sheets in individuals from populations in Chihuahua and Michoacan. Mexico and Texas, USA (Reeder and Reeder, 1966). A second type of male sterility in *B. chondrosioides* was identified in the course of the studies performed for this dissertation. This male sterile type is referred to in this dissertation as the MS male sterile type and has sterile, highly reduced, yellow anthers visible to the naked eye that make no pollen and do not exert from the floret (Figure 2.1).

This chapter describes experiments performed to characterize differences in the distribution of male sterility, the morphology and phenotype of reproductive characters of hermaphrodites and the two male sterile types, and to investigate the mode of inheritance of male sterility in *B. chondrosioides*. The distribution of male sterility in *B. chondrosioides* was investigated by examining male sterility in the field among and within populations in Arizona, Mexico and Texas. The distributions of the sexes within small regions of populations of *B. chondrosioides* were mapped to test the hypothesis that the distribution of male sterility was random. High variation among populations and non-random distribution of male sterility within populations can indicate cytoplasmic male sterility (Sun and Ganders. 1986; Frank, 1989; Gouyon *et al.*, 1991; Manicacci *et al.*, 1997). If gynodioecy is due to cytoplasmic factors in *B. chondrosioides*, it is expected that the proportion of male sterility

will vary largely among populations, even among proximal sites (Manicacci *et. al.* 1997; Taylor *et al.*, 1999).

Experiments were also performed to identify the mode of inheritance of male sterility in *B. chondrosioides*. Despite the rarity of gynodioecy in the grasses (Connor, 1979; 1981), male sterility has been documented in most domestic grasses and is most commonly due to either cytoplasmic male sterility (CMS) or simple recessive nuclear inheritance (Kaul, 1988). Environmental effects (Heslop- Harrison, 1959; Kaul, 1988) and complex genetic systems composed of either dominant or recessive nuclear genes, or both can also cause male sterility in grasses (Kaul, 1988). This study investigated if the MA and MS male sterile types are due to genetic causes, are related or independently inherited, and, if genetic, how both are inherited. These questions were addressed by testing a model of simple nuclear recessive and dominant inheritance on the distribution of sex in the progeny of individuals of known sex type grown from seed collected in the field. It was hypothesized that simple recessive nuclear inheritance cause male sterility and that the MS type represents an intermediate heterozygous type between MA male sterility and hermaphroditism. Alternatively, independent inheritance of each male sterile type and rejection of a model of simple nuclear inheritance would indicate complex genetic inheritance or cytoplasmic male sterility

Lastly, this chapter describes investigations performed to determine how male sterile types of *B. chondrosioides* differ morphologically from each other and hermaphrodites and to explore how male sterility is maintained by examining floral phenotypes and traits that may be correlated with reproductive success. Investigations of variation in floral and

morphological phenotypes due to the sex type of the individual were performed by testing the hypotheses that there are no significant differences in floral and morphological traits among individuals of different sex types. To determine if there were significant differences between the offspring of male sterile parents and the offspring of hermaphrodites, a null hypothesis of no difference was tested on the data taken on seed size and mass, proportion of seeds germinating, and survival to flowering offspring. Those traits with significant difference among sex types may indicate how male sterility is maintained within populations of *B. chondrosioides*.

Bouteloua chondrosioides is a good species in which to investigate male sterility as it is the only gynodioecious species in the genus *Bouteloua*, *sensu* Columbus (1999), and is closely related to species with a range of mating systems including hermaphroditism, dioecy and monoecy (Gould, 1979; Columbus, 1999). By investigating the natural distribution, possible genetic basis, the possible inheritance mechanisms, and means of maintenance of male sterility in *B. chondrosioides*, our body of knowledge about gynodioecy and how male sterility is maintained in the grasses is increased. These investigations lay the foundation for future studies investigating the selective pressures that have given rise to the wide number of mating systems in *Bouteloua*.

Materials and methods:

Field studies: Flowering in *B. chondrosioides* begins 1-4 weeks after the onset of rainfall in July or August and ends 1-2 weeks after rainfall ceases in September through mid-November (Zahn, personal observation). Field studies were performed during the flowering season of *B. chondrosioides* to determine the average proportion of male sterile plants within six Arizonian populations, three Texan populations and two Mexican populations at locales where *B. chondrosioides* is one of the dominant species, based on proportion of ground cover (Figure 2.2; Zahn, personal observation). The Texas populations examined were diploid while the Arizona and Mexico populations were tetraploid based on the results of flow cytometery (Gould, 1979; Chapter 4). Because the non-flowering plant parts and occurrence of several co-occurring *Bouteloua* species are indistinguishable from *B. chondrosioides* in the field, and there are no significant non-floral morphological differences between individuals of different sex types in *B. chondrosioides*, data were only taken on flowering plants. Florets from 2 to 10 spikes were dissected per individual , and from different inflorescences when multiple inflorescences were present within an individual, to determine the sex type of an individual and to examine if sex was constant within an individual. Plants were surveyed in the field in Arizona, Texas and Mexico, when flowering, July through mid November. All populations surveyed were large and were estimated to contain more than 10,000 individuals based on the geography of the locale, the area of continuous distribution of plants and the average flowering plant density (Zahn, personal observations).

In order to map the distribution of flowering plants of different sexes in the field, plots were laid out using a tape measure and string to denote an area ranging from 8-225m^2 for surveying. This plot was then divided into square meters, making a grid, and the sex and relative location of each flowering *B. chondrosioides* plant within each square meter was recorded. The distribution of plants within these grids were measured at every locale but Dragoon and Fort Bowie. At the Canelo, SRER and TRR sites, permanent markers were established so that measurements of the distribution of individuals were taken at the exact same spot each year. The amount of sampling and the size of the total grid area depended on the number of sites visited in a season, the number of plants flowering at a site, the length of the flowering season, and the flowering plant density. Random sampling, where randomly selected plants within the population had their sex recorded, was performed in 1998 on two Mexican and the three Texan populations and in 1999 on six Arizonian populations. This random sampling covered 0.5-1 km^2 of the study population and was performed by sexing random plants along a single directional gradient to prevent overlapping counts. Random sampling was initiated in 1998 to maximize the number of plants able to be observed at a single site and to eliminate potential inaccuracies due to the concentration of particular sex types in small areas. Confidence intervals of the proportion of male sterility observed at the 95% confidence level were calculated based on the number of plants sampled at each site (Milton, 1992).

Sex type expression in the greenhouse: An autoclaved soil mix composed of 6 parts perlite, 5 parts peat moss, and 3 parts Arizona sand with the appropriate amount, approximately 10 Tbsp per 7 kg, of 14-14-14 K-N-P slow release fertilizer (Osmocote) (J. T. Columbus, personal communication) was used to grow all plants in this study. *B. chondrosioides* plants were either transplanted from the field in Arizona and Texas to the greenhouse or grown from seed collected in Arizona, Mexico and Texas in a southwestern facing greenhouse. In order to maintain a 16-hour day, consistent with the light conditions experienced during a typical flowering season, supplemental artificial lighting was provided for the plants that were being grown to investigate the inheritance of male sterility. All other plants were grown under natural light conditions of Tucson, AZ. The daily temperature within the greenhouse ranged from 32° to 20°C. Drip irrigation watered plants with 1 gallon-per-hour emitters for five minutes, three times every other day.

Plants were maintained for up to 6 years in the greenhouse. Except for those plants used in the investigation of the inheritance of male sterility, plants in the greenhouse were surveyed every two months in 1996, 1997, 1998 and 1999 and four times a year in 2000 and 2001 in order to determine if sex type expression was constant when flowering for individual plants. In addition, in order to determine if sex type expression was constant within a genet, twenty plants were vegetatively propagated, moved to a second greenhouse, and re-evaluated independently. The maximum and average life spans of individuals of *B. chondrosioides* are currently unknown, but individuals have been maintained in a greenhouse for up to six years (Zahn, personal observation).

Experimental crosses to investigate the inheritance of male sterility were attempted by manually transferring pollen from the stamens of hermaphrodites to stigmas on the same individual and a male sterile individual from the same population. In addition, pollen was transferred from a hermaphrodite to both a hermaphrodite and a male sterile individual from another population of same ploidy level. None of these manual pollen transfers resulted in seed set, despite multiple pollen transfers. Initially, crosses of *B. chondrosioides* individuals in the greenhouse were planned to determine if self-pollination could occur and to determine the inheritance of male sterility among progeny from crosses of male sterile individuals and hermaphrodites. These experiments had to be cancelled due to an inability to successfully obtain seed from controlled crosses.

Common garden experiment: A common garden experiment was performed to determine if the expressed level and types of male sterility differed from field observations when plants were grown in a novel, stressful, outdoor Tucson environment. Seeds were collected from eight sites in the summer of 1998 and germinated and planted in July, 1999. No more than three seeds from any single maternal line of unknown sex were germinated. When the seedlings had successfully produced coleoptiles and first true leaves greater than 1 cm long, they were planted in a flat in an 8X3 orientation, totaling 24 plants per flat. Flats were randomly placed in groups of 6 in a covered, fenced enclosure with an opaque roof but no walls, surrounded by chicken wire to exclude animals at the University Agriculture Center in Tucson and were watered daily. Data on sex expression of individuals were collected until the end of October, 1999.

Inheritance of male sterility: During field studies in 1998, fruiting spikes were collected from individuals in the field at Alpine and DMSP, Texas. These two populations were chosen because they represented the greatest differences in the levels of male sterility observed in the field within a region, 10 and 37%, respectively. In order to investigate the inheritance of male sterility in *B. chondrosioides*, caryopses were isolated from these spikes in the fall of 1998. The caryopses, which are composed of the seed and a thin pericarp, from here on will be referred to as "seeds." Twenty or more seeds were germinated from each of 15 parents from Alpine and 15 parents from DMSP on moist paper towels in petri dishes in the greenhouse. No more than ten seed could be isolated from spikes collected in the field at Alpine, so no MA male sterile parents from Alpine were used in this experiment. Seeds were germinated from one MS male sterile seed parent and 14 hermaphroditic seed parents from Alpine and seed from two MS male sterile seed parents, six hermaphroditic seed parents and seven MA male sterile seed parents from the DMSP population. All seed parents from both Alpine and DMSP were assigned random family identification numbers 1-30 and the offspring were then coded by family, position on the greenhouse bench, and number of plants in that family. In all, 1308 seeds were germinated during the first week of May 1999 on moist towels. As seeds successfully germinated, individuals with coleoptiles and first true leaves greater than 1 cm long were planted in individual 4"X 4" pots. The bench on which the seedlings were located was divided, from west to east, into four sections referred to as "blocks." Seedlings were randomly assigned to one of four blocks within the greenhouse, with an overflow area for 200 plants that did not fit in the original four blocks. A total of

1074 seedlings were planted. Plants were rotated within their blocks every month and dead plants removed. Data was collected on seedling survival over the first month of the experiment. Plants from the overflow were incorporated within the blocks to maintain a constant plant density after the removal of the dead seedlings. Plants were randomly distributed within the greenhouse blocks with regards to the sex of the seed parent, family, sex of the parent and population of origin. Once flowering began, data was taken daily from late July, 1999 to November 15, 1999. The date of floral initiation; floral sexual maturity, the date when the stigmas or stamens exerted; sex type, whether the plant was hermaphroditic, MA male sterile or MS male sterile; the number of tillers per individual at floral initiation; the height and number of inflorescences, at both floral initiation and sexual maturity; and the number of spikelets on the mature inflorescence were recorded daily from the end of July when the first plants began flowering until November, 15, 1999. The time between floral initiation and sexual maturity and the overall change in height were calculated from these measurements. The sex of the plant was evaluated for every new inflorescence. Sex type expression data was collected bi-monthly after the 15th of November, 1999 until October 1, 2001.

Floral fitness: Inflorescences of *B. chondrosioides* have 9-11 densely packed spikelets. each with 8-12 spikes. The spikes are composed of a single sterile floret and single fertile floret enclosed in a pair of glumes (Gould, 1979). Fertile hermaphroditic florets have a single ovary with two stigmas and styles and three stamens (Gould, 1951).

The stigma length, ovary length, ovary width, stamen length and stamen width of MA, MS and hermaphroditic individuals were measured using a micrometer and converted to mm to document phenotypic differences. Measurements were made on 44 plants collected in the field in Arizona, ten plants grown in the common garden from seed collected in Mexico, and 229 plants grown in the greenhouse from seed collected in Texas. Care was taken to examine spikelets that were sexually mature, but not yet developing seeds. Anthers from individuals of the MS male sterile type were always dissected and examined to determine if pollen could be found within these anthers. In addition, remnant anthers in MA male sterile individuals, the reduced anthers in MS male sterile individuals and anthers of hermaphrodites were stained with iodine-potassium iodine (IKI) stain. In 1999, fruiting spikes were collected in the field in Arizona from 21 hermaphrodites, 32 MA male sterile individuals and six MS male sterile individuals, and the number of seed per spike was counted. Measurements were also taken on the number of fertile spikelets per spike from 1-5 spikes per plant. The number of spikelets per spike were counted in 24 plants collected in the field in Arizona, 13 hermaphrodites, ten MA male sterile individuals and one MS male sterile, 11 plants grown in the common garden from seeds collected in Mexico, seven hermaphrodites, one MA male sterile and three MS male sterile individuals, and 26 plants grown in the greenhouse from seeds collected

in Texas, 19 hermaphrodites, five MA male sterile individuals and two MS male sterile individuals.

Three to five seeds were collected in the field in Arizona, from 12 hermaphrodites, ten MA male sterile individuals, and four MS male sterile individuals, and in the field in Texas from 12 hermaphrodites, eight MA male sterile individuals, and from two MS male sterile individuals. The seed length, width and mass were measured. Seed mass was determined by weighing seeds in groups of 3-5 and calculating the mean in mg.

Data from this study was analyzed by hand for Z-tests and χ^2-tests (Milton, 1992) or using JMP version 3.2.1 (1997 SAS institute) with outliers identified by JMP, and removed. P values of Z-scores were obtained from Milton (1992). For the χ^2-tests, the appropriate test statistic was entered in the chi-square calculator at http://www.stat.ucla.edu to determine the P value.

Results:

Male sterility in the field: The results from field observations of the levels of male sterility at populations in Arizona, Mexico and Texas demonstrated that the MA type of male sterility was found in all populations at frequencies that ranged from 0 to 37.9%, depending on year, number of plants sampled and sampling method. The MS type was rarer, as it did not occur at all in three populations at all, and occurred at lower frequencies than the MA type, ranging from 0 to 11.6%, depending on year, number of plants sampled and sampling method (Tables 2.1-2.3). No variation in sex type was ever documented within an individual examined in the field. The data from three populations in Arizona that were surveyed multiple years were used to test of if both types of male sterility occurred at consistent levels over time. The level of MA male sterility significantly changed over time at only the TRR population, while the level of MS male sterility significantly differed over time at all populations (Table 2.4).

To determine if the frequency of MA male sterility significantly differed with the frequency of MS male sterility a null hypothesis of no difference in the relative proportion of each type of male sterility from each type of data collected was tested using Z-tests comparing the relative proportions of each male sterile type. A regression analysis was also performed to determine if the frequency of MA male sterility in the field is correlated with the frequency of MS male sterility. These tests demonstrated that MA and MS male sterility were not significantly correlated (r^2=0.04, F=1.28, df=1, p<0.27) and occurred at significantly different frequencies among all observations (Z=26.83, p<0.0001), and from all observations in Arizona (Z=5.63, p<0.0001), Mexico (Z=54.14, p<0.0001), and Texas

($Z=52.02$, $p<0.0001$). Those populations where MA and MS male sterility occurred at significantly different frequencies are noted in Tables 2.1-2.3.

Distribution of male sterility in the field: From those populations observed in the field that recorded the distribution of sex in flowering individuals, the least squares mean density of flowering plants measured within grids ranged from 0.63±0.62 to 37.00±2.07 plants per square meter (Table 2.5). There were no significant correlations between plant density and either type of male sterility. There were significant changes in density over time at Canelo ($F=41.85$, $df=1$, $p<0.0001$), SRER ($F=13.40$, $df=2$, $p<0.0001$) and TRR ($F=67.71$, $df=2$, $p<0.0001$).

A G^2 squared likelihood ratio test was used to test if the distribution of sex types mapped within grids was random (Table 2.6). All Texas and Mexican populations had significantly non-random distribution of male sterility within grids. When both grids and random sampling were used to survey the proportion of male sterility, different sampling methods resulted in a significantly different observed proportion of MA male sterility at all but one site, and MS male sterility at three of seven sites (Table 2.7).

The website of the Western Regions Climate Center provided the average monthly rainfall data at <www.wrcc.dri.edu/summary/climsaz.html> for Canelo, SRER and Pena Blanca (data not shown). For the years that these sites were studied, there were no significant correlations between the level of MA or MS male sterility and flowering plant density, total seasonal rainfall or the relative amount of seasonal rainfall.

Variation in male sterility among regions and populations: Given that the distribution of male sterile plants is clustered, not random, different sampling methods yielded different observations of the frequency of male sterility (Table 2.7). Analyses of differences in levels of male sterility between regions and populations were performed by comparing observed levels of male sterility either only within grids or only based on results from random sampling. In order to compare the amount of male sterility observed among the regions of Arizona, Mexico, and Texas, the level of male sterility from all field data collected using random sampling were compared from Arizona in 1999 and from Mexico and Texas in 1998. The data collected in all grids in 1998 among regions. as well as among populations within regions by year were also compared to each other. These data were used to test the null hypothesis that MA and MS male sterility were constant across the Arizona, Mexico and Texas regions and among populations in these regions using a χ^2 test (Table 2.8).

Consistency in sex type: Of those plants observed in the greenhouse to determine if sex was constant, no variation was noted in sex type in any individual where multiple spikes and florets were scored numerous times in the greenhouse. A plant's sex type, including those plants that were vegetatively cloned, was determined to be constant within an individual and over time. This was true even after plants experienced unintentional drought conditions brought about by failure of the irrigation lines.

Common garden experiment: Growing plants in a common garden in Tucson, Arizona demonstrated that plants from Arizona, Texas and Mexico gave rise to all sex types that were able to mature and flower in a single season in a novel environment (Table 2.9). This environment was deemed stressful due to the high average seedling mortality of 76.7%. Seedling mortality among populations ranged from 27 to 97.5% and significantly differed among populations (χ^2= 45.46, df=6, p<0.0001). Although MS male sterility was not observed in the field at Durango, it was recovered in the common garden. Less than 20 offspring per population survived to flower, with the exception of the DMSP population (Table 2.9). The low number of offspring recovered in this experiment prevented testing the hypothesis that male sterility in the common garden was not significantly different from that observed in the field.

Inheritance of sex: The results from planting seeds collected in the field at Alpine and DMSP from parents of known sex type to investigate the inheritance of sex type demonstrated that MA and MS male sterility are independently inherited. No MA line had offspring that were MS and vice versa (Figure 2.3). Nor were any intermediate types of either male sterile type observed. The eight MA parents gave rise to 45-75% MA male sterile offspring, averaging 59.4%. The two MS parents gave rise to 40 and 62% MS male sterile offspring, averaging 51.8%. Three hermaphroditic parents gave rise to an average of 17.2% MA male sterile offspring, ranging from 8 to 24%, while one hermaphroditic parent gave rise to 17.7% MS male sterile offspring. Overall, seed from the DMSP population gave rise to 33.3% MA male sterile individuals and 9.0% MS male sterile individuals. Seed from Alpine gave rise to 0.6% MA and 9.3% MS male sterile individuals.

The distribution of sex types among the offspring of parents of known sex type from the DMSP population were examined using a model of simple nuclear recessive inheritance of male sterility to determine if the inheritance of either the MA or MS male sterile type fit this model. For this model, male sterile parents were presumed to be recessive homozygotes at a single locus, aa. As the MS type did not represent an intermediate type of MA male sterility, hermaphroditic plants that gave rise to MA male sterile offspring were hermaphroditic heterozygotes, Aa. Since there cannot be aa X aa (female X female) crosses, the population cannot be in Hardy-Weinberg equilibrium. The expected number of male sterile progeny from a male sterile parent will be ½ the frequency of Aa individuals in the population divided by 1- the total frequency of male

sterile individuals (aa individuals) in the population. Additionally, ¼ of the frequency of Aa individuals squared divided by 1- the total frequency of male sterile individuals in the population will be male sterile. A diagram of this model is presented in Figure 2.4. For MA individuals the model of simple recessive inheritance was rejected (χ^2=55.7, df=9, p<0.0001) under the assumption that the total proportion of MA male sterility recovered, 33.3%, explained the proportion of aa individuals from DMSP. This model was also rejected using frequencies of aa of 32.7, 36.7, and 28.7%, the values of MA male sterility collected from all sampling, random sampling, and sampling within the grid. A model of simple nuclear recessive inheritance was also rejected for the MS type of male sterility (χ^2 = 37.07, df= 3, p<0.0001) using frequencies of aa individuals of 9%, the total proportion of MS male sterility obtained from seeds collected in DMSP. This model was also rejected using frequencies of aa of 1.3, 0.9, and 1.8%, the values of MA male sterility collected from all sampling, random sampling, and sampling within the grid. These tests were not able to be performed on the Alpine population due to an insufficient number of families that gave rise to male sterile offspring. Because three hermaphroditic seed parents gave rise to MA offspring and one hermaphroditic parent gave rise to MS offspring, dominant nuclear inheritance was immediately rejected for either type of male sterility.

To determine if these populations were at equilibrium, the expected frequency of each male sterile type at equilibrium was calculated (Couvet *et al.*, 1986; Manicacci *et al.*, 1998) using the equation:

Expected frequency of females = $\frac{F*O_{MS} + (1-F)*O_H}{F*S + (1-F)}$

F is frequency of male sterile individuals; O_{MS}= frequency of male sterile offspring of male sterile individuals; O_H= frequency of male sterile offspring of hermaphrodites and S=male sterile fecundity using the relative number of seed per sex parent from Arizona (data presented in Figure 2.8B). Assuming that survival was the same for the offspring of each sex, the data from DMSP resulted in expected values of 31.2% MA male sterile offspring and 8.2% MS male sterile offspring (Manicacci *et al.*, 1998; this study). This test could not be performed on the Alpine population due to the lack of MA male sterile parents and the lack of hermaphrodites that gave rise to MS male sterile offspring. Paired-t-tests rejected the hypothesis that the proportions of each type of male sterility recovered in the greenhouse were significantly different from what was seen in the field for both Alpine and DMSP.

A general linear model demonstrated that the sex of the parent plant explained almost half of the variation among the offspring in sex type (r^2=0.45, Wald-χ^2=380.5, $p<0.0001$). The siblings of an individual explained half of the variation in an individual in sex type (r^2=0.51, Wald-χ^2=421.8, $p<0.0001$). The distribution of sex among the progeny was also significantly correlated with the population where the seeds were collected (r^2=0.13, Wald χ^2=80.5, $p<0.0001$). However, this was not significant when the variation from unequal distribution of the sexes was removed from this model by nesting.

Offspring survival to flowering: Those plants that were evaluated to determine the inheritance of male sterility, were also examined to determine if differences were seen in survival among the progeny of plants of known sex. Data on the average seed germination rate, seedling survival one month after planting, plant survival after the first season in the greenhouse (1^{st} of July - 15^{th} of November) and plant survival over the entire experiment (1^{st} of July, 1999 -1^{st} of October, 2001) by parental sex type and population are presented in Table 2.10. The location of plants within the greenhouse did not significantly affect the probability of plant survival at any stage. The number of seeds germinated per family averaged 43.6±11.7 and significantly differed among families (χ^2=90.4, df=29, p<0.0001). Successful germination occurred in 82.1% of all seeds. An average of 35.8±12.1 seeds were planted per family and the number of seeds per family significantly differed among families (χ^2=138, df= 28, p<0.0001). There was no significant differences in the proportion of seedlings planted among families, parents of different sexes or populations.

The population that seeds were collected from had a significant effect on seedling survival over the first month (F=7.04, df=1, p<0.013), the proportion of plants that flowered during the first season (F=18.14, df=1, p<0.0002), and the probability of plants flowering (F=21.62, df=1, p<0.0001) (Table 2.10). Parental sex type had a significant effect on the probability of plants flowering, as the offspring of MS plants were more likely to survive and flower than hermaphrodites (Tukey/Kramer test, p<0.05) even when differences among populations and sexes were nested in the analyses (F=5.42, df=2,

$p<0.034$) (Table 2.10 and Figure 2.5). No significant effect of survival was seen in MA individuals when differences among populations and sexes were nested in the analyses.

Flowering during the first typical flowering season, over the period the 1st of July through the 15th of November in 1999, ranged from 0 to 73.7% of seedlings planted per family. There were no significant differences among families in survival to flowering, when nested among populations and parental sex type. The number of individuals flowering over the entire experiment, July, 1999 - October, 2001, ranged from 0 to 79% of seeds per family and did significantly differ among families, even when nested by population and parental sex type ($F=1053.88$, $df=25$, $p<0.024$).

Sex type differences: Measurements of floral and reproductive characters that were taken on individuals of different sex types were used to test null hypotheses of no significant differences in the size of sex organs among individuals of different sex types with an ANOVA. The results from these measurements and tests are presented in Table 2.11 and Figures 2.6- 2.8. Pollen mother cells were observed within the anthers of hermaphrodites at the time just prior to the emergence of the spikelets from the grass sheath that contains the developing inflorescence. Pollen grains were observed in all sexually mature anthers, just prior to anther dehiscence, in hermaphrodites but never in MS nor MA male sterile types. Examination of stained anthers demonstrated that the hermaphrodite anthers had neat and ordered cells with regular nuclei in the epidermis of the microsporangium at 40X magnification. The highly reduced stamens of MS male sterile individuals had no observable cellular structure in any of the 4 cell layers found in the microsporangia and no nuclei could be seen. The MA male sterile anther remnants had no measurable developed microsporangia and were composed of an unorganized mass of cells at the end of the filament.

Ovary and stigma length significantly differed between MA male sterile individuals and both MS male sterile individuals and hermaphrodites (Tukey/Kramer test, $p<0.05$) but not between MS male sterile individuals and hermaphrodites. The number of spikes per spikelet in hermaphrodites and MS male sterile individuals, over all populations, did not significantly differ from each other but MA male sterile individuals had significantly fewer spikes per spikelet than both hermaphroditic and MS male sterile individuals ($F=5.26$, $df= 242$, $p<0.006$) (Tukey/Kramer $p<0.05$) (Figure 2.8A). The length of seeds

from MS male sterile individuals and hermaphrodites did not differ significantly from each other, but both were shorter than the seeds of MA individuals (Tukey/Kramer $p<0.05$) (Table 2.11; Figure 2.7). The width of seeds from MA and MS male sterile individuals were not significantly different from each other, but both were significantly wider than hermaphrodites (Tukey/Kramer $p<0.05$) (Table 2.11; Figure 2.7). Seed mass ranged from 4-16 mg. MS parents had significantly heavier seeds than MA male sterile individuals and hermaphrodites. In turn, MA parents had significantly heavier seeds than hermaphroditic parents (Tukey/Kramer $p<0.05$) (Table 2.11; Figure 2.8C). The average number of seeds per spike collected in the field from Arizona populations ranged from 0-6. Hermaphrodites averaged 1.29 ± 0.52 seeds per spike, MA male sterile individuals averaged 1.28 ± 0.51 seeds per spike and MS male sterile individuals averaged 0.98 ± 0.78 seeds per spike (Figure 2.8B). Seed set in MA male sterile individuals and hermaphrodites were not significantly different from each other but both set significantly more seed per spike than MS male sterile individuals (Tukey/Kramer $p<0.05$)(F ratio=6.25, $df=1579$, $p<0.002$).

Other reproductive characters: For the investigation of the inheritance of male sterility, data were taken on the offspring of parent of known sex type to identify any phenotypes associated with the family, location within the greenhouse, sex of the parent and sex of the individual that could explain the maintenance of male sterility in *B. chondrosioides*. Appendix A presents the data taken on non-floral and non-reproductive traits in the greenhouse from the 1st of July to the 15th of November 1999. Most of these results were in accordance with those of other grass species (S. Smith, personal communication) and demonstrated no correlation with sex type. In order to determine if traits were correlated with sex, analyses were performed that removed any significant variation due to the location within the greenhouse, family, sex of the parent, and population by nesting the data. Through these analyses, it was demonstrated that MS male sterile plants took a significantly longer time to reach both floral initiation ($F=2.73$, $df=15$, $P<0.0006$) and floral maturity ($F=2.89$, $df=15$, $p<0.0003$), than what was seen in hermaphrodites (Tukey/Kramer, $p<0.05$) (Figure 2.9A). MS plants also had significantly taller inflorescences than hermaphrodites, but only at floral maturity ($F=3.96$, $df=4$, $p<0.004$) (Tukey/Kramer, $p<0.05$), and MS male sterile inflorescences were not significantly taller than the inflorescences of MA male sterile individuals (Figure 2.9B).

Discussion of chapter 2:

Inheritance of sex: While these studies were unable to determine to what extent the environment may influence sex type expression, repeated surveys of individuals in the greenhouse demonstrated that sex phenotype was constant within an individual over time. If sex determination is due to environmental effects, either those signals that would cause these effects were not encountered in the greenhouse, or these effects are a component of development and fixed at a set time at or before the maturity of the first inflorescence. The common garden experiment was performed to test environmental effects, but the high mortality makes the data on sex type expression from this experiment suspect. The rotation of plants within the greenhouse was performed to reduce any plant density effects since most plant growth substances can cause male sterility, including ethylene (Sawhney and Shukla, 1994). However, this rotation may have confounded any attempts to determine if there were environmental effects due to plant density that may have affected the expression of male sterility. Further tests examining the effect of the environment, especially extremes in soil moisture, day length, and temperature, on clones of individuals of each of the three sex types might help determine if sex type expression in *B. chondrosioides* is affected by the environment.

Despite being unable to fully investigate if sex type expression is affected by the environment, the data obtained from the DMSP population support a primarily genetic inheritance of sex. This is supported by the fact that the frequency of both types of male sterility observed in the greenhouse were not statistically different from that expected at equilibrium (Couvet *et al.*, 1986; Manicacci *et al.*, 1998) or from the levels of male

sterility observed in the field. The independent inheritance of both male sterile types, and the fact that male sterile parents gave rise to male sterile offspring at a higher ratio than hermaphroditic parents also substantiate that the inheritance of male sterility in *B. chondrosioides* is primarily genetic (Couvet *et al.*, 1986).

If the heritability of sex is high, the rejection of simple recessive or dominant nuclear inheritance demonstrates that male sterility in *B. chondrosioides* has a complex genetic inheritance. While the inheritance of male sterility in *B. chondrosioides* could be due to multi-genic causes, the independent distribution of the two male sterile types observed among the progeny of male sterile parents, the 40-75% male sterility recovered in offspring of male sterile parents of both types, and the recovery of both types of male sterility, in separate, hermaphroditic lines suggest a nucleo-cytoplasmic inheritance of male sterility with nuclear restorers. The two independently inherited male sterile phenotypes with significantly different distributions within and among populations in *B. chondrosioides* are similar to what has been seen in populations of the cytoplasmically male sterile *Daucus carota*, *Plantago lanceolata* (Van Damme, 1983), and *Silene vulgaris* (Charlesworth and Laporte, 1998). In these species, at least two different male sterile phenotypes due to unique mitochondrial mutations have been identified (Van Damme, 1983; Van Damme, 1986; Charlesworth and Laporte, 1998). Non-random distribution of male sterility in *B. chondrosioides* is supported by observations of clustering of male sterile plants in the field (Zahn, personal observation), the non-random distribution of male sterile plants within grids in all populations with higher than 10% male sterility, the significant differences between regions and populations within Arizona

and Texas, and the significant differences in the frequency of male sterility observed in grids when compared to random sampling. High variation in the frequency of male sterile individuals in the field within and between populations and the non-random distribution of male sterility are characters used to indicate cytoplasmic male sterility in other species (Couvet *et al.*, 1986; Frank, 1989; McCauley and Taylor, 1997; Manicacci *et al.*, 1998; Delph and Carroll, 2001).

Moreover, the GLM based on the sex of the maternal parent demonstrated that the maternal parent produced just under half of the sex type variation among the offspring (r^2= 0.45, $p<0.0001$). If male sterility is due to CMS and restored by nuclear alleles, the effect of the maternal parent on sex type expression will be diluted by the paternal parent's genetic contribution to sex type via the nuclear restorers and explains why the maternal parent does not explain 100% of the sex of the offspring. In cases of nuclear inheritance of male sterility, the expected proportion of male sterile offspring will be directly correlated to the proportion of male sterile individuals within the population. In the DMSP population where male sterility was 36%, no male sterile parent would be expected to give rise to more than 38% male sterile offspring under the assumption that each hermaphrodite, whether heterozygous or homozygous for the dominant non-male sterile alleles, is equally likely to pollinate each seed on a male sterile individual. While population structure could affect these assumptions, simple nuclear recessive inheritance is not supported by the data obtained in the greenhouse.

If crosses could be performed they might be informative. Hermaphroditic siblings of male sterile individuals of each type of male sterility should be crossed with a male

sterile sibling. In addition, hermaphrodites with male sterile siblings should be self pollinated, if possible. If selfing does not work, two hermaphroditic individuals from maternal lines that give rise to the same form of male sterility should be crossed. In addition, a hermaphrodite from a maternal line that has given rise to MA male sterility should be crossed with a MS male sterile individual and the reciprocal cross between a hermaphrodite from a maternal line that has given rise to MS male sterility and a MA male sterile individual should also be performed. These proposed crosses, diagramed in Figure 2.10, should help determine the mode of inheritance and, if male sterility is due to CMS, give a preliminary indication of the number and genetic determination of nuclear restorers required to restore male fertility in these lines.

Distinctness of each sex type: The MA and MS male sterile types and hermaphrodites were demonstrated to be distinct phenotypes, based on anther length and phenotype and ovary and stigma length (Figure 2.7, Table 2.11). The floral organs of MA male sterile individuals were distinct from both hermaphroditic and MS male sterile individuals on the basis of ovary length, stigma length and the phenotype of the remnant anthers which never reached a measurable length, and were different phenotypically from MS male sterile anthers in both shape and color (Figure 2.1).

There was little evidence supporting that anther length is a continuous traits despite some slight overlap in anther length between individuals that are MS male sterile and hermaphroditic individuals (Figure 2.6). The overlap in anther size observed is likely due to error created by the methods of conversion from measurements taken with the micrometer and converted to mm and the problem of standardizing the precise age of the anther in hermaphrodites. When multiple anthers were measured within an individual the average variance among multiple measurements made on MS male sterile individuals and hermaphroditic individuals were very similar when the average difference in anther size was taken into account. However, the variance from measurements of hermaphroditic anthers was almost 75% higher than the variance of measurements of MS anthers, even when the average difference in anther size was taken into account. The separation of anther morphology as distinct qualitative traits are also supported by the significantly different average anther length in MS male sterile individuals compared to hermaphroditic individuals (Table 2.11), and their distinct cellular phenotype. In addition, the MS male sterile individuals always lacked pollen mother cells or pollen

grains when examined, but upon close inspection, pollen mother cells and pollen grains could be found in the anthers of all hermaphroditic anthers examined depending on the developmental stage in which they were examined. Plasticity in anther length needs to be further investigated as does the proportion of fertile pollen produced by hermaphrodites. especially those related to male sterile individuals.

The degree of plasticity in flowering traits in *B. chondrosioides* need to be further examined, as fitness levels could co-vary with the level of male sterility (McCauley *et al.*. 2000). The floral traits investigated may have a strong environmental- or frequency-dependent component similar to that observed in *Plantago lanceolata*, which has demonstrated that significant differences in seed weight and reproductive biomass were obtained in only some environments (Van Damme and Van Delden, 1984).

Male sterility in the field: The data imply that MS male sterility is more evenly distributed within regions and among populations than the MA male sterile type. As MS male sterility was found at low levels within populations, studies are needed to determine if these are due to a constant mutation rate or a result of the maintenance of this male sterile type. Potential adaptations in MS male sterile individuals were observed in floral timing and height, compared to the MA and hermaphroditic sex types from DMSP and Alpine, in the greenhouse. The low frequency of MS male sterility indicates that, if it is maintained within populations and is due to CMS, it is most likely older than the MA type, because in cytoplasmically male sterile plants, the frequency of male sterile individuals is expected to decrease with population age (Delph and Carroll, 2001). Alternatively, based on the low frequency and relatively even distribution among populations, MS male sterility could be due to complex nuclear inheritance.

The results demonstrating that there were differences in the level of MS male sterility in Arizona populations over time were surprising when compared to the observation that male sterility in individuals grown from seed in the greenhouse collected from DMSP were not significantly different from one generation to the next. While it is possible that changes did occur over time, it is more likely that the results demonstrating change over time in Arizona are due to sampling errors. These errors most likely originated because of sampling at different times within the respective flowering seasons and/or plasticity in timing of flowering. These theories are supported by differences in flowering density that were seen over time at these sites (Table 2.5) and the fact that MS male sterile individuals demonstrated significantly different floral timing from hermaphrodites in the greenhouse.

Furthermore, the levels of male sterility within a small site may also be affected by yearly differences in seed dispersal, seedling establishment, and mortality.

The significant differences observed in the levels of male sterility in the field depending on sampling method were initially surprising, but less so when the non-random distribution of male sterile plants was documented in Mexico and Texas in 1998. The actual level of male sterility within populations is most likely closer to the amount detected by random sampling than that detected within the grids because of this non-random distribution of male sterility. Although most other studies of male sterility in the field have randomly sampled a large percentage of the population or sampled every individual at a single site, sampling every individual within a population of *B. chondrosioides* is not practical. Most other gynodioecious species tend to have relatively small local populations of a hundred or fewer individuals (Forcioloi *et al.*, 1998; Gigord *et al.*, 1998; Hidalgo-Fernandez *et al.*, 1999; McCauley *et al.*, 2000) but thousands of *B. chondrosioides* plants can occur on a single hillside (Zahn, personal observation).

In gynodioecious plants, once male sterility is established within a population, selection occurs to differentiate female and male traits to produce the segregation of characters by sex type (Webb, 1999) and is attributed to either an increase in female function or pleiotropic effects of sex determining genes (Van Damme, 1984). Those differences that were significant among sexes, the increased size and weight of seeds in both MA and MS individuals, along with the increase in pistil size in MA individuals, in *B. chondrosioides* could be explained as selection towards an increase in female function (Webb, 1999). In addition, the observed changes in height and timing of maturation in

MS male sterile individuals could have resulted from selection optimizing pollination (Connor, 1973).

Another unexpected result was that unlike other gynodioecious plants (Couvet *et al.*, 1986), there was no significant difference in seed germination between the offspring of male sterile parents and the offspring of hermaphrodites. This was unpredicted, as higher offspring quality was expected, due to the increased weight and size of both MS and MA male sterile seeds relative to hermaphrodite seeds. Because these experiments were performed in the greenhouse, it is not known if and how seedling establishment and survival to flowering would differ among the sexes in their natural environment. Studies of the gynodioecious *Plantago lanceolata* have demonstrated that male sterile individuals have a greater survival rate in the field and tend to live longer, especially in harsher environments, compared to hermaphrodites (Van Damme and Van Delden, 1982; Van Damme and Van Delden, 1984). If a similar system is in operation in *B. chondrosioides*, it was not detected in the greenhouse.

In conclusion, investigations of the distribution, inheritance, and maintenance of polymorphisms is the basis for the study of evolutionary biology, as it allows us to directly examine natural selection at work (Thompson *et al.*, 1998). Genetic polymorphisms that have readily discernible effects on the phenotype provide model systems for the evolutionary dynamics of natural populations. By studying the occurrence and phenotypes of male sterile plants in *B. chondrosioides*, a foundation has been laid for future studies investigating how polymorphisms in plant mating systems evolve and how they affect a species' genetic diversity.

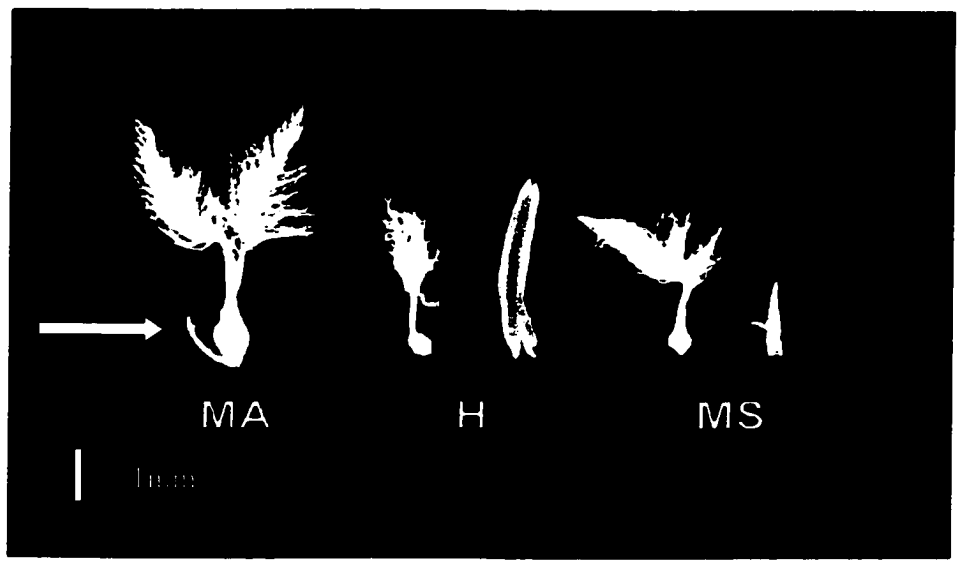


FIGURE 2.1, Sex types of *B. chondrosioides*: Dissected florets of a MA male sterile individual, hermaphroditic individual (H) and a MS male sterile individual. All three are near the developmental stage where the stigmas and stamens exert. A single stamen (of 3 per floret) for the hermaphroditic and MS individual were removed from their filaments and placed to the right of the pistil. The arrow points to the remnant anther on the filament (still attached to the base of the ovary) of the MA male sterile form.

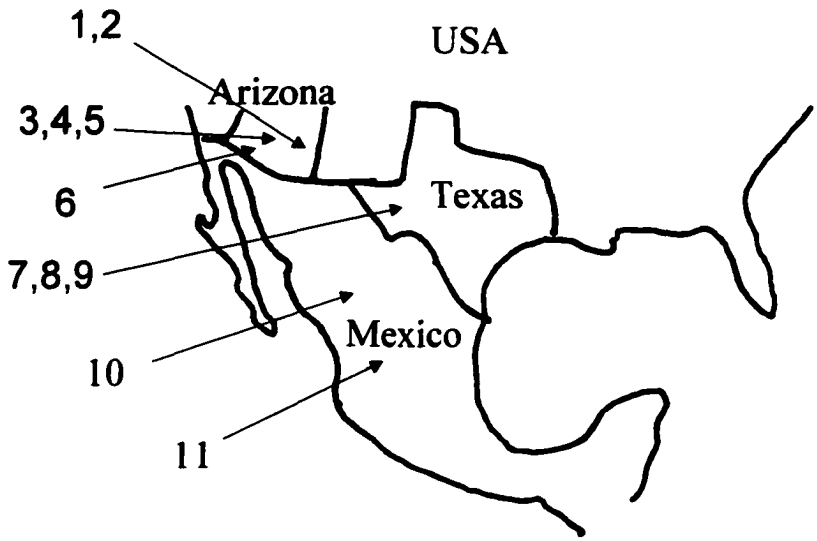


FIGURE 2.2. Populations surveyed for this study: 1) Dragoon, AZ (32:01 N 110:02 W). 2) Ft. Bowie, AZ, (32:08 N 109:26 W) 3) Canelo, AZ (31:29 N 110:33 W), 4) Santa Rita Experimental Range (SRER), Sahaurita, AZ (31:53 N 110:53 W), 5) Appleton-Whittell Research Ranch of the National Audubon Society (TRR), Elgin, AZ (31:39 N 110:31 W) 6) Pena Blanca, AZ (31:20 N 110:56 W) 7) Sul Ross Hill, Alpine, TX (30:21 N 103:39 W), 8) Chihuahuan Desert Research Institute (CDRI), TX (30:56 N 103:83 W), 9) Davis Mountain State Park (DMSP), TX (30:42 N 104:05 W), 10) Durango, MX, (23:90 N 104: 25 W) 11) Aguascalientes, MX (22:25 N 102:50 W). Locations given are estimates based on map position and USGS latitude and longitude positioning available at: <http://geonames.usgs.gov/pls/gnis/web_query.gnis_web_query_form>

TABLE 2.1, Field observations of male sterility in Arizona populations

Year	Location	Sampling	N	# MA	#MS	%MA	%MS
1997	Canelo	Grid	715	17	22	2.4%	3.1%
1997	SRER*	Grid	662	0	3	0.0%	0.5%
1997	TRR*	Grid	698	3	10	0.4%	1.4%
1998	Pena Blanca	Grid	142	2	3	1.4%	2.1%
1998	SRER	Grid	316	2	3	0.6%	0.9%
1998	TRR	Grid	514	4	4	0.8%	0.8%
1999	Canelo****	Grid	647	20	2	3.1%	0.3%
1999	SRER	Grid	907	0	0	0.0%	0.0%
1999	TRR	Grid	725	2	1	0.3%	0.1%
1999	Canelo****	Random	706	70	6	9.9%	0.8%
1999	Dragoon***	Random	100	9	0	9.0%	0.0%
1999	Ft. Bowie*	Random	99	3	0	3.0%	0.0%
1999	Pena Blanca	Random	200	6	10	3.0%	5.0%
1999	SRER	Random	489	2	4	0.4%	0.8%
1999	TRR***	Random	239	12	1	5.0%	0.4%

*=denotes populations with significant Z-scores between the proportions of MA and MS male sterility (*=$p<0.05$, **=$p<0.01$, ***=$p<0.001$, ****= $p<0.0001$)

TABLE 2.2, Field observations of male sterility in Texas populations in 1998

Location	Sampling	N	# MA	#MS	total	%MA	%MS
Alpine**	Grid	69	19	8	69	27.5%	11.6%
CDRI****	Grid	377	143	7	377	37.9%	1.9%
DMSP****	Grid	453	130	8	453	28.7%	1.8%
Alpine****	Random	741	75	7	741	10.1%	0.9%
CDRI****	Random	454	71	1	454	15.6%	0.2%
DMSP****	Random	472	173	4	472	36.7%	0.8%

*=denotes populations with significant differences between the proportions of MA and MS male sterility (*=$p<0.05$, **=$p<0.01$, ***=$p<0.001$, ****= $p<0.0001$)

TABLE 2.3. Field observations of male sterility in Mexico populations in 1998

Location	Sampling	N	# MA	#MS	total	%MA	%MS
Aguascalientes****	Grid	370	83	4	370	22.4%	1.1%
Durango****	Grid	207	60	0	207	29.0%	0.0%
Durango****	Random	191	33	0	191	17.3%	0.0%

*=denotes populations with significant differences between the proportions of MA and MS male sterility (*=$p<0.05$, **=$p<0.01$, ***=$p<0.001$, ****= $p<0.0001$)

TABLE 2.4, Change over time in the frequency of male sterility in grids in three Arizona populations

Population	Years Observed	MA Test statistic	P	MS Test statistic	P
Canelo	2	2.4	<0.12	4.9	<0.03
SRER	3	2.9	<0.8	3.8	<0.05
TRR	3	3.8	<0.05	10.5	<0.01

TABLE 2.5, Average density of flowering plants within grids by region, year and population

Region	Population	Year	N	m^2 sampled	Plants per m^2
Arizona	All	1997	2075	445	4.66
	All	1998	972	246	3.95
	All	1999	2279	325	7.01
	Canelo	1997	715	180	3.97
	Canelo	1999	647	60	10.78
	PB	1998	142	16	8.88
	SRER	1997	662	225	2.94
	SRER	1998	316	150	2.11
	SRER	1999	907	225	4.03
	TRR	1997	698	40	17.45
	TRR	1998	514	80	6.43
	TRR	1999	725	40	18.13
Mexico	All	1998	577	40	14.43
	Aguascalienetes	1998	370	10	37.00
	Durango	1998	207	30	6.90
Texas	All	1998	899	240	3.75
	Alpine	1998	69	110	0.63
	CDRI	1998	377	100	3.77
	DMSP	1998	453	30	15.10

*=denotes significant differences in density of flowering plants among regions and populations (*=$p<0.05$, **=$p<0.01$, ***=$p<0.001$, ****= $p<0.0001$)

TABLE 2.6, G^2 likelihood ratio test of random distribution of male sterility within grids

Population	Test Statistic	df	P
Aguascalientes	41.61	18	<0.0013
Alpine	90.52	46	<0.0001
Canelo (1997)	184.51	236	<0.9944
Canelo (1999)	132.28	116	<0.1433
CDRI	271.5	126	<0.0001
DMSP	123.85	48	<0.0001
Durango	74.37	23	<0.0001
Pena Blanca	26.65	30	<0.90
SRER (1997)	51.95	274	<1.00
SRER (1998)	39.28	300	<1.00
TRR (1997)	54.34	78	<0.98
TRR (1998)	50.81	142	<1.00
TRR (1999)	19.45	78	<1.00

TABLE 2.7, Differences in the observed proportion of male sterility based on sampling method

	MA		MS	
Population	Test Statistic	P	Test Statistic	P
Alpine	3.17	<0.001	2.75	<0.003
Canelo	5.76	<0.001	1.34	<0.09
CDRI	7.37	<0.0001	2.24	<0.023
DMSP	2.59	<0.005	1.22	<0.11
Durango	2.81	<0.003	0.00	<0.50
SRER	1.42	<0.08	2.01	<0.033
TRR	3.32	<0.0005	0.64	<0.37

TABLE 2.8, Chi-squared test of differences in the proportion of male sterility among and within populations in Arizona, Mexico and Texas by year and sampling method

				%MA		% MS	
Population	Sampling	df	Year	Test statistic	P	Test statistic	P
All	Grid	1	1998	261.2	<0.001	11.3	<0.001
All	Random	1	1998/1999	46.7	<0.001	1.6	<0.02
Arizona	Grid	1	1997	24.0	<0.001	14.7	<0.001
Arizona	Grid	1	1998	0.8	<0.4	2.1	<0.15
Arizona	Grid	1	1999	37.2	<0.001	2.5	<0.12
Arizona	Random	4	1999	59.3	<0.001	29.4	<0.001
Mexico	Grid	1	1998	0.4	<0.4	0.1	<0.5
Texas	Grid	1	1998	6.00	<0.02	23.8	<0.001
Texas	Random	1	1998	103.50	<0.001	5.9	<0.02

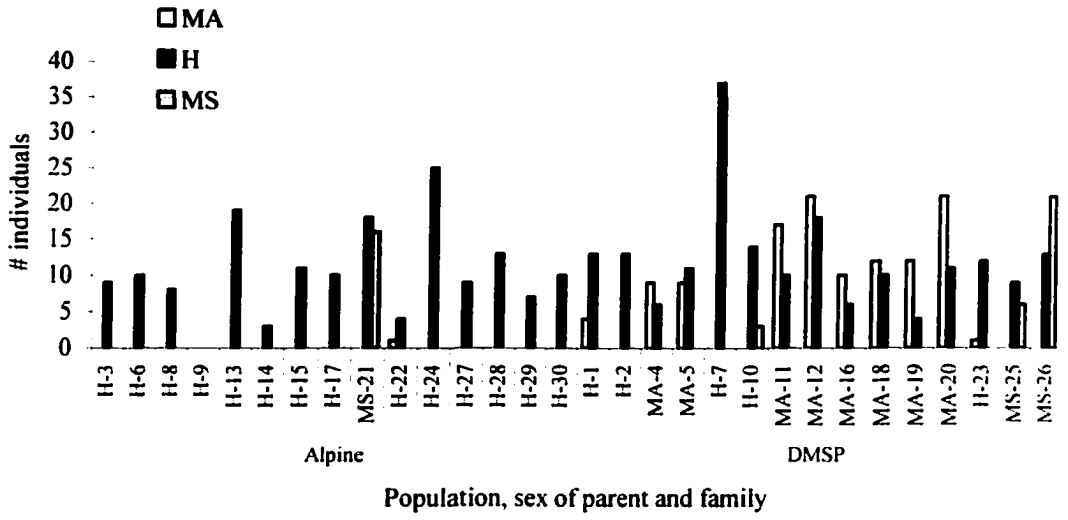


FIGURE 2.3, Distribution of sex types in the greenhouse: This graph displays the distribution of sex types among the offspring of seed parents of MA male sterile, MS male sterile and hermaphroditic (H) sex types from populations in Alpine and Davis Mountain State Park (DMSP), Texas.

There are two possible crosses that give rise to male sterile individuals under the assumption that male sterile individuals are aa and that hermaphrodite individuals are either Aa or AA

	A	a
a	Aa	aa
a	Aa	aa

	A	a
A	Aa	Aa
a	Aa	aa

The expected frequency of aa individuals in the next generation=

$$\frac{1/2 \text{ (the frequency of aa X Aa crosses)} + (1/4 \text{ the frequency of Aa X Aa crosses})}{(1 - \text{the frequency of aa individuals in the population})}$$

FIGURE 2.4, A model of simple recessive genetic inheritance: This model was tested on the offspring of parents of known sex from the DMSP population

TABLE 2.9, Mortality and sex types recovered in the common garden experiment

Population State or country	Canelo Arizona	SRER Arizona	TRR Arizona	Aguascalienetes Mexico	Durango Mexico	Alpine Texas	CDRI Texas	DMSP Texas
Seeds planted	34	151	125	92	121	115	41	115
% Mortality	88.2%	88.1%	84.8%	85.9%	87.6%	95.7%	75.6%	47.0%
# flowered	4	18	19	13	15	5	10	61
#MA	0.0	2.0	6.0	0.0	3.0	1.0	0.0	20.0
#MS	0.0	0.0	1.0	0.0	1.0	0.0	0.0	2.0
% MA	0.0%	11.1%	31.6%	0.0%	20.0%	20.0%	0.0%	32.8%
% MS	0.0%	0.0%	5.3%	0.0%	6.7%	0.0%	0.0%	3.3%

TABLE 2.10, Seed germination, seedling survival, and flowering due to the sex of the parent and population of origin

Parent Sex	Population	% germination	% survival 1 month	% flower by 11/15/99	% flower by 10/01
H	Alpine	79.7 +/- 4.1%	68.7 +/- 3.5	19.0 +/- 3.9%	31.6 +/- 3.8%
	DMSP	84.9 +/- 6.3%	79.9 +/- 5.4%	40.1 +/- 6.0%	50.2 +/- 5.8%
MA	Alpine	N/A	N/A	N/A	N/A
	DMSP	81.9 +/- 5.8%	82.7 +/- 5.0%	44.2 +/- 5.5%	63.9 +/- 5.4%
MS	Alpine	100 +/- 15.4%	72.9 +/- 13.2%	45.8 +/- 14.6%	57.6 +/- 14.3%
	DMSP	75.7 +/- 10.9 %	79.9 +/- 9.4%	61.3 +/- 10.3%	75.6 +/- 10.1%
All sexes	Alpine	81.1 +/- 3.9%	69.0 +/- 3.3%	20.8 +/- 4.0%	33.3 +/- 4.1%
All sexes	DMSP	82.2+/-3.9%	81.2+/-3.2%	44.8+/-4.0%	60.0+/-4.1%

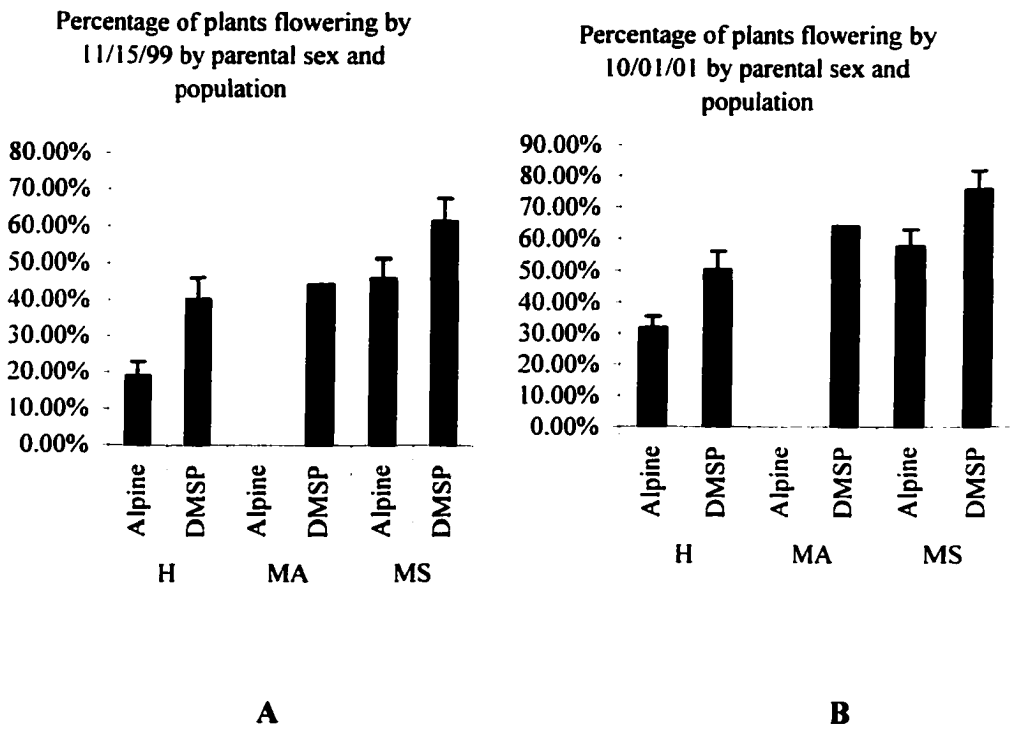


FIGURE 2.5. Percentage of plants flowering in the greenhouse: These graphs show the proportion of plants surviving to flower between 7/1/99 and 11/15/99 by parental sex type and population (A) and proportion of plants surviving to flower between 7/1/99 and 10/01/01 by parental sex type and population (B).

TABLE 2.11, Floral organ measurements from plants from Arizona, Mexico and Texas +/- SE

	Region	H	MS	MA	d.f.	F	P
Stigma	Arizona	3.11 +/- 0.16	N/A	3.8 +/- 0.15	40	9.72	<0.003
length in mm	Mexico	2.05 +/- 0.29	2.54 +/- 0.56	2.92 +/- 0.79	9	0.81	<0.485
	Texas	3.02 +/- 0.10	2.55 +/- 0.12	3.96 +/- 0.11	212	40.54	<0.001
	Average	2.97 +/- 0.09	2.55 +/- 0.11	3.90 +/- 0.10	263	48.39	<0.001
Ovary	Arizona	0.73 +/- 0.04	0.83 +/- 0.17	1.09 +/- 0.04	43	22.33	<0.001
length in mm	Mexico	0.67 +/- 0.09	0.90 +/- 0.18	0.83 +/- 0.25	9	1.12	<0.478
	Texas	0.76 +/- 0.03	0.72 +/- 0.03	1.05 +/- 0.03	226	36.41	<0.001
	Average	**0.75 +/- 0.02**	**0.72 +/- 0.03**	**1.05 +/- 0.02**	**279**	**53.94**	**<0.001**
Ovary	Arizona	0.46 +/- 0.02	0.50 +/- 0.10	0.58 +/- 0.02	42	7.97	<0.001
width in mm	Mexico	0.40 +/- 0.04	0.44 +/- 0.08	0.58 +/- 0.11	9	0.90	<0.459
	Texas	0.45 +/- 0.01	0.47 +/- 0.01	0.57 +/- 0.01	228	21.77	<0.001
	Average	**0.45 +/- 0.01**	**0.47 +/- 0.01**	**0.57 +/- 0.01**	**280**	**31.82**	**<0.001**
Stamen	Arizona	3.54 +/- 0.13	1.19 +/- 0.57		20	15.92	<0.001
length in mm	Mexico	2.81 +/- 0.24	2.21 +/- 0.45		8	1.57	<0.384
	Texas	3.43 +/- 0.07	2.05 +/- 0.08		141	178.03	<0.001
	Average	**3.41 +/- 0.06**	**2.05 +/- 0.08**		**171**	**198.22**	**<0.001**
Stamen	Arizona	0.57 +/- 0.03	0.25 +/- 0.13		20	5.88	<0.025
width in mm	Mexico	0.47 +/- 0.05	0.35 +/- 0.09		8	5.19	<0.050
	Texas	0.52 +/- 0.01	0.32 +/- 0.01		140	116.57	<0.001
	Average	**0.53 +/- 0.01**	**0.32 +/- 0.01**		**170**	**139.33**	**<0.001**
Seed	Arizona	2.57 +/- 0.03	2.80 +/- 0.05	2.89 +/- 0.04	125	21.25	<0.001
length in mm	Texas	2.77 +/- 0.02	2.85 +/- 0.05	2.82 +/- 0.03	356	2.29	<0.113
	Average	**2.71 +/- 0.02**	**2.84 +/- 0.04**	**2.84 +/- 0.02**	**460**	**12.88**	**<0.001**
Seed	Arizona	0.9 +/- 0.02	1.00 +/- 0.03	1.05 +/- 0.02	125	17.64	<0.001
width in mm	Texas	0.89 +/- 0.07	0.97 +/- 0.02	0.94 +/- 0.01	345	11.07	<0.001
	Average	**0.89 +/- 0.007**	**0.97 +/- 0.01**	**0.96 +/- 0.009**	**461**	**26.18**	**<0.001**
Seed	Arizona	0.77 +/- 0.03	0.96 +/- 0.05	1.02 +/- 0.03	125	12.48	<0.001
weight in mg	Texas	0.85 +/- 0.02	1.30 +/- 0.10	0.90 +/- 0.06	108	11.63	<0.001
	Average	**0.81 +/- 0.02**	**1.06+/- 0.04**	**0.95 +/- 0.03**	**232**	**14.12**	**<0.001**

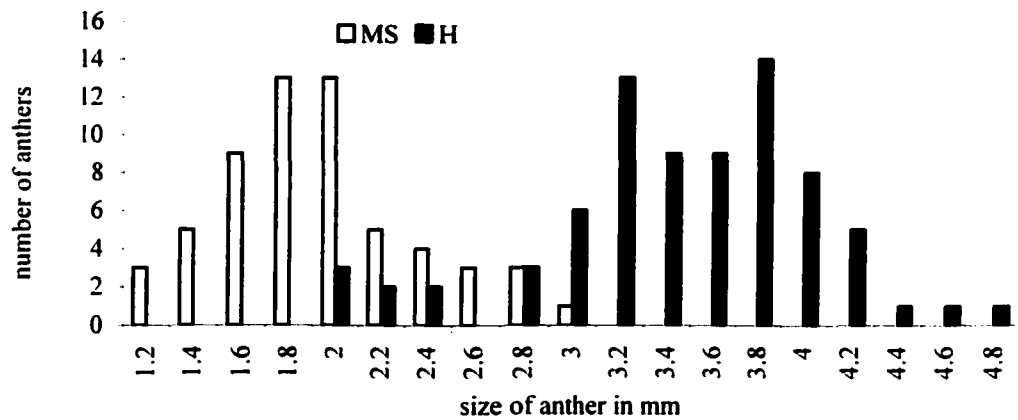


FIGURE 2.6, Stamen length in hermaphrodites and MS male sterile individuals: Measurements were taken on 59 MS male sterile (≤3mm long) and 77 hermaphroditic stamens (mostly > 3mm long) from individuals grown in the greenhouse from seed collected from individuals of known sex type from Texas. Not shown are MA male sterile anther remnants that are always under 0.5mm long.

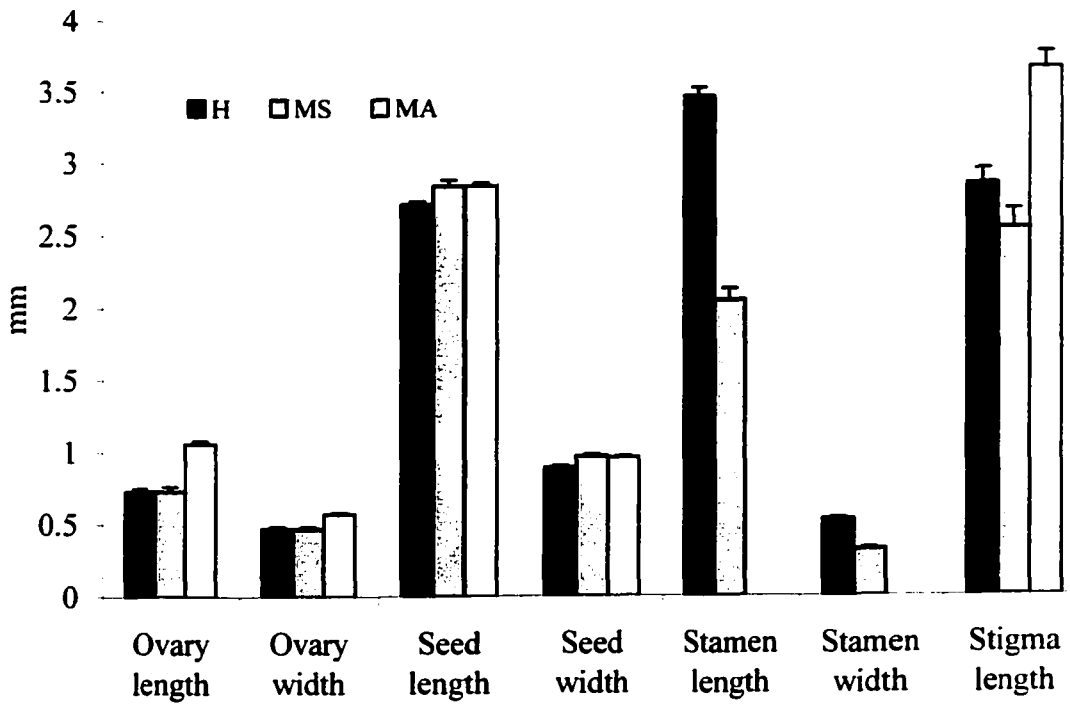


FIGURE 2.7, Floral organ and seed size: This graph displays the overall sizes in mm of MA male sterile, MS male sterile and hermaphrodite plants, plus SE, in ovary length, ovary width, seed length, seed width, stamen length, stamen width and stigma length. $p<0.0001$ for all comparisons among sex types.

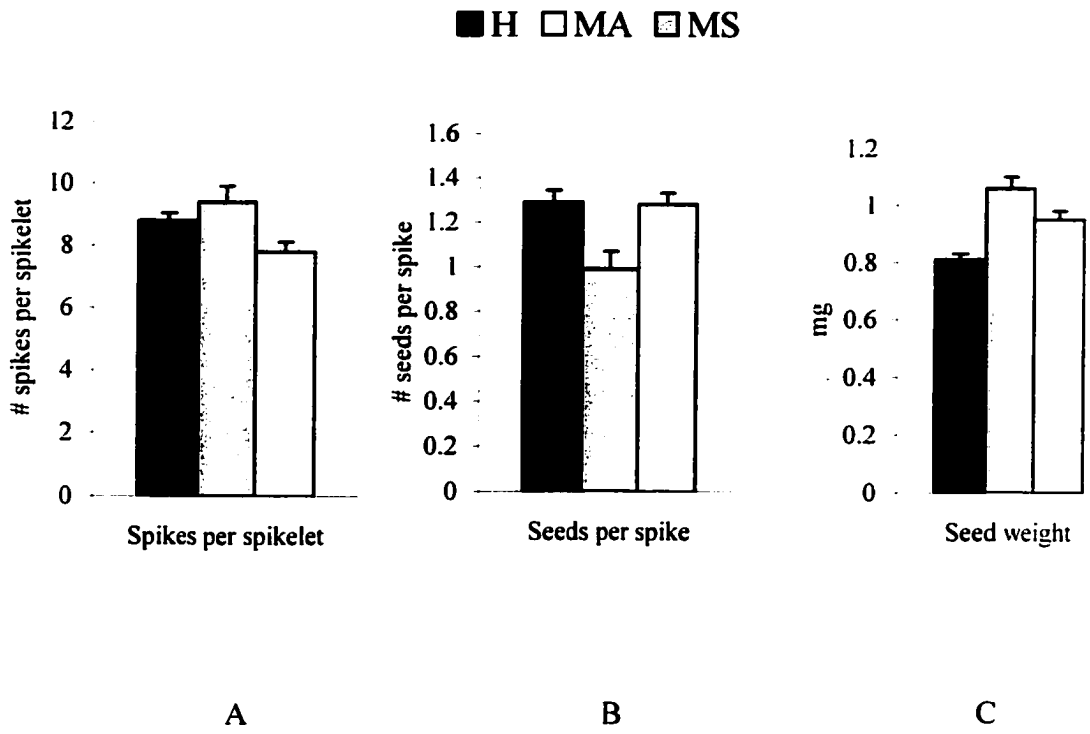
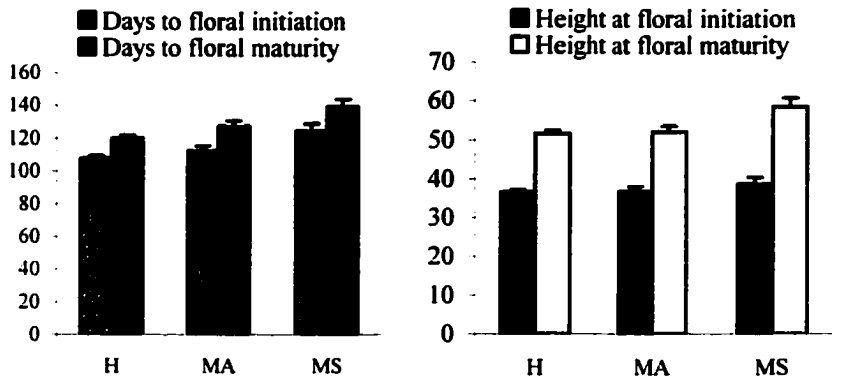


FIGURE 2.8, Spikes per spikelet, seeds per spike, and average seed weight: The results from measurements of the number of spikes per spikelet (A), seeds per spike (B), and average seed weight (C) for hermaphrodite, MS and MA male sterile individuals are shown. All values differ significantly among sexes. (A. $p<0.001$, B. $p<0.002$, C. $p<0.001$)



A B

FIGURE 2.9, Height and timing of flowering among different sex types: These graphs show the significant differences in time for plants to initiate flowering and reach floral maturity in days between hermaphroditic (H) , MA and MS male sterile individuals ($P<0.001$)(A), and non-significant differences among sex types in height in cm at time of floral initiation, but significant differences in height in cm at time of maturity between hermaphroditic (H) , MA and MS male sterile individuals ($P<0.004$) (B).

1.) Cross a male sterile (either MA or MS) with a hermaphrodite with no male sterile siblings from the population (Black= MA line; White= MS line)

If male sterility is due to CMS, and there are no restorers, all progeny will be male sterile.

2.) Hermaphrodites that are siblings of male sterile individuals (either MA or MS) are crossed to male sterile individuals

Should be informative in number of restorers needed for male fertility if male sterility is due to CMS. If male sterility is due to nuclear inheritance should help demonstrate the mode of inheritance.

3.) Hermaphrodites that are siblings of male sterile individuals (either MA or MS) are self pollinated

Should be informative in number of restorers needed for male fertility if male sterility is due to CMS. If male sterility is due to nuclear inheritance should help demonstrate the mode of inheritance.

4.) Hermaphrodites that are siblings of MS male sterile individuals are crossed to MA male sterile individuals. Hermaphrodites that are siblings of MA male sterile individuals are crossed to MS male sterile individuals.

If male sterile types are independent, with unique restorer alleles, MA parents will have only MA male sterile offspring and MS male sterile parents will have only MS male sterile offspring.
If male sterility is due to nuclear inheritance should recover both MA and MS male sterile offspring from both crosses.

FIGURE 2.10, Proposed crosses: The crosses should be used to investigate further the mode of inheritance of male sterility among the offspring of maternal lines of known sex type.

MITOCHONDRIAL DNA DIVERSITY IN THE GYNODIOECIOUS *BOUTELOUA CHONDROSIOIDES* (POACEAE)

Chapter 3

Summary of chapter 3: Mitochondrial genome variability was studied in the gynodioecious grass *Bouteloua chondrosioides* which is hypothesized to be male sterile due to cytoplasmic male sterility. Data from previous studies have demonstrated several characters indicative of cytoplasmic male sterility in *B. chondrosioides* including independent inheritance and distribution of two male sterile types, called the MA and MS male sterile types, variation in the distribution of male sterility among and within populations, predominately non-random distribution of male sterility in the field, and 50% or greater average number of male sterile offspring from male sterile parents. This chapter describes studies performed to determine if mitochondrial DNA RFLPs or haplotypes are correlated with male sterility.

Analyses of mitochondrial DNA organization using Southern hybridization with probes for the mitochondrial genes *ATPA*, *COB*, *COXI*, *COXII* and *COXIII* revealed a high level of mitochondrial polymorphism: 19 unique mitochondrial haplotypes, known as mitotypes, were detected in 30 maternal lines. This diversity is high relative to that reported in other studies of gynodioecious plants. The distribution of RFLPs alleles observed after probing with the *ATPA*, *COXII* and *COXIII* genes were significantly non-random and demonstrated significant structure among lines expressing different sex types. Analyses of these data suggest that specific mitochondrial variants are associated with the majority of, but not all, maternal lines that produce MA male sterile offspring. No mitochondrial types were identified that were correlated with MS male sterility or male fertility.

Introduction to chapter 3: Gynodioecy is a mating system in plants where populations are composed of both male sterile and hermaphroditic individuals. Gynodioecy is commonly inherited through the cytoplasm and modified by nuclear genes, a condition known as cytoplasmic male sterility (CMS) (Charlesworth, 1981; Frank, 1989; Saumitou-Laprade *et al.*, 1994; Elkonin and Tyrnov, 1999; Srivastava, 2000). Studies to date demonstrate that male sterility due to CMS is not due to a genetic loss or dysfunction but results from the accumulation of toxic proteins in the anthers (Saumitou-Laprade *et al.*, 1994; Conley and Hanson, 1995; Wise *et. al*, 1999). In species in which the genes causing male sterility are known, these proteins have been demonstrated to result from the transcription and translation of chimeric sequences in the mitochondrial DNA (mtDNA) caused by the fusion of a gene or genes either from the main or alternate respiratory pathway with an open reading frame (Hanson, 1991; Schnable and Wise, 1998; Srivastava, 2000). The gene fusions that result in chimeric sequences are created by recombination at repetitive or inter-genic loci in the plant mitochondria (Hanson, 1991; Mackenzie and McIntosh, 1999). Male sterile loci situated near recombinational repeats have been identified in both maize and Petunia (Hanson, 1991; Srivastava, 2000).

In most cases of CMS, nuclear genes have been identified that that restore male fertility in individuals carrying male sterile cytoplasms (Schnable and Wise, 1998). The genes that restore male fertility are diverse and can affect all stages from DNA replication to modification of the protein (Saumitou-Laprade, *et* al., 1994). Studies have demonstrated that most unique male sterile cytoplasmic types have male fertility restored by a unique nuclear restorer or set of restorer alleles and that individuals may carry genes

that restore more than one CMS cytoplasmic type (Hanson and Conde, 1985; Frank, 1989; de Haan, *et al.*, 1997a).

The high phenotypic diversity observed in CMS types, even within a single species, is explained by multiple recombination events that create distinct chimeric regions within the mitochondrial genome (Conley and Hanson, 1995; Schnable and Wise, 1998). Repetitive loci within the plant mitochondria can generate sub-genomic mitochondrial plasmids that recombine and regenerate into a full mitochondrial genome with a new genetic organization (Hanson, 1991; Saumitou-Laprade *et al.*, 1994; Srivastava, 2000). In cases of ongoing recombination, polymorphic mitochondrial alleles will be observed within an individual (Andre *et al.*, 1992). Multiple mitochondrial organizational types have been detected as multiple alleles within a single individual in maize (Andre *et al.*, 1992; Fauron *et al.*, 1995). If these mitochondrial variants are not removed from the germ line by selection or drift they can be passed on to the next generation and propagated within a population (Atlan and Couvet, 1993).

The mitochondrial rearrangements associated with male sterility can vary from only 5% of the mitochondrial genome to a complete rearrangement of the mitochondrial genome (Dohmen, *et al.*, 1994). Because of the variation observed in mitochondrial rearrangements, RFLPs correlated with male sterility may not identify the locus or loci causing male sterility (Fauron *et al.*, 1995). However, species with CMS are expected to demonstrate high variation in mtDNA RFLPs at the population level because of the positive correlation between male sterility and mitochondrial genome rearrangements (Ronfort *et al.*, 1995). Since mtDNA polymorphisms are the result of mitochondrial

recombination (Budar and Pelletier, 2001) and such rearrangements are often correlated with male sterility (Hanson, 1991), surveys of mtDNA sequences in plants of differing sex types can determine if a correlation between sex and mtDNA restriction fragment length polymorphisms (RFLPs) exists to further corroborate CMS within a species (Ronfort, *et al.*, 1995). The examination of correlations between mtDNA RFLPs and male sterility have been used to identify the unique cytoplasmic types associated with male sterility in natural populations of *Beta vulgaris* (Saumitou-Laprade *et al.*, 1993; Cuguen et al., 1994), *Daucus carota* (Ronfort *et. al.*, 1995), *Plantago lanceolata* (de Haan *et al.*, 1997b), *Rosmarinus officinalis* (Hidalgo-Fernandez *et al.*, 1999) and *Thymus vulgaris* (Belhassen *et al.*, 1991; Belhassen *et al.*, 1993).

Based on the previous successes in correlating male sterility with mitochondrial RFLPs in other plant species, a study of mtDNA variation in *Bouteloua chondrosioides*, a perennial, gynodioecious, grass with two male sterile types, was performed. If male sterility is due to CMS in *B. chondrosioides*, it is expected that high levels of mitochondrial diversity will be detected and that correlations between mitochondrial RFLPs and sex type expression will be observed (Belhassen *et al.*, 1991). In order to avoid problems due to examining individuals with restorer alleles, individuals from maternal lines were used and the sex expression of individuals from that line were assayed. The genes used for probes were the apocytochrome b gene (*COB*), subunit alpha of ATP synthase (*ATPA*), and subunits I, II and III of cytochrome oxidase (*COXI; COXII; COXIII*). These genes were chosen because they have been associated with cytoplasmic male sterility in other grass species (Hanson, 1991; Sadoch and Goc, 1997;

Rajeshwari, *et al.*, 1994; Saumitou-Laprade *et al.*, 1994; Sane et al., 1996; Seth *et al.*, 1996). PCR primers were designed for maize sequences that are highly conserved between maize and rice. It was assumed that if genes were highly conserved between the highly divergent grasses maize and rice, they were less likely to have diverged between the more closely related maize and *B. chondrosioides* (Kellogg, 2000).

Male sterility in *B. chondrosioides* is hypothesized to be due to CMS because it demonstrates characteristics that are similar to those seen in other cytoplasmically male sterile plants. These traits include a significant variation in the levels of male sterility among populations, highly structured populations with non-random distribution of male sterile individuals, more than 50% male sterile offspring from male sterile parents and two independently inherited male sterile types (Zahn, chapter 2). The two male sterile types in *B. chondrosioides* are referred to as the MA and MS male sterile types. The MA type has, instead of anthers borne on filaments, filaments that end with saggitate anther remnants that make no pollen and are aborted early in development (Reeder and Reeder, 1966; Zahn, Chapter 2). The MA type has been found in all populations examined to date in the USA and Mexico at highly variable frequencies, ranging from 0.3-37.9% (Reeder and Reeder, 1966; Zahn, Chapter 2). The MS type has highly reduced, yellow anthers that make no pollen (Zahn, chapter 2). The MS male sterile type has been observed in most populations surveyed and tends to be rarer and more constant in its frequency, which ranges from 0-12%, than the MA type (Zahn, Chapter 2). The two male sterile types co-occur in most populations but no correlation was found between the frequencies of each male sterile type in the field (Zahn, chapter 2). The MA and MS type

were demonstrated to be independently inherited through the examination of progeny resulting from maternal lines of known maternal sex type which established that no maternal line gave rise to both MA and MS male sterility (Zahn, chapter 2; Table 3.1).

Cytoplasmic male sterility has been well documented in many domestic grasses (Kaul, 1988). However, the maintenance of male sterility within natural populations of a species as a gynodioecious mating system is rare in the grass family (Connor, 1979). Despite the rarity of gynodioecy, studies have investigated the inheritance and genetics of male sterility in *Cortaderia* and *Poa* species (Connor, 1973; Connor and Charlesworth, 1989; Anton and Connor, 1995). Despite the inability to rule out CMS as the cause of male sterility in gynodioecious *Cortaderia* species, no studies have addressed mitochondrial genome variability in non-domestic grasses. This chapter presents work that examines if a relationship exists between mitochondrial RFLPs and male sterility by looking at the amount and pattern of variation of the mitochondrial genome in 30 individuals of *Bouteloua chondrosioides* descended from lines with known sex type expression.

Materials and methods:

Plant material: In 1998, fruiting spikes of *B. chondrosioides* from hermaphrodites, MA male sterile individuals and MS male sterile individuals were collected from the field at Davis Mountain State Park (DMSP) (30:42:00 N 104:05:00 W) and fruiting spikes from hermaphrodites and one MS male sterile individual were collected from the field at Alpine, Texas (30:21:30 N 103:39:38 W). Caryopses were isolated from these spikes in the fall of 1998. The caryopses, which are composed of the seed and a thin pericarp, from here on will be referred to as "seeds." Seeds were isolated in the fall of 1998 and germinated on moist paper towels in the spring of 1999 in a greenhouse at the University of Arizona, Tucson (see Chapter 2 for greenhouse conditions). Thirty maternal lines, defined as a seed parent of known sex type and all offspring grown from seed collected on that individual, were utilized for these experiments, fifteen each from Alpine and DMSP. All seed parents from both Alpine and DMSP were assigned random family identification numbers 1-30 and the offspring were then coded by maternal line, location within the greenhouse, and number of plants in that family. As seeds successfully germinated, individuals were planted in 4"X 4" pots in an autoclaved soil composed of 6 parts perlite, 5 parts peat moss, and 3 parts sand with the appropriate amount (10 Tbsp per approximately 7 kg) of 14-14-14 K-N-P slow release fertilizer (Osmocote). The distribution of sex types recovered among the progeny of the seed parents of known sex type is given in Table 3.1.

DNA isolations: As it is not necessary to isolate mtDNA in order to identify differing cytotypes (Saumitou-Laprade *et al.*, 1993), total DNA was isolated from leaves as described by (Huff *et al.*, 1993) from at least two individuals per family. If more than one sex type was expressed within a maternal line, DNA was isolated from at least one individual of each sex type. The exception to this was maternal line 22, which only had one MA male sterile individual flower. This individual died before leaf tissue for DNA isolation could be collected.

Restriction of total DNA: 1 to 5μg total DNA for each family was digested overnight in 40μL at 37°C with 20 units of restriction endonuclease, either *Bam* HI, *Eco* RI, *Hind* III, or *Pst* I; the appropriate 1X buffer; and BSA when recommended by the manufacturer; and was brought up to volume with double distilled water (ddH_2O). Digestions were performed on at least one individual per family with at least two enzymes, one enzyme per digest. In lines with both hermaphrodites and male steriles, DNA was extracted and digested from at least one individual of each sex type.

Southern blotting: Total DNA digests were run out on a 0.8% agarose gel in TAE buffer (40mM Tris, 20mM NaAcetate, 2mM EDTA). Gel fixation, Southern blotting and probing onto a positively charged nylon membrane were performed as directed in the DIG Application Manual for Filter Hybridization obtained from the Roche website: <http://biochem.roche.com/prodinfo_fst.htm?/prod_inf/manuals/dig_man/dig_toc.htm.> After blotting overnight, the DNA was UV-cross linked to the membrane.

DNA probes: Primers were designed for the *ATPA*, *COB*, *COXI*, *COXII*, and *COXIII*, probes using the computer program Primer 3 (Rozen and Skaletsky, 1998). Primer sequences and length when amplified for maize are given for each primer pair (Table 3.2).

Amplification of the probes incorporated non-radioactive Digoxigenin-11-dUTP (DIG-11-dUTP) into PCR products. PCR reactions mixed 0.5μM each of the appropriate primer pair, 1μL of total *B. chondrosioides* DNA from line 7, a line in which no male sterility was observed in 37 flowering individuals, 2mM dATP, dCTP, dGTP and 1.3mM dTTP and 0.7mM alkali-labile DIG-11-dUTP, pH 7.0, 1X reaction buffer (Gibco-BRL), 3mM $MgCl_2$, and 1 unit taq DNA polymerase (Gibco-BRL) and sterile ddH_2O to 30μL volume on ice. An unlabeled control PCR was also performed, using 2mM of dTTP and no DIG-11-dUTP. The PCR reaction was performed in a Perkin Elmer Cetus DNA Thermal Cycler after an initial denaturing of the DNA at 94°C for 5 minutes. The 30 cycle PCR reaction included 1 minute of denaturing at 94°C, 1 minute of annealing at 60°C, 1 ½ minutes of elongation at 72°C and ended with an additional elongation of 10 minutes at 72°C and chilling to 4°C for at least 10 minutes. 2μL of each reaction was run out on a 1% agarose gel with EtBr along with size standards and visualized under UV light to confirm amplification of the probe.

Hybridization: The membrane was prehybridized and hybridized at 68°C, as directed.

RFLP detection: Detection of RFLPs was performed as directed, with the exception that instead of using a NBT solution, 3mg of powdered NBT per 10mL was mixed into the detection buffer before adding to the membrane. After alleles were visible on the blot, the membrane was rinsed in TE (0.1M Tris, 0.01M EDTA, pH 7.0) and the banding pattern scanned into Adobe Photoshop. Alleles were scored based on relative position (see Figure 3.2), as no DIG labeled standard was available. Rough visual estimates of size were performed by comparing the blot to the photo of the agarose gel with a Kb DNA ladder with a maximum size allele of 12Kb (Stratagene) that was taken before Southern blotting. In order to confirm that there was no variation in allelic patterns for individuals from the same maternal parent two individuals from each family were enzymatically digested, once with *Bam* HI and once with *Eco* RI, run out side by side on a gel, and probed with *ATPA* (Figure 3.1).

DNA sequencing: The regions used as probes for *ATPA* and *COXII* and *COXIII* were PCR amplified for each sex type, isolated using a QIAquick PCR purification kit according to manufacture's directions and sequenced using the appropriate primers given in Table 3.2. Sequencing was performed at the Genomic Analysis and Technology Core at the Arizona Research Laboratory Division of Biotechnology on the University of Arizona campus.

Data analyses: MtDNA RFLPs were organized by the size of the restriction fragment detected when probed by the mitochondrial genes (example given in Figure 3.1). Each allele, resulting from the differing size alleles detected on the blot after detection of the hybridized probe, was numbered from 1-5 based on the relative size of the allele, from largest to smallest fragment size. The RFLPs resulting from multiple digests from a single probe were given a haplotypic designation from A-J. These haplotypes were further combined to create mitotypes that were also given letter designations ranging from A-Q.

Allelic data was entered into JMP (version 3.2.1, 1989-1997, SAS Institute, Inc.) and Arlequin ver 2.000 (Schneider, *et al.*, 2000). A G^2 likelihood ratio test was used to test the null hypothesis that mtDNA RFLP sequences were randomly distributed with regard to sex type expression within a line based on each probe/enzyme combination, the total allelic pattern resulting from probing multiple enzymatic digests and the mitotypes (results presented in Table 3.4). These data, for the probe/enzyme combinations, the total allelic pattern resulting from probing multiple enzymatic digests and the mitotypes were randomized 10 times and analyzed with regards to sex. These randomizations demonstrated that the probability of a type II error was <0.05.

Probing multiple individuals within a line in which a single individual had multiple RFLP alleles was not always successful. In order to determine which allele should be used for scoring and analyses, the data were analyzed with all possible combinations of alleles and by excluding the individual with multiple alleles to determine how this affected the significance of the test.

When these tests were performed on all individuals with multiple alleles, only one probe/enzyme combination (*CoxIII*/*Hind* III) had either significant or non-significant G^2 likelihoods depending on the allelic combination used for analyses. The allelic pattern resulting in the lowest P value was assumed for subsequent analyses of this enzyme/probe combination because the majority of combinations for this enzyme/probe combination demonstrated significant differences among sex type as well as when analyses were performed that excluded this individual. For all other combinations, or when individuals with multiple alleles were removed altogether from the data set, there were no differences in the overall significance of the distribution of alleles concerning sex or population.

Results:

MtDNA variation: To determine if male sterile lines demonstrated unique mtDNA variation, 30 maternal lines consisting of either only hermaphroditic individuals, hermaphrodites and MA male sterile individuals, or hermaphrodites and MS male sterile individuals, were digested with restriction enzymes, Southern blotted, and hybridized to non-radioactively labeled *ATPA*, *COB*, *COXI*, *COXII* and *COXIII* probes. Most probes hybridized to large, <5kb, single copy DNA fragments with the exception of the *COXII/Pst* I probe/enzyme combination, which showed a monomorphic allele composed of multiple bands, and those individuals that showed multiple alleles.

Four probe/enzyme combinations, *COB/Bam* HI, *COB/Eco* RI, *COXI/Hind* III and *COXI/Pst* I had one monomorphic allele. These monomorphic probe/enzyme combinations were uninformative and excluded from further analyses. The *ATPA/Eco* RI probe/enzyme combination resulted in two different size alleles, the *COXI/Bam* HI probe/enzyme combination resulted in three different size alleles, the *ATPA/Bam* HI, *ATPA/Hind* III, *COXII/Bam* HI, *COXII/Hind* III, and *COXIII/Bam* HI probe/enzyme combination resulted in four different size alleles and the *COXIII/Hind* III probe/enzyme combination resulted in five different size alleles (Table 3.3).

Each maternal line, in which 2-4 individuals were evaluated, showed consistent RFLP alleles, no matter the individual's sex type, with the exceptions of individuals that had multiple alleles. Multiple alleles were scored in nine maternal lines, in six of eight different probe/enzyme combinations, and represented 4% of individuals screened. Only one individual showed multiple alleles for more than one probe/enzyme combination.

There were no correlations between sex type expression and number of RFLP alleles in an individual as multiple alleles were observed in individuals of all three sex types.

In those cases where an individual showed multiple alleles, another individual from the same family was scored and analyzed. When a second individual, from the same line, was scored for the same probe/enzyme combination, it was demonstrated that multiple alleles were due to the individual and not the maternal line, as no more than one individual surveyed within a line had multiple alleles. All individuals with multiple alleles had at least one allele in common with other individuals from the same line surveyed with the same enzyme/probe combination, with one exception. Two individuals in line 8, in which all progeny were hermaphroditic, surveyed with the *COXIII/Hind* III probe enzyme/combination, gave different allelic patterns. In one individual from line 8, two alleles, of which both were found in other lines with this enzyme/probe combination, were observed. A second individual from line 8 also examined with the *COXIII/Hind* III probe enzyme/combination showed a single unique allele, not seen in any other individuals or lines surveyed. Analyses were performed using both alleles from the double individual and the unique allele, and excluding line 8. None of these changes in the data entered into analyses due to the anomalous individuals affected the results or significance of the analyses. Due to mortality in the greenhouse, no other individuals were available for DNA analyses from this line.

From the combinations of all probe/enzymes, 19 unique mitotypes were created. Three mitotypes were observed in multiple lines. Mitotype "A", was observed in two lines from DMSP with MA male sterility, mitotype "C" was observed in three lines from

Alpine, three of which were hermaphroditic and one of which expressed MA male sterility, and mitotype "D" was observed in three lines from DMSP with MA male sterility. G^2 likelihood tests testing the null hypothesis that the distributions of mitotypes were randomly distributed among lines of different sex types failed to reject the null hypothesis.

Analyses of the alleles for each probe/enzyme combination and combinations of all RFLPs per probe were also performed using a G^2 likelihood test in JMP (version 3.2.1, 1989-1997, SAS Institute, Inc.). An AMOVA, using these haplotypes, tested if there were significant differences in mtDNA alleles for each probe/enzyme combination for the three sex types and full mitotypes in Arlequin ver 2.000 (Schneider, *et al.*, 2000). The results of both tests supported that the distribution of RFLPs, were non-random in lines containing different sex types for the regions encompassing the probes *ATPA*, *COXII* and *COXIII*.(Tables 3.4 and 3.5). The resulting F_{ST}s demonstrated that the distribution and type of RFLPs found in hermaphrodite and MS lines were significantly different from those observed in MA lines ($p<0.05$). However, no significant differences between the MS and hermaphroditic lines were detected.

Because the *ATPA*, *COXII* and *COXIII* probes resulted in non-random association with sex phenotype, the regions of each gene used as a probe were PCR amplified in individuals of each sex type and sequenced to determine if there were base pair or sequence level differences among the sexes. Sequencing demonstrated no differences among the sex phenotypes, as the sequenced regions were monomorphic in *B. chondrosioides* individuals.

Discussion of chapter 3: The results from this study suggests that mtDNA RFLPs are correlated with the MA male sterile type in *B. chondrosioides*. The results of the G^2 likelihood tests support a significantly non-random mitochondrial distribution among the sexes for most probe/enzyme combinations. The average of all probe/enzyme combinations resulted in moderate to large F_{ST} values among the three sex types which also indicate that the distribution of RFLPs among the sexes is not random. Eight of eleven MA lines shared at least one unique haplotype for the *ATPA*, *COXII* or *COXIII* probes. If these sequences are linked to a mitochondrial region causing male sterility, it is likely that male sterility in these lines are due to the same underlying genetic factors because it is unlikely that each mtDNA pattern represents a unique CMS type (de Haan, *et al.*, 1997a).

The associations between mitochondrial mutations and MA male sterility were not complete because three of eleven MA male sterile lines had RFLPs more similar to hermaphrodites than to other MA male sterile individuals. Furthermore, there was no support that MS male sterile lines were correlated with unique mtDNA RFLPs. The fact that not all MA male sterile individuals had similar mitochondrial RFLP patterns suggests that if MA male sterility is due to CMS, either multiple mutations have caused the MA male sterile phenotype, or there has been recombination around the region causing MA male sterility that has disrupted the linkage between the RFLP markers used in this study and the loci causing male sterility. Both scenarios are possible because male sterility is often associated with new mitochondrial variants (Conley and Hanson, 1995) and regions of recombination (Hanson, 1991). In addition, multiple genetic CMS types giving rise to

the same phenotype have been documented in *Origanum vulgare* (Keyr-Pour, 1980). *Plantago lanceolata* (de Haan, *et al.*, 1997b) and *Thymus vulgaris* (Belhassen *et al.*, 1991).

The MS male sterile type is not supported as a unique CMS type by this study, although its phenotype is more similar, than that of the MA type, to the phenotypes observed in domestic grass species with CMS (Kaul, 1988). The fact that the MS lines each had a different mitotype suggests that if MS male sterility is due to CMS, either there are multiple genes causing this phenotype that are of multiple recent origins and establishment of this sterile phenotype, or high levels of recombination have occurred, disrupting any linkage with the mitochondrial genes used in this study (Belhassen *et al.*, 1993). Genetic crosses and/or protein assays are recommended for future studies to help determine if MS male sterility is due to CMS and if chimeric proteins are expressed in MS male sterile anthers compared to male fertile anthers.

Because no line from a male sterile seed parent resulted in all male sterile offspring (Table 3.1), if CMS is causing male sterility in *B. chondrosioides*, nuclear restorers are found in these populations. It is possible that lines that produced only hermaphroditic progeny contain a male sterile cytoplasm and nuclear restorers. If this is occurring it may complicate any attempt to link sex and mitotypes. However, the observed significantly non-random distribution of RFLPs among lines of different sexes, suggests that if this is occurring, it is at a low enough frequency to still allow the detection of correlations between mitotypes and sex.

There were no significant differences among sex types over mitotypes, and this is likely due to a lack of power in these studies. This study would be more conclusive if more individuals from maternal lines of known sex type from a single population were surveyed. In addition, the results obtained from this study could have been affected by population structure and the distribution of maternal relatives within the population. Greater sampling of individuals from a maternal line of known sex type within a single population would most likely aid in determining if the distribution of mitotypes among individuals is due to sex type. If all MA plants are closely related, the observed similarities in mtDNA RFLPs may simply be due to maternal relationships and not the cause of male sterility. Intensive sampling within multiple small areas would help determine how the role of seed dispersal and population structure affect the observed patterns of mtDNA polymorphism. In other plant species with CMS, male sterile lines have been demonstrated to be more diverse than hermaphroditic lines (Hidalgo-Fernadez, *et al.*, 1999), which was not seen in *B. chondrosioides*. Sampling more individuals within a single population will clarify if the observed high number of alleles within hermaphroditic lines is due to sampling error from the differences in sex type distribution among the two populations examined for this study.

The observation of 19 mitotypes in 30 lines is more diverse that the amount of polymorphism observed in other species (reviewed in Hidalgo-Fernandez *et al.*, 1999) including *Beta vulgaris* (Saumitou-Laprade, *et al.*, 1993), *Daucus carota* (Ronfort *et al.*, 1995), and *Plantago lanceolata* (de Haan, *et al.*, 1997b), *Rosmarinus officinalis* (Hidalgo-Fernandez *et al.*, 1999), and *Thymus vulagris* (Belhassen *et al.*, 1993). Possible

explanations for this high level of mitochondrial variation include a high mitochondrial recombination rate, or the maintenance of ancestral polymorphisms (Hidalgo-Fernandez *et al.*, 1999). The relative lack of shared mitotypes between populations, however, does not support that mitotype variability in *B. chondrosioides* is due to the maintenance of ancestral polymorphisms.

The observation of multiple alleles in 4% of individuals surveyed, and the fact that the three probes that had a non-random distribution among lines of different sexes [*ATPA*, *COXII*, and *COXIII*] had monomorphic sequences, there is support that new RFLPs arise through the creation of new cytotypes through mitochondrial recombination. The multiple alleles observed within individuals support this theory because they most likely are due to active recombination. However, further studies are needed to determine if the frequency of multiple mitochondrial RFLPs within an individual is the same in the field as in the greenhouse. The majority of mitochondrial rearrangements are expected to have no selective values (McKenzie and McIntosh, 1999). Based on this, if the 4% observed rate of mitochondrial polymorphism within individuals is constant in the field, the large populations (greater than a thousand individuals) observed in *B. chondrosioides* (Zahn, personal observation), relative to the smaller populations (less than a hundred) in other gynodioecious species, (Forcioloi *et al.*, 1998; Gigord *et al.*, 1998; Hidalgo-Fernandez *et al.*, 1999; McCauley *et al.*, 2000), may explain the high mitotypic diversity. Under the infinite alleles model (Hartl and Clark, 1997), there is a greater probability of the establishment of any single new, neutral, mitotypic type in a large population if there

is a constant mitochondrial recombination rate, and if that mitochondrial recombination is selectively neutral (Hartl and Clark, 1997).

The results from this study demonstrate that the mitochondrial genome of *Bouteloua chondrosioides* is quite diverse in a small part of its range. It of interest to determine if the degree of mitochondrial diversity documented in this study is constant throughout the range of *B. chondrosioides*. Besides a more detailed study of the MS male sterile type, investigations of other populations are needed to explore if the *ATPA*, *COXII*, and *COXIII* genes are correlated with the MA male sterile type in other populations. Understanding the relationship between male sterility and mitochondrial polymorphisms in *Bouteloua chondrosioides* will add to our understanding of how gynodioecy arose and is maintained in this species.

TABLE 3.1, Distribution of sex among seed parents and their offspring in the lines used in this stu

Popualtion	Maternal line	Sex of parent	# MA	# MS	# flowered	% MA	% MS
Alpine	3	H			9	0.0%	0.0%
Alpine	6	H			10	0.0%	0.0%
Alpine	8	H			8	0.0%	0.0%
Alpine	9	H			0	0.0%	0.0%
Alpine	13	H			19	0.0%	0.0%
Alpine	14	H			3	0.0%	0.0%
Alpine	15	H			11	0.0%	0.0%
Alpine	17	H			10	0.0%	0.0%
Alpine	21	MS		16	34	0.0%	47.1%
Alpine	22	H	1		5	20.0%	0.0%
Alpine	24	H			25	0.0%	0.0%
Alpine	27	H			9	0.0%	0.0%
Alpine	28	H			13	0.0%	0.0%
Alpine	29	H			7	0.0%	0.0%
Alpine	30	H			10	0.0%	0.0%
DMSP	1	H	4		17	23.5%	0.0%
DMSP	2	H			13	0.0%	0.0%
DMSP	4	MA	9		15	60.0%	0.0%
DMSP	5	MA	9		20	45.0%	0.0%
DMSP	7	H			37	0.0%	0.0%
DMSP	10	H		3	17	0.0%	17.6%
DMSP	11	MA	17		27	63.0%	0.0%
DMSP	12	MA	21		39	53.8%	0.0%
DMSP	16	MA	10		16	62.5%	0.0%
DMSP	18	MA	12		22	54.5%	0.0%
DMSP	19	MA	12		16	75.0%	0.0%
DMSP	20	MA	21		32	65.6%	0.0%
DMSP	23	H	1		13	7.7%	0.0%
DMSP	25	MS		6	15	0.0%	40.0%
DMSP	26	MS		21	34	0.0%	61.8%

TABLE 3.2, PCR primers for probes

Gene	Primers	Length of sequence in maize
Apocytochrome b	TGGGGCAAGTATGTTCTCA TAACAAATGGTGCCTCCACA	750 bp
ATP synthase subunit A	CGTTGGCTTATCCGTCAGTC GTGGTTGTTTGGGCACTTCT	177 bp
Cytochrome c oxidase subunit I	TGGTGGGAATCATCAACTTT GGCGTAAGCATCTGGATAAT	1197 bp
Cytochrome c oxidase subunit II	AGGGTGATTTAAGTGCAACG CATCACATTTGACACCTGA	1643 bp
Cytochrome c oxidase subunit III	ACGTTGGAAGGGCATCATAC GAAGAGGGATTTCCCAAGGA	198 bp

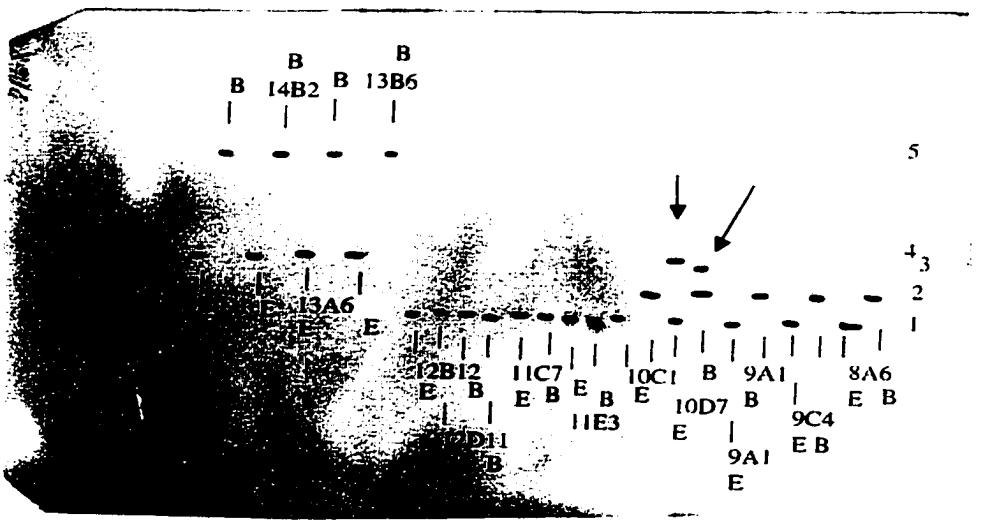


FIGURE 3.1, A Southern blot probed with *ATPA*: This Southern blotted nylon membrane demonstrates five unique bands (labeled vertically 1-5 on the right) from DNA digested with *Bam* HI (B) and *Eco* RI (E) and probed with a Digoxigenin-labeled probe, *ATPA*. The bottom of the blot shows the designation of the two individuals per line and enzyme used for that digest. Vertical lines match the individual to the band. Family of unknown sex, #9; MA families, #11 and 12; MS family, #10; and hermaphroditic, families, #8,13,14,15. Both populations are also represented on the gel: Alpine families are #8, 9, 13,14,15; and DMSP families are #10,11,12. Individuals 12 D11 and 11C7 are MA, and individual 10C1 is MS. All other individuals are hermaphrodites or are of unknown sex. Band intensity was manually increased for better visibility using Adobe Photoshop© version 5.0. Arrows indicate the multiple bands seen in individual 10D7 relative to individual 10C1.

TABLE 3.3, RFLP alleles for polymorphic probe/enzyme combinations

Population	Line	expressed Sex in line	Bam HI *ATPA*	Eco RI *ATPA*	Hind III *ATPA*	Bam HI *COXI*	Bam HI *COXII*	Hind III *COXII*	Bam HI *COXIII*	Hind III *COXIII*	all Mitotype
Alpine	3	H	2	1	4	2	3	3	4	4	C
Alpine	6	H	2	1	4	2	3	3	4	?	?
Alpine	8	H	2	1	4	2	3	3	4	3, 4 / 5	?
Alpine	9	?	2	1	?	2	3	?	?	4	E
Alpine	13	H	**4**	2	1	?	**4**	**3**, 4	2	3	M
Alpine	14	H	**4**	2	1	2	3	1, **3**	**3**	1	K
Alpine	15	H	2	1	?	2	3	3	4	4	E
Alpine	17	H	2	1	4	2	3	3	4	4	C
Alpine	21	H/MS	**4**	2	1	2,3	3	?	**3**	3	L
Alpine	22	H/MA	2	1	4	2	3	3	4	4	C
Alpine	24	H	3	2	2	2	3	3	**3**	1	N
Alpine	27	H	2	1	4	1	3	3	?	4	B
Alpine	28	H	2	1	4	2	**2**	3	4	4	F
Alpine	29	H	2	1	3, **4**	3	1	2	**3**	3	G
Alpine	30	H	2	1	2	?	**2**	2	1,**2**,4	4	Q
DMSP	1	H/MA	1	1	1	2	1	**1**	1	**2**, 4	A
DMSP	2	H	2	1	4	2	?	2	4	4	H
DMSP	4	H/MA	1	1	1	2,3	1	?	1	**2**	?
DMSP	5	H/MA	1	1	1	2	1	**1**	1	**2**	A
DMSP	7	H	2	1	4	?	?	3	?	1	I
DMSP	10	H/MS	2	1	1	2	3	3	4	3	J
DMSP	11	H/MA	**1**	1	1	2	?	?	4	**2**	S
DMSP	12	H/MA	**1**	1	1	2	1	**1**	1	3	D
DMSP	16	H/MA	?	1	1	2	1	**1**	1	3	?
DMSP	18	H/MA	**1**	1	1	2	1	**1**	1	3	D
DMSP	19	H/MA	3	2	2	2,3	3	1,**3**	2	4	O
DMSP	20	H/MA	**1**	1	1	2	1	**1**	1	3	D
DMSP	23	H/MA	2	1	2	2	3	3	4	4	P
DMSP	25	H/MS	**1**	1	3	2	?	?	4	4	R
DMSP	26	H/MS	2	1	4	1	3	3	4	4	B

TABLE 3.4, G^2 likelihood test of signficant difference in RFLP distribtion among three sex types

Gene	Enzyme	df*	$\chi 2$	P
ATP synthase subunit A	all	12	31.6	<0.0016
	Bam HI	6	20	<0.0028
	Eco RI	2	0.9	<0.6334
	Hind III	6	16.5	<0.0113
Cytochrome c oxidase subunit I	*Bam* HI	4	6.4	<0.1746
	all	10	18.2	<0.0514
Cytochrome c oxidase subunit II	*Bam* HI	6	15.9	<0.0188
	Hind III	4	18.1	<0.0012
Cytochrome c oxidase subunit III	all	16	28.3	<0.0293
	Bam HI	6	19.9	<0.0029
	Hind III	6	15.4	<0.0175

*= degrees of freedom in the model calculated by multiplying the number of alleles -1 by the number of sexes -1.

TABLE 3.5, Differentiation among sex types at each and all RFLP loci

	Between sexes:		Within sexes:			Fixation indices:	
Locus	SSD	Va	SSD	N	Vb	FST	P-value
BATPA	2.56	0.12	6.05	26	0.24	0.34	0.00098
EATPA	0.12	-0.01	4.02	27	0.15	-0.07	0.83675
HATPA	2.27	0.10	6.69	26	0.27	0.28	0.00293
BCOXI	0.56	0.00	4.61	24	0.20	0.01	0.31280
BCOXII	2.02	0.10	5.18	23	0.24	0.31	0.00782
HCOXII	2.12	0.13	4.36	23	0.20	0.39	0.00391
HCOXIII	1.22	0.03	8.18	26	0.33	0.09	0.10948
BCOXIII	2.02	0.09	6.99	25	0.29	0.23	0.00489
All	12.89	0.58	44.43	27	1.85	0.24	0.00000

SSD= Sum of squares
Va= Variance between sexes
Vb= Variance within sexes

THE OCCURRENCE OF POLYPLOIDY IN THE GYNODIOECIOUS *BOUTELOUA CHONDROSIOIDES* (POACEAE)

Chapter 4

Summary of chapter 4: In order to determine if male sterility is related to ploidy in the gynodioecious grass *Bouteloua chondrosioides*, the ploidy level of hermaphrodite and male sterile plants, of both types found in this species, were determined. Ploidy levels were determined by performing flow cytometry on tissue from seedlings grown from seeds collected from populations in Arizona, Mexico and Texas. Populations in Texas, USA and Aguascalientes (near Calvillo) and Michoacan, Mexico were diploid; populations in Arizona, USA and Aguascalientes (near Rincon de Romos), Durango, and Zacatecas, Mexico were tetraploid; and populations in Jalisco, Mexico had both diploid and tetraploid individuals. In addition, one individual from Jalisco was octoploid. There were no triploid or pentaploid individuals identified. High mortality occurred in the seedlings in which ploidy was determined and only one MA male sterile individual was seen among the ten seedlings that survived and flowered from tetraploid populations, while the remaining four individuals were hermaphroditic. Six male sterile individuals, representing both the MA and MS types, survived and flowered from a diploid population, and ten individuals were hermaphroditic.

Additional investigations also examined whether tetraploid populations in *B. chondrosioides* resulted from a single polyploid event followed by range expansion, or if tetraploids are descended from multiple polyploid lineages. This was done by constructing a phylogenetic tree from polymorphic chloroplast DNA (cpDNA) sequences to determine the relatedness of populations of different ploidy levels. Phylogenetic analyses of cpDNA sequences demonstrated that tetraploid populations are not all

descended from a single lineage, and these results suggest that multiple polyploid lineages are found in *B. chondrosioides*.

Introduction to chapter 4: Polyploidy is the existence of individuals within a species or genus possessing duplications of the haploid nuclear genome (X) resulting in multiples greater than two of the base chromosome number (Stebbins, 1950; 1971; Leitch and Bennett, 1997). To illustrate: diploid individuals have a base chromosome number of 2X, and polyploid individuals have a base chromosome number of 3X or larger (4X, etc..). Polyploidy is widespread in angiosperms and may occur in up to 75% of all grass species (Keeler, 1998).

The effects of polyploidy on rates and types of evolution may be quite large and cause significant and rapid changes in chromosome organization, Mendelian inheritance and gene silencing in a species (Song *et al.*, 1995; Leitch and Bennett, 1997; Soltis and Soltis, 1999; Otto and Whitton, 2000). In addition, polyploidy may result in an increase in heterozygosity and allelic diversity (Segraves *et al.*, 1999). Recently it has been demonstrated that many polyploid species have evolved from repeated polyploidy, in which each instance of chromosome doubling is referred to as a polyploid event (Soltis and Soltis, 1995,1999; Leitch and Bennett, 1997). However, the number of species with multiple polyploid events and the frequency of multiple polyploid origins in most taxa are currently unknown and studies documenting multiple origins of polyploid lineages are currently lacking (Segraves *et al.*, 1999). Additionally, recent studies have demonstrated that the evolution of polyploid species from multiple polyploid events is common (Soltis and Soltis, 1995,1999; Leitch and Bennett, 1997; Van Dijk and Bakx-Schotman, 1997 Keeler, 1998).

Bouteloua chondrosioides is a perennial, gynodioecious grass of the arid grasslands in southern Arizona and southwestern Texas and Mexico ranging through Central America. Individuals of *B. chondrosioides* are either diploid (2X=20) or tetraploid (4X=40), and one aneuploid individual has been noted (2X=22) (Reeder and Reeder, 1966; Gould, 1979). In initial studies of gynodioecy in *B. chondrosioides*, no male sterility was observed in Arizona which led to the suggestion that the occurrence of male sterility within populations may be correlated with the ploidy level of the population (Reeder and Reeder, 1966). One objective of this study was to investigate if male sterility in *B. chondrosioides* is correlated with polyploidy.

Polyploidy is common in the genus *Bouteloua*, *sensu* Columbus (1999), but, other than *B. chondrosioides*, no *Bouteloua* species has a gynodioecious mating system. *Bouteloua* contains a number of diclinous mating systems, in which male and female function are found in separate flowers either within or among individuals, including monoecy, andromonoecy, gynodioecy, and dioecy (Columbus, 1999). Many *Bouteloua* taxa with diclinous mating systems are polyploid or have multiple ploidy levels (JR Reeder, personal communication; Gould, 1979; Huff *et al.*, 1993). Most hermaphroditic *Bouteloua* species also are polyploid or have multiple ploidy levels. Of 28 *Bouteloua* species, where X=10 (Jacobs, 1986), 16 have one ploidy level that is either diploid or tetraploid (2X=20, 4X=40), two have two ploidy levels and are either diploid and tetraploid (2X=20, 4X=40) or are tetraploid and hexaploid (4X=40, 6X=60), and 10, including *B. chondrosioides*, have more than three documented ploidy states including aneuploid counts (Gould, 1979; J Reeder personal communication), where particular

chromosomes are present in extra or fewer copies instead of having chromosome numbers that are a direct multiple of the base chromosome number. Ploidy level has not been linked to mating system in the well studied gynodioecious *Bouteloua dactyloides* (formerly *Buchloë dactyloides*) which has populations that are either diploid, tetraploid or hexaploid (Huff *et al.*, 1993; Yin and Quinn, 1995).

Other investigations suggest that *B. chondrosioides* is male sterile due to cytoplasmic male sterility (CMS) (Zahn, Chapters 2 and 3). Cytoplasmic male sterility in plants is not associated with polyploidy except in cases of allopolyploidy, the formation of a polyploid lineage with genetically different chromosome sets that results from a hybridization event between different species. Hybridization is known to result in CMS in many species (Kaul, 1988). Allopolyploidy can cause CMS because hybrid nuclei lacking the proper restorer alleles can acquire male sterile cytoplasms resulting in CMS (Kaul, 1988; Bretagnolle *et al.*, 1998). However, allopolyploidy can also disrupt CMS because species-specific chromosomal changes can result that restore male fertility (Mann and Endo, 1991; Leitch and Bennett, 1997). In the case of polyploids derived from autopolyploid events, individuals that have an increase in their haploid set of chromosomes by a multiple of more than 3, the ancestral cytoplasmic and genomic conditions are expected to be maintained within the polyploid lineage (Mann and Endo, 1991; Bretagnolle *et al.*, 1998). It is not known if tetraploid individuals in *B. chondrosioides* are due to allopolyploidy or autopolyploidy, and these questions are beyond the scope of the current study.

Besides *Bouteloua chondrosioides*, the only other gynodioecious grasses are found in the genera *Poa* and *Cortaderia* (Connor, 1979). Like *Bouteloua*, both *Cortaderia* and *Poa* have diverse diclinous mating systems as well as hermaphroditism (Connor, 1983; Anton and Connor, 1995). All *Cortaderia* species, no matter their mating system, are polyploid (Connor, 1983). The ploidy levels in gynodioecious *Poa* species are not known but many *Poa* species, including dioecious species, are polyploid (Anton and Connor, 1995). In some species of both *Cortaderia* and *Poa*, apomixis (asexual seed formation) has replaced gynodioecious sexual reproduction and has resulted in monomorphic all female populations (Connor, 1979; 1983). Male sterility in polyploid grasses has been linked to apomixis (Connor, 1979). It is not known if *B. chondrosioides* has apomictic reproduction. If male sterility in *B. chondrosioides* is an apomictic reproductive system similar to the ones in *Poa* and *Cortaderia*, based on the correlation between polyploidy and apomixis, male sterility can be hypothesized to be correlated with tetraploidy.

Instead of counting chromosomes in pollen mother cells (PMCs), which can only be done in hermaphrodites because male sterile individuals make no pollen (Reeder and Reeder, 1966; Zahn, Chapter 2), flow cytometry was performed to estimate chromosome number in male sterile individuals of *B. chondrosioides*. Flow cytometry has become a common means for estimating DNA content and ploidy levels in plants (Amsellem *et al.*, 2001). It is a much more rapid technique for ploidy level estimation than root tip or pollen mother cell isolation (Amsellem *et al.*, 2001). It is expected that in polyploids, the C-value, or total amount of haploid or un-replicated DNA within a cell, should increase

by a factor equal to that of the chromosomal duplication (Bennett *et al.*, 2000). The actual DNA content within a plant species is not dependent on chromosome number, although within a species or genus each doubling of the base chromosome number is expected to lead to a two-fold increase in DNA content (Bennett, *et al.*, 2000). Therefore, sampling individuals of *B. chondrosioides* of known sex with flow cytometry can determine if there are different ploidy levels in individuals of different sexes.

Initially it was planned that flow cytometry would be performed on sexually mature plants of known sex collected in the field or grown from seed in the greenhouse and common garden. Obtaining flow cytometric readings that were interpretable was especially difficult in mature plants. Only a few diploid individuals from Texas resulted in reading that were useful for this study, due to high background interference in mature plants, especially in tetraploid individuals. However, flow cytometry was successful when performed on seedlings. Therefore, instead of performing flow cytometry on adult plants of known sex, seedlings were analyzed using flow cytometry and their sex was recorded when they flowered in the greenhouse.

Besides examining if polyploidy is correlated with sex expression, this study seeks to test if the distribution and origin of polyploid *Bouteloua chondrosioides* is similar to that seen in the dioecious *Bouteloua dactyloides*. It has been demonstrated that the current distribution of tetraploid and hexaploid populations in *B. dactyloides* can each be traced back to one of two geographically distinct, and marginalized, diploid populations (Huff *et al.*, 1993; Peakall *et al.*,, 1995). This supports the sequence of events proposed by Stebbins (1971) explaining the development and establishment of polyploids. This model

states that tetraploids arise through either allo- or autopolyploidy with adaptations that buffer them against environmental changes. Adaptive characters resulting from polyploid events have been documented in plant species and include increases in cell, plant and seed size, changes in size, shape and number of stems and leaves, and changes in the timing of flowering relative to the progenitor diploid (Stebbins, 1950; Otto and Whitton, 2000). In addition, polyploids and the progeny of mating among polyploid lineages of independent origin may be able to adapt to a new environment or be competitively superior to the progenitor diploid individuals (Soltis and Soltis, 1995; Segraves *et al.*, 1999). Over time, natural selection acts on these polyploid adaptations and is expected to increase the frequency of tetraploids, which will displace diploid plants. Eventually, any diploids that remain are geographically restricted and become increasingly rare over time, and eventually go extinct. The distribution of ploidy in *B. dactyloides* supports this model (Huff, *et al.*, 1993; Peakall *et al.*, 1995). While there are significant phenotypic differences among individuals within and among *B. chondrosioides* populations, no specific morphological differences associated with ploidy level have been identified.

In order to test if the distribution of polyploidy resulted from a single range expansion, similar to that observed in *B. dactyloides*, or multiple polyploid events, chloroplast DNA (cpDNA) sequences were utilized to create a phylogenetic tree of individuals from tetraploid and diploid populations to determine if tetraploid populations were monophyletic. CpDNA sequences are useful for studies of phylogeography in plants as the chloroplast genome is effectively haploid, multi-copy and non-recombining

(Van Dijk and Bakx-Schotman, 1997). CpDNA has previously demonstrated variability among populations of *B. chondrosioides* (JT Columbus, personal communication). Utilizing cpDNA avoids problems in phylogenetic analysis that are due to a lack of informative variation resulting from the slow rate of evolution in coding regions in plant mitochondrial DNA (Laroche, *et. al.*, 1997), or that are due to the examination of paralogous nuclear genes with different evolutionary paths which can be present in polyploid individuals. Studying cpDNA diversity among populations both allows the examination of how populations are related to each other and the inference of historical information (Van Dijk and Bakx-Schotman, 1997; Soltis and Soltis, 2000). This study of cpDNA sequence diversity will test the hypothesis that tetraploidy evolved once in *B. chondrosioides* and will be used to test if the ancestral diploid populations(s) can be identified.

Materials and methods:

Plant materials: In 1998, fruiting spikes of *B. chondrosioides* from hermaphrodites, MA male sterile individuals and MS male sterile individuals were randomly collected in the field in Arizona, and Texas, USA and Aguascalientes, Durango, Jalisco, Michoacan and Zacatecas, Mexico. Voucher specimens were collected at these sites and will be deposited in the herbarium at the University of Arizona, Tucson. Caryopses were isolated from these spikes in the fall of 1998. The caryopses, which are composed of the seed and a thin pericarp, from here on will be referred to as "seeds." Seeds were germinated on moist paper towels in 1999 and 2000 in a greenhouse at the Ecology and Evolutionary Biology greenhouse on the University of Arizona campus, Tucson (see Chapter 2 for greenhouse conditions). In addition, mature, flowering, individuals from DMSP and SRER were transplanted from the field to the greenhouse.

The numbers of individuals that had seed grown and analyzed are given in Table 4.1. Multiple seeds per individual were germinated on moist paper towels and planted. As seeds germinated, two seeds from each individual were planted in an 7X6 distribution in a flat in 6.5cm of autoclaved soil mix (described in Chapter 2). Flats were kept in the middle of a southwest-facing greenhouse and watered three times daily by misters for five minutes per watering event.

Chromosome counts: Chromosome counts were performed on two hermaphroditic individuals each from those collected from the field from DMSP, Texas and SRER, Arizona. Inflorescences from two individuals from each population were collected just previous to stamen exertion; placed in a solution of one part glacial Acetic Acid and two parts 95% ethanol for twenty four hours at room temperature; placed in 70% ethanol; stamens were dissected to isolate pollen mother cells (PMCs); stained with acetocarmine stain; and chromosomes were identified and counted using a phase contrast microscope (Huff and Palazzo, 1998; J Reeder, personal communication, S Smith, personal communication).

Flow cytometry: When seedlings reached the 3-5 leaf stage, 1 or 2 leaves 5-8 cm long were collected and chopped into fine pieces approximately 1X1mm with Wilkinson Sword Classic Double Edge razor blades in 1mL of buffer of 100mM Tris Base, 2.5 mM $MgCl_2 \bullet 6H_2O$, 85 mM NaCl, 0.1% Triton X-100 and 4µg/mL of DAPI. The chopped tissue was soaked in buffer for 1-5 minutes, filtered through a 53-µm mesh to remove leafy material, and an additional 1mL of buffer rinsed through the mesh. The DNA content of these samples were analyzed using a Partec PCC (Cell Counter Analyser) (Partec, Münster, Germany) flow cytometer operated at 488 nm.

Previously published chromosome counts of pollen mother cells (Gould, 1979) were used as a guide to hypothesize that all individuals from Arizona were tetraploid (4X=40) and all individuals from Texas were diploid (2X=20). This hypothesis was tested and confirmed by examining individuals from populations from the Santa Rita Experimental Range (SRER) and Pena Blanca, AZ and from Alpine and Davis Mountain State Park (DMSP), Texas. Once it was established that there was no variation within samples from Arizona (N=26) and Texas (N=30) populations, previously analyzed individuals from SRER and DMSP were included as standards for counts of Mexican populations of unknown chromosome number. Leaf tissue from 10 sexually mature individuals including hermaphrodites (N=6) and both male sterile forms from Texas (N=2 for each); from DMSP were also able to be surveyed using this technique.

PCR and sequencing: DNA was extracted from plants, as described (Huff *et al.*, 1993). Universal primers for trnH [tRNA-His (GUG)] and trnK [tRNA-Lys (UUU) exon 1] and trnS [tRNA-Ser (GGA)] and trnT [tRNA-Thr (UGU) exon 1] were used for PCR amplification and sequencing of polymorphic cpDNA regions of *B. chondrosioides* (Demesure *et. al.*, 1995). PCR reactions mixed 0.5μM each of the appropriate primer pair, 1μL of *B. chondrosioides* DNA, 2mM dATP, dCTP, dGTP and dTTP, 1X reaction buffer (Gibco-BRL), 3mM $MgCl_2$, and 1 unit taq DNA polymerase (Gibco-BRL) and sterile ddH_2O to 30μL volume on ice. The PCR reaction was performed in a Perkin Elmer Cetus DNA Thermal Cycler after an initial denaturing of the DNA at 94°C for five minutes. The thirty cycle PCR reaction included one minute of denaturing at 94°C, one minute of annealing at 60°C, one and one-half minutes of elongation at 72°C and ended with an additional elongation of ten minutes at 72°C and chilling to 4°C for at least 10 minutes. 2μL of each reaction was run out on a 1% agarose gel with EtBr along with size standards and visualized under UV light to confirm amplification of the DNA sequence. PCR products were isolated using a QIAquick PCR purification kit according to manufacturer's directions and sequenced using the appropriate primers diluted to 3pmol/μL. Sequencing was performed at the Genomic Analysis and Technology Core at the Arizona Research Laboratory Division of Biotechnology on the University of Arizona campus.

Phylogenetic analyses: Sequences were entered in the BLAST database at <http://www.ncbi.nlm.nih.gov/BLAST/> for initial alignment with previously published maize (*Zea mays*) and rice (*Oryza sativa*) sequences. Supplementary alignment was performed manually using GeneDoc (Nicholas and Nicholas, 1997) on 568 bases from each sequence of *B. chondrosioides*, maize and rice. Data was exported into text format from GeneDoc, and converted into Nexus format at: <http://bcf.arl.arizona.edu/tools/convert.html>.

Parsimony analyses were carried out on each alignment of the trnH-trnK and trnS-trbT sequences as well as using a conditional combination of both sequences (Soltis and Soltis, 2000) using PAUP* version 4.0b8a for Macintosh (PPC)(Swofford, 1999). To find the shortest tree(s) a heuristic search of 1000 taxon addition replicates were performed under the criteria that one tree was held at each step during stepwise addition, tree-bisection-reconnection (TBR) branch-swapping was performed, the MULPARS option was in effect, the steepest descent option was in effect, branches having maximum length zero were collapsed to yield polytomies, topological constraints were not enforced and the outgroups specified were maize and rice. Bootstrap analyses were conducted using 1000 re-sampling replicates and the same tree search procedure as described above.

Results:

Chromosome counts: The chromosome counts demonstrated that the two individuals from Arizona had 40 chromosomes and were tetraploid, and the two individuals from Texas had 20 chromosomes and were diploid. These counts were in accord with previous chromosome counts from these regions.

Flow cytometry: The results of flow cytometric analysis of isolated nuclei were summarized in histograms of their DNA content with two peaks corresponding to the pre-DNA replication G_0/G_1 nuclei and the post-DNA replication G_2 nuclei of *Bouteloua chondrosioides*. Figure 4.1 presents the fluorescence peaks that result from flow cytometry of a diploid individual from Texas and a tetraploid individual from Arizona. The peaks on the histograms presented in Figure 4.1 represent the total fluorescence of each sample, a function of the DNA content of the nuclei examined. Two peaks are seen in each histogram. The first, taller, peak on the x-axis is the 2C peak which is determined by the G_0/G_1 DNA content within the nuclei of the cells. The second, smaller, peak, approximately twice the DNA content of the first peak on the x-axis, are the 4C nuclei that are found within cells in the G_2 part of the cell cycle and that have doubled their DNA in preparation for mitosis and cell division.

The resulting data from flow cytometry demonstrated that individuals from SRER, Arizona had approximately twice the DNA content of individuals from DMSP, Texas. These individuals were used as standards and the ploidy levels of populations in Mexico were analyzed relative to these two populations. The results from this study demonstrated that all individuals examined from Arizona, were tetraploid (number of plants=26), and that all individuals from Texas were diploid (number of plants=40). The results of flow cytometry of plants from Mexico are given in Table 4.2.

Sex expression in plants of known ploidy level: High mortality and a low frequency of flowering plants complicated efforts to demonstrate or reject a correlation between male sterility and ploidy level. All ten sexually mature individuals from DMSP with successful flow cytometry were diploid, regardless of sex type. In addition, 16 of the diploid DMSP grown from seeds and analyzed with flow cytometry flowered. Of these individuals, ten were hermaphrodites, five were MA male steriles and one was MS male sterile. From tetraploid individuals surveyed in Arizona and Mexico, five hermaphrodites flowered from the SRER population, one MA male sterile individual and two hermaphrodites flowered from the tetraploid population just south of Durango, and two hermaphrodites flowered from the Zacatecas population.

Phylogenetic analysis of chloroplast sequences: PCR amplification of the trnH-trnK and trnS-trnT primer pairs and sequencing of the resulting bands of 1930 bp and 1268 bp were done for each diploid and tetraploid population. The amplified sequences of trnS-trnT from two individuals from Texas populations, one from Alpine and one from DMSP, had no significant similarity to sequences of any taxa in the NCBI database. The sequencing of PCR amplified DNA using the trnK primer failed twice for the individual from Alpine. The sequence of these two individuals were also very different from all other sequences for the first 70 bases of trnH. However, the sequence obtained, using the trnH primer, had significant similarity to other plant chloroplast genes in the NCBI database.

Populations were pooled by region resulting in phylogenetic analyses of two populations from Aguascalientes, one population from Arizona, two populations from Durango, three populations from Michoacan, two populations (three individuals total) from Texas and one population from Zacatecas. No DNA was collected for this study from individuals from Jalisco because of 100% mortality of those seedlings examined with flow cytometry in the greenhouse. Maize and rice were the designated outgroups.

For the trnH-trnK sequence, nine trees having 404 steps were found. The consensus tree, along with bootstrap values is presented in 4.2. For the trnS-trbT sequence, seven most parsimonious trees of 1366 steps were found. The consensus tree along with bootstrap values is presented in Figure 4.3. In both trees, most clades are not strongly supported by bootstrap analyses greater than 80%. The only supported grouping from the trnH-trnK tree is a clade containing two Texas individuals (from different populations).

From the trnS-trbT tree this clade is also supported and is a sister group to a Michoacan population. This monophyletic clade in turn is supported as sister group to a second Michoacan population.

Combining the two sequences resulted in three most parsimonious trees of length 1783 (Figure 4.4). The consensus tree demonstrated strong support for the clade of the two Texas populations and Michoacan populations and, furthermore, that this clade is the sister taxon to the tetraploid Aguascalientes population. There was also strong support that the other Texas population was a sister taxon to the Arizona population. The remaining taxa on all trees resulted in a polytomy and had undefined relationships among each other.

Discussion of chapter 4:

Study of the relationships between sex and ploidy: No correlation between sex type and ploidy level was supported by this study based on the fact that ten diploid individuals and one tetraploid individual were male sterile. In addition, male sterility was observed in all populations in which ploidy was examined with flow cytometry. Two populations in Mexico, determined to be tetraploid in this study, the Aguascalientes population north of Rincon de Romos and the Durango population, just south of Durango City, each had more than 20% male sterility. This contradicts Reeder and Reeder's hypothesis that male sterility would only be found in diploid populations. Unfortunately, this conclusion is only weakly supported due to the inability to determine ploidy in most sexually mature plants combined with the high mortality of individuals in which ploidy was determined. It is recommended that more individuals be sampled from a single tetraploid population in future studies to conclusively demonstrate that male sterile individuals, of both types, found in *B. chondrosioides*, can be both diploid and tetraploid.

It has been suggested that polyploidy could result in dioecy, the full separation of sexes, due to the breakdown of incompatibility systems and selection for new adaptations against inbreeding (Miller and Venable, 2000). This path is only possible in species with incompatibility systems and it is unclear how polyploidy affects other mating system traits that may be subject to selection (Make, 1992; Miller and Venable, 2000; Brunet and Liston, 2001). In addition, incompatibility is maintained in polyploid lineages in the grasses (Hayman, 1992). It is not known if *B. chondrosioides* has incompatibility systems, but they have been observed in other *Bouteloua* species (Connor, 1979). Mating

systems can also be structured such that diploid plants are dioecious or gynodioecious whereas polyploid plants are andromonoecious, monoecious or gynomonecious, as seen in annual *Mercurialis* species (Euphorbiaceae) (Durand and Durand, 1992).

The fact that male sterility in *B. chondrosioides* may be cytoplasmic in nature (Chapter 2 and 3), indicate that ploidy level should have little effect on sex expression, if polyploids are not of hybrid origin. If both polyploid individuals and male sterility are due to hybridization between species, we might expect to see only polyploid male steriles. This is not supported, as all populations, whether tetraploid or diploid, had some degree of male sterility that varied widely among populations (Chapter 2). The highest levels of male sterility in *B. chondrosioides* were observed in the diploid Texas populations followed by the tetraploid Mexican populations, with the lowest levels observed in the tetraploid Arizona populations (Chapter 2) suggesting that the distribution of sex is independent of ploidy.

Distribution of ploidy: The populations with either exclusively diploid or tetraploid counts from this study were in accord with earlier chromosome counts (Table 4.2). The lack of odd level ploidy (triploid, pentaploid etc.) within any populations, especially the Jalisco population, suggests that gene flow between ploidy levels is rare or does not exist, or occurs only though meiotic disjunction resulting in a even number of chromosomes in the offspring. One point of interest from this study is the difference in ploidy levels observed in samples from near Rincon de Romos in Aguascalientes, Mexico. The site of the original population studied in 1966 was visited in the fall of 1998 to study male sterility but no *B. chondrosioides* individuals were found. Instead, the nearest site, located a mile and half away on an active mine, was sampled instead. An earlier study demonstrated that the population sampled in this region was diploid (Reeder and Reeder, 1966), while the flow cytometry performed on samples from this general area in 1998 demonstrated that the plants were tetraploid. This may indicate that there initially were two close populations of different ploidy levels or that the original diploid population has gone extinct and been replaced, either through displacement or re-colonization by the tetraploid population. Polyploid individuals may be more adaptable and better able to colonize new habitats relative to their progenitor diploid populations or species (Van Dijk and Bakx-Schotman, 1997; Keeler, 1998; Amsellem *et al.*, 2001). Further studies of ploidy levels in nearby populations may elucidate what happened to the 1966 diploid population. In addition, greater sampling of nearby regions may also help determine if the distribution of ploidy is related to specific ecological sites and/ or geography.

The mixed ploidy levels observed in Jalisco were unexpected based on previous counts that suggested that populations of *B. chondrosioides* do not have both ploidy levels within a single population. The distribution of multiple ploidy levels within a single population can impact population structure and affect gene flow between populations (Keeler and Davis, 1999). Because of the expected competitive advantage found in polyploids, mixed ploidy levels are expected to only occur in areas of environmental heterogeneity or where only limited crosses can occur between ploidy levels (Segraves *et al.*, 1999). It would be of interest to determine if there are characters associated with polyploidy in *B. chondrosioides*, and if they are constant among all tetraploid populations.

Because previously published chromosome counts of *B. chondrosioides* include results from chromosome counts from only a few individuals per population, it is difficult to determine the true distribution of ploidy within a population. Other *B. chondrosioides* populations may have mixed ploidy levels that were not detected because of high mortality in seedlings of polyploid individuals. When siblings of octoploid individual were germinated to establish if this cytotype was constant within the offspring of the same parent, all died before they could be sampled with flow cytometry.

Phylogenetic distribution: Populations from Aguascalientes, Durango, Michoacan, (Mexico) and Texas (USA) demonstrated high levels of cpDNA diversity over relatively small distances. If polyploidy had arisen only once, it is expected that all polyploid lineages would be a monophyletic clade associated with only one diploid lineage. This was not seen. However, if multiple colorizations of tetraploid lineages occurred, we might also expect patterns of cpDNA diversity similar to those found in this study. This implies that ancestral polymorphisms have been maintained within these populations, supporting a replacement of the diploids by tetraploids rather than a single colonization event. More studies covering more of the range of *B. chondrosioides* will be needed to further confirm this observation.

The cpDNA sequence polymorphisms resulted in phylogenies that were not informative of the geographical distribution of these populations. The trnS-trbT tree demonstrated high support that a tetraploid population was the sister group to a clade of two diploid populations. Furthermore, this relationship was maintained in the combined tree along with high support that a pair of diploid and tetraploid taxa were sister groups. The consensus tree composed of both genes demonstrates that at least three separate polyploid events have occurred. These data, along with the discovery of an octoploid individual, support that tetraploid individuals have arisen multiple times and that tetraploid populations have been established more than once.

Recent advances in genome analysis have demonstrated the importance of studying polyploidy as a facet of the evolution and the evolutionary potential of a species. The results from these experiments highlight the need for more studies of the distribution and

origin of polyploidy within a single species in order to better understand the population genetics and effects of polyploidy (Keeler and Davis, 1999). Studies of species with multiple polyploid events and establishment give us insight into the ecology and evolutionary factors associated with polyploids (Keeler and Davis, 1999). These factors are very important in light of new genetic data that indicates that polyploidy is a frequent stage in many plants' evolutionary history (Soltis and Soltis, 1999). Studies of natural populations representing transitory stages are of immense value to scientists delving into the evolutionary history of a species.

TABLE 4.1, Origin and number of seedling grown for flow cytometry

Population	Latitude	Longitude	Seeds grown
Alpine, TX	30:21 N	103:39W	16
DMSP, TX	30:42 N	104:05 W	20
Pena Blanca, AZ	31:20 N	110:56 W	16
SRER	31:53 N	110:53 W	21
Aguascalientes, MX[1]	22:13 N	102:24 W	27
Aguascalientes, MX[2]	22:01 N	103:50 W	27
Durango, MX[3]	23:90 N	104:75 W	61
Durango, MX[4]	23:80 N	104:50 W	36
Jalisco, MX[5]	21:15 N	102:10 W	31
Jalisco, MX[6]	21:24 N	101:59 W	28
Michoacan, MX[7]	20:25 N	101:90 W	12
Michoacan, MX[8]	20:13 N	101:58 W	57
Zacatecas, MX[9]	21:50 N	103:20 W	21

1=N of Calvillo; SW of Aguascalientes on RT 70

2=N of Rincons de Romos on RT 45

3=S of Durango city on RT 45

4=N of Nombre de Dios on RT 45

5=NE of Lagos de Moreno; near of Paso de Cuarenta on RT 80

6= SE of Lagos de Moreno; on RT 45

7=S of La Piedad de Cabadas on RT 37

8=N of Morelia on RT 15

9=N of Moyahua de Estrada on RT 54

TABLE 4.2, Distribution of chromosome counts of *Bouteloua chondrosioides*

Location	Type of count	chromosomes[a]	#[b]	Reference(s)
USA				
Arizona	PMC	40	2	Gould, 1979; Zahn, this study
	Flow cytometery	40	26	Zahn, this study
Texas	PMC	20	2	Gould, 1979; Zahn, this study
	Flow cytometry	20	40	Zahn, this study
Mexico				
Aguascalientes[1]	PMC	20		Reeder and Reeder, 1966
	Flow cytometry	20	26	Zahn, this study
Aguascalientes[2]	PMC	20		Reeder and Reeder, 1966
	Flow cytometery	40	12	Zahn, this study
Chihuahua	PMC	22; 40		Gould, 1979; Reeder and Reeder, 1968
Chiapas	PMC	40		Gould, 1979
Durango	PMC	40		Gould, 1979
Durango[3]	Flow cytometery	40	30	Zahn, this study
Durango[4]	Flow cytometery	40	29	Zahn, this study
Jalisco	PMC	20		Reeder and Reeder, 1966
Jalisco[5]	Flow cytometery	20; 40; 80*	14	Zahn, this study
Jalisco[6]	Flow cytometery	20; 40*	12	Zahn, this study
Michoacan	PMC	20		Gould, 1979
Michoacan[7]	Flow cytometery	20	8	Zahn, this study
Michoacan[8]	Flow cytometery	20	32	
Oaxaca	PMC	40		Gould, 1979
Zacatecas	PMC	20; 40		Gould, 1979
	Flow cytometery	40	23	Zahn, this study

Populations are numbered the same as in Table 4.1

a- number of chromosomes estimated based on results from flow cytometery

b-number of seedlings that were successfully analyzed with flow cytometery

*=Jalisco population 5 had four diploid individuals, nine tetraploid individuals and one octoploid individual. Jalisco population 6 had eight diploid individuals and four tetraploid individuals

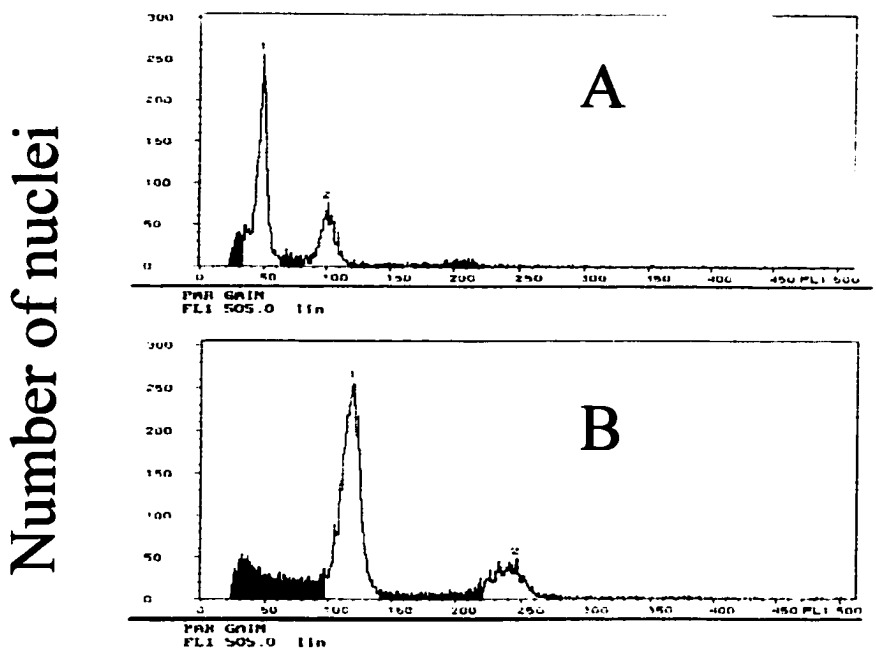


FIGURE 4.1, Flow cytometric peaks: Results of flow cytometry for a seedling from DMSP Texas (A) and seedling from SRER Arizona (B). Peak 1 for Arizona is approximately twice as large as the Texas individual, with twice the DNA content. Therefore the Texas individual is diploid (2N=20) and the Arizona individual is tetraploid (2N=40).The second peak on each graph represents those cells that are in the synthesis (S) phase of the cell cycle and have undergone DNA doubling but have not yet finished the mitotic division.

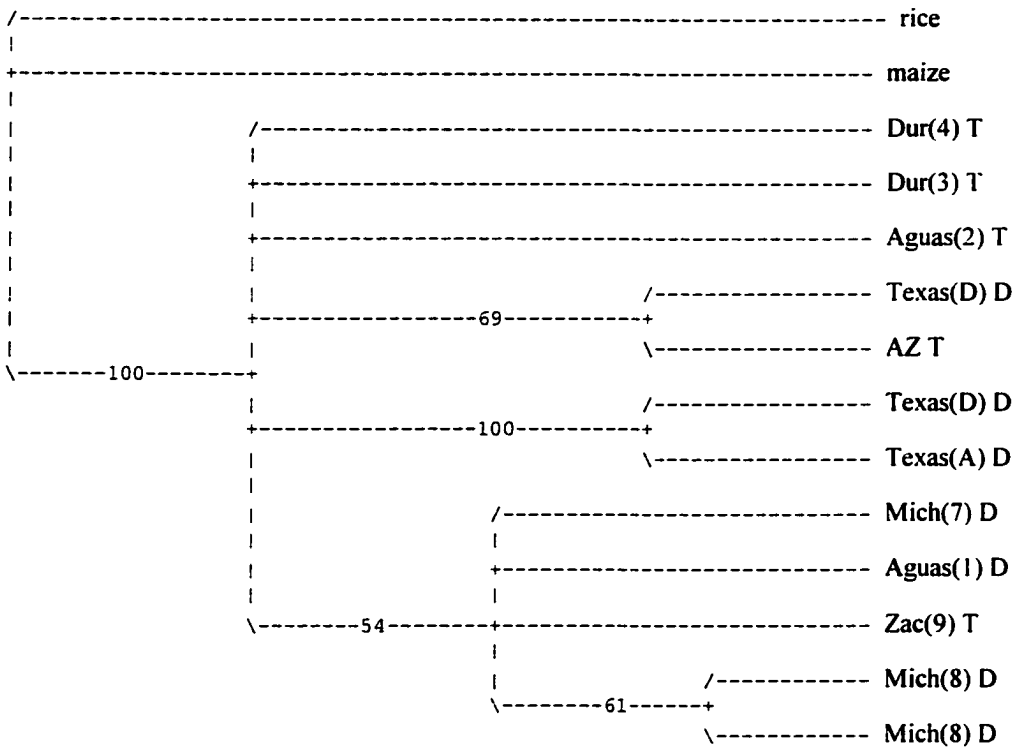


FIGURE 4.2, Consensus tree of trnH and trnK sequences: Consensus of nine most parsimonious trees for the sequence obtained from PCR amplification of the region between trnH and trnK (tree length 404; consistency index= 0.8787, retention index=0.7678). Terminal taxa and their ploidy levels are the same as in Table 4.1, letters represent multiple individuals surveyed. Aguas = Aguascalientes; AZ = Arizona; Dur = Durango; Mich = Michoacan; Texas(A) = Alpine, Texas; Texas(D) = DMSP, Texas; Zac= Zacatecas.

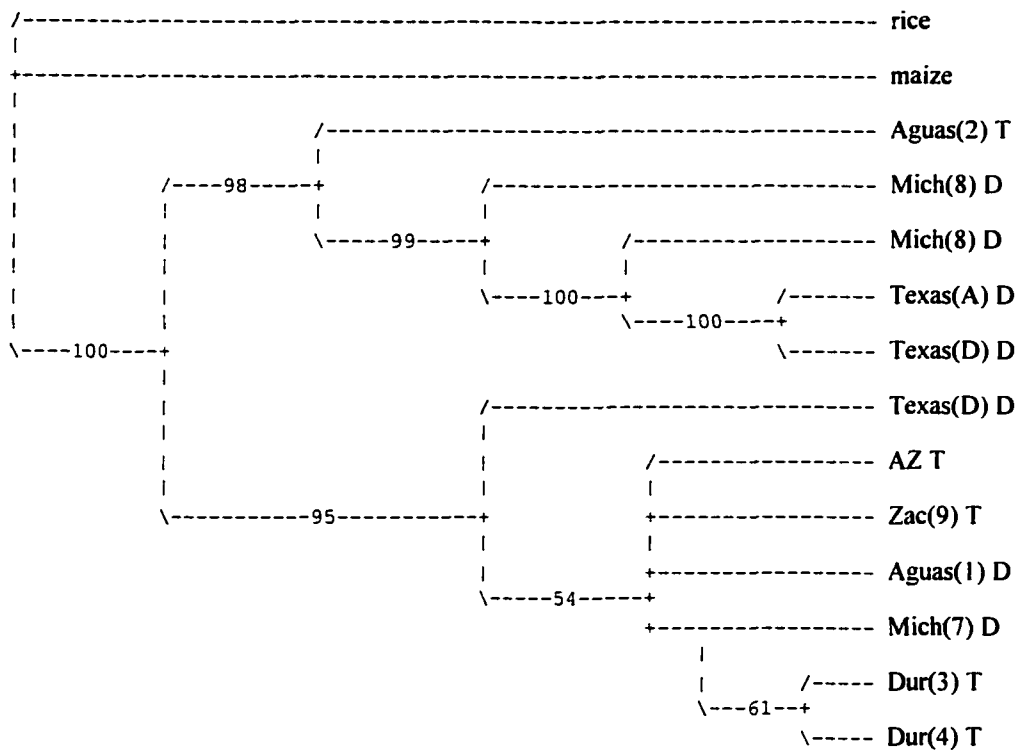


FIGURE 4.3, Consensus tree of trnS and trbT sequences: Consensus of seven most parsimonious trees for the sequence obtained from PCR amplification of the region between trnS and trbT (tree length 1366; consistency index=0.880, retention index=0.759) . Terminal taxa and their ploidy levels are the same as in Table 4.1, letters represent multiple individuals surveyed. Aguas = Aguascalientes; AZ = Arizona; Dur = Durango; Mich = Michoacan; Texas(A) = Alpine, Texas; Texas(D) = DMSP, Texas; Zac= Zacatecas.

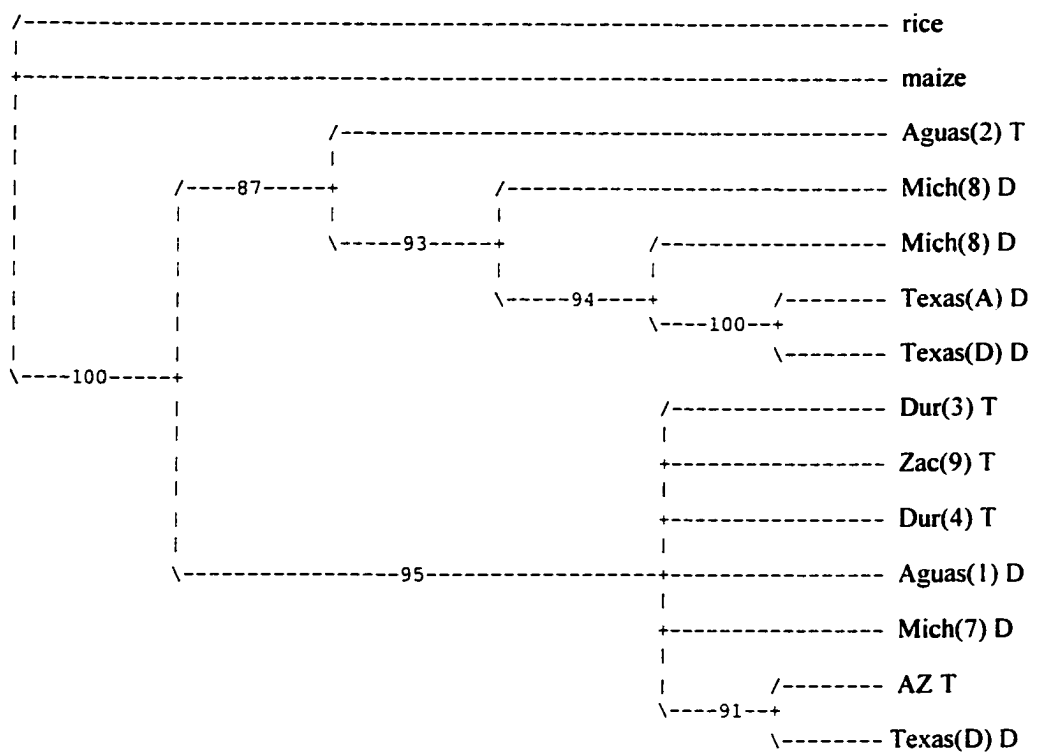


FIGURE 4.4, Consensus tree of both trnH and trnK and trnS and trbT sequences: Consensus of three most parsimonious trees for the sequences obtained from PCR amplification of both the region between trnH and trnK and trnS and trbT (tree length 1783; consistency index= 0.873, retention index=0.746) . Terminal taxa and their ploidy levels are the same as in Table 4.1, letters represent multiple individuals surveyed. Aguas = Aguascalientes; AZ = Arizona; Dur = Durango; Mich = Michoacan; Texas(A) = Alpine, Texas; Texas(D) = DMSP, Texas; Zac= Zacatecas.

SUMMARY, CONCLUSIONS AND FUTURE DIRECTIONS

In the literature, the maintenance of male sterility within a population is reduced to the question of how individual fitnesses are affected in male sterile individuals when their reproductive fitness is effectively halved, relative to hermaphrodites, by the loss of male function. The genetic determination of male sterility is of great interest since the mode of inheritance can significantly affect the maintenance of male sterility within a population. The most common cause of male sterility is cytoplasmic male sterility (CMS). Another common form of male sterility is simple nuclear recessive inheritance. CMS has been determined to be caused by defects in mitochondrial respiration. Plants with CMS have pollen production restored by nuclear restorer genes which modify the expression of the proteins that cause male sterility. The combination of cytoplasmic and nuclear genes affecting pollen production makes the determination of the inheritance of male sterility complex. Because of restorer alleles, there can be multiple male sterile cytoplasms each with a unique restorer and male sterile cytoplasms in male fertile individuals. In the case of a population with CMS, all male sterile individuals will have a male sterile cytoplasm and no restorer and hermaphrodites can either have both a male sterile cytoplasm and nuclear restorer or can have a normal cytoplasm. Because of the resulting genetic distribution, certain traits can be indicative of CMS. These traits include non-random distribution of male sterility within a geographically isolated location, high variation in the level of male sterility among populations, multiple male sterile phenotypes, and a high proportion of male sterility in the offspring of a male sterile individual, relative to the proportion of male sterile offspring from restored hermaphrodites. While gynodioecy

is rare in the grasses, both CMS and simple nuclear recessive mutants resulting in male sterility have been documented within crop species that are grasses.

This dissertation focused on experiments that investigated the phenotype, distribution, causes and maintenance of male sterility in the gynodioecious grass, *Bouteloua chondrosioides*. In addition, investigations were performed to investigate how polyploidy affects sex expression and how ploidy is distributed within and among populations. The results from these studies suggest that male sterility in *B. chondrosioides* is most likely due to cytoplasmic male sterility.

Chapter 2 describes observations performed in the field to examine how male sterility is distributed, experiments in the greenhouse and common garden that investigate the inheritance of male sterility, and measurements taken to determine what phenotypic variation occurs among the two male sterile types, MA and MS, that may explain the occurrence of male sterility in the field. Observations in the field were used to document the distribution of male sterility in *B. chondrosioides*. A common garden experiment was performed to determine if the levels of male sterility observed in the field could be recovered in a novel environment. The greenhouse experiment examined the distribution of male sterility among the offspring of individuals of known sex type. These data were used to test a model of simple genetic inheritance. Measurements of individuals from the field and greenhouse were used to test if there were significant differences between the sex types that might explain the maintenance of male sterility in the field.

The field studies demonstrated that the proportion of male sterility observed in the field was highly variable and significantly different among the regions of Arizona,

Mexico, and Texas, and significantly differed among populations within Arizona and Texas and that the distribution of male sterility within populations in Mexico and Texas was non-random. The distribution of male sterility among the offspring of individuals of known sex type rejected models of simple nuclear recessive or dominant inheritance. In addition, the same proportion of male sterile individuals as those observed in the field was recovered in the offspring. This experiment demonstrated that there are two unique male sterile types that are independently inherited, as no maternal seed parent had both types of male sterility in their offspring. While this experiment was not conclusive in determining the inheritance of sex type in *B. chondrosioides*, the data demonstrated that male sterile individuals could be recovered in the greenhouse at frequencies not significantly different from those seen in the field. In addition the data suggest that male sterility is most likely due to cytoplasmic male sterility, although a complex multigenic nuclear inheritance could not be ruled out. Due to high mortality, the common garden experiment failed to be useful in determining the effect of the environment on sex type expression but did demonstrate that individuals of each sex type could grow to sexual maturity from seed within three months. In addition, no characters such as increased seed germination or increased flower number were identified that could explain the maintenance of male sterility in nature, although some that were indicated as possibly varying among the sex types included seed set, seed size, seedling survival and establishment, height at sexual maturity and timing of flowering. Additional characters could be involved in the development of male sterility but were not identified in these experiments.

Chapter 3 describes experiments that further investigated if male sterility in *B. chondrosioides* is best explained by cytoplasmic male sterility by surveying mitochondrial DNA RFLPs in 30 maternal lines where the sex expression within the family line was known. Multiple individuals were analyzed from each family, focusing on individuals of different sex types in the family lines that had both hermaphrodites and male sterile individuals, were surveyed. These investigations resulted in 19 unique mitochondrial haplotypes in 30 maternal lines, which is high relative to the diversity reported in other studies of gynodioecious plants suggesting that there may be natural selection for diversity in the mtDNA genome. There were unique alleles seen only in 8 of 11 MA male sterile lines which shared specific RFLPs. In addition most enzyme/probe combinations were not distributed randomly with regards to the sex type expressed within a maternal line. No mitochondrial types were identified that were correlated with MS male sterility or male fertility. The distribution of mtDNA RFLPs were significantly non-random among lines of different sex types, but because sample size was small there was not enough power in these experiments to determine if there are significant differences among mitotypes. These results demonstrated that there are specific mitochondrial variants associated with some maternal lines that produce MA male sterile offspring which is consistent with the hypothesis that male sterility is the result of mtDNA rearrangements. This analysis helps rule out the possibility that these rearrangements are just random events, although not in the case of MS male sterility. However, it is possible that the same mutation, yet to be determined, causes MS male sterility in these populations.

This research could be extended by examining if similar genes are associated with MA male sterility in different geographical regions than Texas. In addition, intensive sequencing and surveys of individuals of the MA male sterile type, compared to MS male sterile individuals and hermaphrodites, may identify regions with changes unique to all MA male sterile lines that differ from MS male sterile and hermaphroditic lines.

Chapter 4 describes experiments performed to examine if polyploidy is associated with male sterility in *B. chondrosioides* using flow cytometery. In addition, it was tested whether tetraploid populations in *B. chondrosioides* resulted from a single polyploid event followed by range expansion, or if tetraploids are descended from multiple polyploid lineages. To determine the relatedness of populations of different ploidy levels, a phylogenetic tree was constructed from polymorphic chloroplast DNA (cpDNA) sequences. Flow cytometry of cells was not possible on most mature plants. Instead, seedlings from different populations were examined and grown in the greenhouse to determine their sex. High mortality occurred among seedlings in which ploidy was determined and only 28 individuals from this study (of 252 total examined) survived to flower. Of these, 7 MA individuals, 1 MS individual and 10 hermaphrodites flowered from one of the diploid populations (of the 106 plants deemed diploid). From three tetraploid populations 1 MA individual and 9 hermaphrodites flowered (of the 120 plants deemed tetraploid). In addition 10 mature individuals from Davis Mountain State Park, Texas, representing all three sex types, were able to be surveyed and all of them were diploid. This analysis suggests that sex is not correlated to ploidy. From these flow cytometric surveys, two populations demonstrated mixed ploidy levels, while the rest

were either solely diploid or tetraploid. In addition, one individual from one of these mixed populations was octoploid. There was no evidence of triploid or pentaploid lines. Phylogenetic analyses of cpDNA sequences demonstrated that tetraploid populations are not all descended from a single lineage.

The results from these studies suggest that the cause of male sterility in *B. chondrosioides* is primarily genetic, not associated with ploidy level, and is probably not due to simple recessive or dominant nuclear inheritance. Combined with the non-random distribution of male sterility in the field, the significant variation among populations within regions, and the association between most MA male sterile lines and mtDNA RFLPs, these studies support the hypothesis that male sterility in *B. chondrosioides* is due to cytoplasmic male sterility. These studies have laid a foundation that may be used to test models of evolutionary and ecological theories in *B. chondrosioides* in the future.

Further studies that might aid in confirming the degree of heritability of male sterility include cloning individuals of different sex types and exposing them to different environmental conditions. This would aid in determining if male sterility can be variable within a mature genet. Additionally, growing seedlings from parents of known sex type in different environmental conditions and analyzing if the distribution of sex significantly differs among the environments would also identify if male sterility has an environmental component.

Crosses could also be performed with cloned individuals to determine the heritability of sex type, how sex types are inherited, and if the patterns of inheritance further support that sex type is maternally inherited. Crosses between ploidy levels should also be done

to determine if polyploidy is a barrier to gene flow in *B. chondrosioides*. If the results of crosses indicate maternal inheritance of male sterility, larger scale surveys of mtDNA polymorphisms within a single population, examination of spatial mtDNA structure and mapping of the mitochondrial regions proximal to those genes that are associated with MA male sterility from my studies and any identified from an examination of protein differences should be performed. In addition, the genetics of male sterility should be further investigated by examining if there are unique proteins in male sterile anther remnants and if these proteins are chimeric proteins. Furthermore, more studies examining the maintenance of male sterility are recommended, including an examination of seed set or other fitness differences between MA and MS male steriles and hermaphrodites under different environmental conditions. These molecular and ecological studies will further delve into the inheritance and maintenance of male sterility and can be used to advance our understanding of the evolution of male sterility in *B. chondrosioides* and perhaps the entire genus, *Bouteloua*.

APPENDIX A

The data taken on timing of inflorescence initiation and maturity, the height at floral initiation and maturity, number of tillers, number of inflorescences and number of spikes from those offspring of parents of known sex type in the greenhouse are presented in Table A.1. Materials and methods and other results from this study are presented in Chapter 2 of the dissertation. These data were analyzed to determine, which of these traits are explained by four factors: the family, the position in the greenhouse referred to as the block, the sex of the parent, and the sex of the individual. If a trait had more than one significant factor, that trait was further analyzed by nesting significant factors to remove the addition variance. In addition, these characters were examined to determine which traits co-vary.

Floral timing: The number of days from seedling germination to floral initiation and floral maturity, defined as the exertion of the stamens or stigmas, were significantly different among plants from different families, their location within the greenhouse, the sex of the parent plant and the sex of the plant. The location of the plant within the greenhouse significantly affected the timing of flowering, as plants in the easternmost part of the greenhouse took significantly longer to initiate flowering and to mature. The time between floral initiation and sexual maturity was not significantly different among blocks within the greenhouse. The time between floral initiation and floral maturity significantly differed among blocks, families, populations and the sex of the plant. There were significant differences among sexes even when the variation due to the block, family, sex of the parent and population were removed, by nesting the data, in the that MS male steriles took a significantly longer time to reach floral initiation ($F=2.73$, $df=15$, $P<0.0006$), floral maturity ($F=2.89$, $df=15$, $p<0.0003$) and that the time between floral initiation and floral maturity were significantly longer ($F=2.26$, $df=15$, $p<0.005$) than in hermaphrodites (Tukey/Kramer, $p<0.05$). As expected, the length of time to floral initiation was significantly correlated with the length of time to sexual maturity ($r^2= 0.98$, $F=11077.12$, $df=1$, $p<0.0001$).

Inflorescence height: The height of the inflorescence at the time of floral initiation and floral maturity were significantly different among families, populations and block, although when the variation due to population was removed by nesting the data from there was no significant difference between families. The location of plants within the greenhouse affected the height of plants, as those on the westernmost side of the greenhouse were significantly taller than plants to the east. Even when this positional variation was removed from the analyses, by nesting, the longer it took plants longer to initiate flowering, the taller they were at both the time of floral initiation (r^2=0.41, F=31.05, p<0.0001), and at the time of sexual maturity (r^2=0.27, F=13.13, p<0.0001). The height of the plant at floral maturity was significantly different among sexes only at maturity where MS plants were significantly taller than both MA male steriles and hermaphrodites (Tukey/Kramer p<0.05). When the variation among families was removed by nesting, the significant differences among sexes were lost, but when the MA male sterile individuals were removed from this analysis, the difference in height between MS male steriles and hermaphrodites was significant (F=3.96, df=4, P<0.004). The height of the inflorescence at floral initiation was significantly correlated with the mature height of the inflorescence (r^2=0.50, F=318.59, p<0.0001). The average change in height from floral initiation to sexual maturity decreased as the time to floral initiation increased (r^2=0.07, F=23.64, p<0.0001). The family was the only fixed factor that significantly affected the overall change in height between floral initiation and floral maturity (F=1.73, df=28, p<0.014). The overall change in height that occurred between floral initiation and sexual maturity was significantly correlated, as expected, to the

height of the plant at the time of sexual maturity (r^2=0.23, F=96.07, p<0.0001). The overall change in height also increased, as expected, with the amount of time between floral initiation and maturity increased (r^2=0.15, F=52.54, p<0.0001).

Floral and inflorescence number: The numbers of inflorescences were negatively correlated with the date flowering initiated (r^2=13.33, F=45.10, df=1, p<0.0001) and were positively correlated with the number of tillers (r^2=11.11, F=36.51, df=1, P<0.0001) and number of spikelets (r^2=12 F=40.81, df=1, p<0.0001). The number of inflorescences did not significantly vary among sexes when the variation among families, populations, or blocks was removed through nesting. The numbers of tillers varied significantly by both family and by block. Removing the variation due to position in the greenhouse by nesting, the average number of tillers at the time of floral initiation significantly decreased with time (r^2=0.09, F=4.16, p<0.0027). The numbers of tillers were also slightly positively correlated with the number of spikelets per inflorescence (r^2=0.06, F=2.47, df=4, p<0.045). The numbers of spikelets per inflorescence were significantly different among sex types but not when nested by family or sex of the parent.

TABLE A.1, Timing of flowering, height of inflorescences, number of tillers, inflorescences and spikelets by family, population, block, sex of the parent plant and sex of the plant

	df	FI	F	P	FM	F	P	Δ FI-FM	F	P	HI	F	P
Family													
Average	28	110.9+/-1.4	1.4	0.078	123.4+/-1.4	1.9	0.004	10.6+/-0.2	1.8	0.009	35.6+/-0.5	1.8	0.010
Pop.													
Alpine	1	108.9+/-2.4	0.5	0.463	122.8+/-2.5	0.1	0.750	9.8+/-0.5	6.8	0.010	38.0+/-0.6	11.0	0.001
DMSP		111.0+/-1.6	0.5	0.463	123.7+/-1.7	0.1	0.750	11.2+/-0.3	6.8	0.010	34.3+/-0.9	11.0	0.001
Block													
A	3	104.5+/-2.5	4.9	0.002	116.9+/-2.6	4.2	0.006	10.3+/-0.5	0.6	0.603	31.9+/-0.9	22.4	0.001
B		108.1+/-2.5	4.9	0.002	123.0+/-2.6	4.2	0.006	10.7+/-0.5	0.6	0.603	35.5+/-0.9	22.4	0.001
C		113+/-3.0	4.9	0.002	125.5+/-2.8	4.2	0.006	11.1+/-0.5	0.6	0.603	40.4+/-0.9	22.4	0.001
D		118.6+/-3.1	4.9	0.002	131.6+/-3.3	4.2	0.006	11.2+/-0.6	0.6	0.603	41.6+/-1.1	22.4	0.001
Parent Sex													
H	2	106.9+/-2.0	4.2	0.016	120.1+/-2.1	3.9	0.021	10.3+/-0.4	2.7	0.066	35.8+/-0.8	1.9	0.148
MA		115.3+/-2.2	4.2	0.016	128.7+/-2.4	3.9	0.021	11.6+/-0.4	2.7	0.066	38.0+/-0.8	1.9	0.148
MS		109.1+/-3.1	4.2	0.016	121.4+/-3.3	3.9	0.021	10.4+/-0.6	2.7	0.066	37.0+/-1.2	1.9	0.148
Plant Sex													
H	2	107.7+/-1.6	7.3	0.001	120.0+/-1.7	9.1	0.001	10.1+/-0.3	7.8	0.001	36.6+/-0.6	0.7	0.476
MA		112.5+/-2.8	7.3	0.001	127.4+/-3.0	9.1	0.001	12.6+/-0.6	7.8	0.001	36.8+/-1.1	0.7	0.476
MS		124.6+/-4.3	7.3	0.001	139.1+/-4.5	9.1	0.001	11.7+/-0.8	7.8	0.001	38.7+/-1.7	0.7	0.476

		HM	F	P	T	F	P	I	F	P	S	F	P
Family													
Average	28	51.5+/-0.7	3.1	0.001	19.9+/-0.5	3.3	0.001	4.33+/-0.2	1.1	0.321	7.2+/-1.4	2.1	0.001
Pop.													
Alpine	1	50.6+/-1.2	3.2	0.074	20.9+/-0.9	1.9	0.165	3.82+/-0.3	4.8	0.028	7.4+/-0.1	14.0	0.001
DMSP		53.0+/-0.8	3.2	0.074	19.4+/-0.6	1.9	0.165	4.62+/-0.2	4.8	0.028	6.7+/-0.1	14.0	0.001
Block													
A	3	46.4+/-1.1	19.0	0.001	21.8+/-0.9	4.4	0.005	4.91+/-0.3	2.5	0.062	7.0+/-0.2	1.1	0.339
B		50.7+/-1.1	19.0	0.001	20.6+/-0.9	4.4	0.005	4.56+/-0.3	2.5	0.062	7.2+/-0.2	1.1	0.339
C		57.1+/-1.2	19.0	0.001	19.1+/-0.9	4.4	0.005	3.94+/-0.3	2.5	0.062	7.2+/-0.2	1.1	0.339
D		57.2+/-1.4	19.0	0.001	16.9+/-1.1	4.4	0.005	3.72+/-0.4	2.5	0.062	7.4+/-0.2	1.1	0.339
Parent Sex													
H	2	51.8+/-1.0	1.5	0.220	18.95+/-0.7	2.2	0.116	4.27+/-0.3	1.4	0.255	6.9+/-0.1	4.0	0.019
MA		51.7+/-1.1	1.5	0.220	20.2+/-0.8	2.2	0.116	4.68+/-0.3	1.4	0.255	7.4+/-0.1	4.0	0.019
MS		54.7+/-1.5	1.5	0.220	21.7+/-1.1	2.2	0.116	3.87+/-0.4	1.4	0.255	7.4+/-0.2	4.0	0.019
Plant Sex													
H	2	51.6+/-0.8	4.5	0.012	19.9+/-0.6	0.9	0.419	4.43+/-0.2	3.9	0.022	7.0+/-0.1	3.2	0.044
MA		52.1+/-1.4	4.5	0.012	20.5+/-1.0	0.9	0.419	4.68+/-0.4	3.9	0.022	7.5+/-0.2	3.2	0.044
MS		58.4+/-2.2	4.5	0.012	18+/-1.6	0.9	0.419	2.85+/-0.6	3.9	0.022	7.5+/-0.3	3.2	0.044

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