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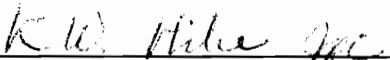
**DNA SYSTEMATIC STUDIES IN THE GENUS BROMUS L. (POACEAE)**

by

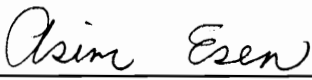
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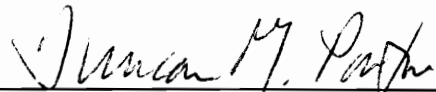
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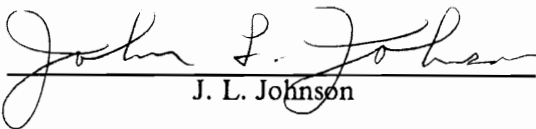
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## DNA SYSTEMATIC STUDIES IN THE GENUS *BROMUS* L. (POACEAE)

by

Michael Pillay

Chairman K. W. Hilu

Biology

(ABSTRACT)

Chloroplast and ribosomal DNA restriction endonuclease analyses were used to assess phylogenetic relationships between different subgenera of *Bromus*, a genus of over 100 diploid and polyploid species. Variation in chloroplast DNA (cpDNA) fragment pattern was examined, initially, in 15 species of subgenera *Festucaria* and *Ceratochloa*. Subsequently, cpDNA restriction site variation was examined in 38 species, using 10 enzymes and filter hybridization experiments. Variation in the ribosomal DNA (rDNA) repeat units was examined in 56 species, using four restriction endonucleases. Generally, higher polyploid species of *Bromus* showed very little or no interspecific variation in chloroplast DNA sequences. For example, nine species of *Ceratochloa* were identical in cpDNA structure. In contrast, diploid species showed various degrees of nucleotide sequence divergence. Cladistic analysis of cpDNA restriction site variation indicated two major lines of evolution within *Bromus*. One clade includes species of *Festucaria*, *Neobromus* and *Ceratochloa* and the other comprises species of *Stenobromus* and *Bromus*. cpDNA trees indicate greater genetic relationships among the subgenera of *Bromus* when compared to results from morphology and cytogenetics. The restriction site data suggests that *Festucaria*, *Ceratochloa* and *Neobromus* are closely related, while *Stenobromus* and *Bromus* are genetically isolated from the other subgenera and appear to be evolutionarily advanced. However, the cpDNA results cannot differentiate species of subgenera *Stenobromus* and *Bromus* since species of both subgenera were interwoven in the same clade. The cpDNA results suggest that additional characters are needed to provide further information on the taxonomy and evolution of *Bromus*. Restriction analysis of rDNA produced a wide variety of patterns. Digestion with *BamHI* produced extensive length heterogeneity within and among species. *EcoRI* digests were not useful for phylogenetic analysis since the enzyme generally cleaves only once per repeat unit. *EcoRI* and *KpnI* endonuclease fragment patterns were used to

identify the number of repeat unit length variants per species. The *BstEII* and *KpnI* patterns were useful in determining relationships at the subgenus level. The unique 2.1 kb *BstEII* fragment of the coding region in subgenera *Festucaria*, *Ceratochloa*, *Neobromus* and *Stenobromus* suggests close genetic relationship among these subgenera. A similar fragment of 1.1 kb was present in subgenus *Bromus* suggesting genetic isolation from the other subgenera of *Bromus*.

# Dedication

To my wife Grace Pillay, our children Delicia, Anton and Alban and to my late parents, Mr. and Mrs. V. Dorasamy Pillay

# Acknowledgements

"How dull it is to pause, to make an end,  
to rust unburnished, not to shine in use"

Alfred Lord Tennyson

Graduate study is a challenge. It becomes an exceedingly greater challenge when in addition to fulfilling an academic task, one has simultaneously to fulfil the normal obligations of family life: of being a caring parent, a loving husband and also the family breadwinner. In order to strive for academic excellence, it becomes extremely difficult to maintain an equilibrium in the familial relationship. On numerous occasions I had to answer silently and painfully the question most often posed by my children. "Where are you going, Daddy"? I am hopeful that eventually they would realize that they were, indeed, the motivation and inspiration of my desire to attain the highest honors in academics. Therefore, my family deserves more appreciative thanks than can be inscribed here for their patience, endurance and understanding. It is with profound gratitude and appreciation that I dedicate this work to my wife, Grace Veronica and our beloved children Delicia, Anton and Alban.

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"for promotion comes neither from the east

nor from the west, nor from

the south, But God is the judge"

Psalms 75:6-7

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# *Introduction and Literature Review*

# Introduction

A phylogenetic system expresses the genealogical relationships between organisms and group of organisms (Hennig 1966). Genealogical relationships are essentially genetic relationships. The central goal of systematic and phylogenetic studies is making inferences about genetic similarities and differences from observations and measurements of characters (Giannasi and Crawford 1986). Since the systematist is concerned with genetic comparisons it would seem ideal to study the genetic material itself. The discovery of the genetic role of DNA has revolutionized studies involving the structure and evolution of genomes for improving and further elaborating the systematics and theories of biological evolution (Antonov 1986). It is known that the chemical composition and fundamental principles of organization of genetic material show the same patterns in all living matter. Therefore DNA can be used as a universal character for comparison of genomes of different organisms. Further, since genomic and organelle DNA are inherited (the former biparentally and the latter uniparentally) through evolutionary time, the material should provide the best insight into the phylogeny of the organisms containing them (Gianassi and Crawford 1986).

The molecular approach in elucidating relationships between plants is becoming increasingly more important in examining groups of organisms which are difficult to study by conventional techniques. The genus *Bromus* and the grasses, in general, are prime examples of organisms in this category.



Evolutionary and systematic studies in *Bromus* are limited and have been restricted to morphological, cytogenetical and serological aspects. Evolution in *Bromus* is based on polyploidy and hybridization within and/or between the different subgenera. This pattern of evolution has generated questions regarding the phylogenetic relatedness of the different intrageneric subdivisions of *Bromus* which remain inconclusive. Stebbins (1956, 1981) presented the most comprehensive treatment of *Bromus*. He divided the genus into seven distantly related subgenera (*Festucaria*, *Ceratochloa*, *Neobromus*, *Bromus*, *Stenobromus*, *Nevskiella* and *Boissiera*), and proposed an evolutionary scheme with information from gross morphology, chromosome size, and analyses of meiotic chromosome behavior from a few interspecific hybrids. The reliability and effectiveness of such data in resolving phylogenetic relatedness are limited by many factors. In the absence of fossils, phylogenetic relations have to be based on observations from extant forms. The principle obstacles here are numerous cases of parallel and convergent evolution of morphological characters in plants (Troitsky and Bobrova 1986), and the high degree of phenotypic plasticity (Clayton 1981). Additional problems include the difficulty of hybridizing species from the different subgenera of *Bromus*. Other limitations include problems associated with interpreting chromosome pairing in hybrids, especially those of polyploid origin, due to asynaptic or desynaptic mutations or re-diploidization of polyploids (Dewey 1982; Jackson 1984; Kaul and Murthy 1985). Hence there is need for new types of data to resolve the phylogenetic relationships among the subgenera of *Bromus*.

Molecular phylogenetic research, although not free from problems, has provided a level of quantitative resolution that is difficult to obtain by conventional methods and, most importantly, has, in a number of instances, provided new and unexpected insights into plant evolutionary relationships (Palmer 1986a). Restriction endonuclease analysis of chloroplast DNA (cpDNA) and ribosomal DNA (rDNA) has proven useful at several taxonomic levels but have been particularly valuable for elucidating relationships among congeneric species.

## *Objectives and Significance*

The focus of this research is the evolution and systematics of the different subgenera of the *Bromus* complex. The objectives of this study were: 1) to use chloroplast and ribosomal DNA restriction endonuclease data to re-evaluate the phylogenetic relationships within *Bromus*; 2) to compare previously proposed systematic relationships with those suggested from molecular data; and 3) to compare the phylogenetic information from ribosomal DNA to that of a chloroplast DNA phylogeny. The phylogenetic scheme proposed by Stebbins (1956, 1981) and shown in Fig. 1 will be used as the framework for this comparison. These objectives will be met by:

1. examining cpDNA restriction fragment analysis to assess the relationships between the subgenera *Festucaria* and *Ceratochloa*.
2. using the presence/absence of specific restriction sites in the chloroplast genome to propose a phylogeny for *Bromus*.
3. making an assessment of rDNA variation and evolution in *Bromus* in a phylogenetic sense (types of informative variation, location of this variation).

The proposed molecular approach is expected to substantiate, modify or disprove the hypothesis proposed by Stebbins (1981), and possibly lead to a different treatment of the genus. The genus *Bromus* also comprises a number of economically important forage species and understanding the phylogenetic relationships between cultivated and wild species should facilitate transfer of desirable genes from wild to cultivated species.

Studying the systematics and evolution of *Bromus* will not only lead to a better understanding of this genus, but also to a better understanding of grass systematics and evolution in general. The taxonomic and evolutionary problems in *Bromus* reflect some of the major problems characteristic

of the grass family in which hybridization and polyploidy have played a major role in their evolution. In this respect, Stebbins (1981 p 377) has stated that “as a vehicle for learning more about the evolution of grasses, the genus *Bromus* presents many fine opportunities”.

# Literature Review

## *The origin, distribution and economic importance and systematics of Bromus*

### Introduction

*Bromus* is a large genus of about 100 diploid and polyploid species that is worldwide in distribution. The genus includes important forage, range and weedy species (Gould and Shaw 1983). The name *Bromus* is derived from an ancient Greek word for food (Wagnon 1952). *Bromus* is thought to have originated in the Miocene in Eurasia (Stebbins 1981). The genus *Bromus* subsequently evolved into a large complex of species which have been grouped in several ways by taxonomists (Table 1). One treatment has divided the species into several different genera. Tsvelev (1976) treated the complex as five distinct genera: *Eubromus*, *Bromus*, *Ceratochloa*, *Neobromus*, and *Bromopsis*. Smith (1970) divided the genus *Bromus* into six sections (*Genea*, *Bromus*, *Ceratochloa*, *Neobromus*, *Nevskiella* and *Pnigma*). Stebbins (1981), taking an intermediate course, followed Smith's (1970)

treatment except that he elevated the six sections to the status of subgenera and placed *Boissiera* in a distinct subgenus instead of placing it within section *Bromus* as Smith proposed. Smith (1970) has indicated that the differences between the sections, though striking, are relatively trivial modifications of fundamentally similar structures and are much more trivial than the characters that define the genus *Bromus* as a whole. However, Stebbins (1981) contends that the subdivisions of *Bromus* are much more strongly separated and more distantly related to each other; consequently, they should be treated as subgenera. Armstrong (1987) has indicated that a treatment as subgenera or distinct genera may be more consistent with treatments of the other groups of grasses.

**Table 1. Taxonomic names applied to *Bromus* indicating sectional, subgeneric and generic treatments.**

Sections	Subgenera	Genera
(Smith 1970)	(Stebbins 1981)	(Tsvelev 1976)
<i>Pnigma</i>	<i>Festucaria</i>	<i>Bromopsis</i>
<i>Bromus</i>	<i>Bromus</i>	<i>Bromus</i>
<i>Genea</i>	<i>Stenobromus</i>	<i>Anisantha</i>
<i>Ceratochloa</i>	<i>Ceratochloa</i>	<i>Ceratochloa</i>
<i>Neobromus</i>	<i>Neobromus</i>	<i>Neobromus*</i>
<i>Nevskiella</i>	<i>Nevskiella</i>	<i>Nevskiella</i>
<i>Bromus**</i>	<i>Boissiera</i>	<i>Boissiera</i>

\* Not found in the USSR

\*\* Included in the section *Bromus* as *B. pumilio*

## Origin of the genus *Bromus*

*Bromus* is considered to occupy an intermediate position between the tribes *Festuceae* and *Triticeae* (*Hordeae*) (Armstrong 1987). It is hypothesized that *Bromus* originated in the Miocene at the time when the *Festuceae* and *Triticeae* were being differentiated from each other (Stebbins 1981). The genus probably arose in Eurasia since the greatest diversity among the diploid species of the genus occurs in western Eurasia. The original ancestors of *Bromus* are extinct, probably because of profound changes in both climate and biota during the Pliocene and Pleistocene. During these epochs, Central Asia experienced elevation of the mountains ranges and plateaus and acquired a continental climate with extreme seasonal fluctuations of temperature (Stebbins 1981). Heavy grazing also exerted strong selection pressure on the original species. Consequently species like the rhizomatous *B. inermis* complex and the annuals belonging to subgenus *Bromus* and *Stenobromus* were favored since they were able to coexist with the herbivores. The combination of these factors probably eliminated the original *Bromus* species (Armstrong 1987). Stebbins (1981) provides the following history for the genus *Bromus*. The primitive species of *Bromus* originated in the Miocene. During the Pliocene, differentiation of the subgenera *Neobromus*, *Ceratochloa*, and *Festucaria* occurred. *Neobromus* and *Ceratochloa* spread to North and finally to South America. The diploid and tetraploid species of these subgenera finally became extinct in Eurasia. The subgenus *Festucaria* spread initially to Africa and then to North America. During the Pleistocene, the differentiation and spread of the subgenera *Bromus* and *Stenobromus* occurred. The building up of the polyploids in *Festucaria* occurred in the Old World and formation of octoploids in *Ceratochloa* took place in the New World.

## Distribution and economic importance of *Bromus*

*Bromus* is most diversified in Europe, but many species have also been described from Asia, Africa, and North and South America (Soderstrom and Beaman 1968 ). Generally, the genus is most widely distributed in the temperate and mountainous regions of the world. In the tropics, the species are mainly restricted to the higher mountains. Some of the species are also distributed in arctic regions (Shear 1900). The seven subgenera of *Bromus* comprise approximately 130 recognized species (Smith 1970). *Stenobromus* contains 10 and subgenus *Bromus* 40 annual or biennial species. The center of diversity of these two sections is in the Mediterranean area. Both subgenera are also widespread in Eurasia and a number of species has been introduced either naturally or by humans into the New World, Africa and Australia (Smith 1972; Kahler et al. 1981). They are considered the most advanced evolutionarily (Armstrong 1987). The two subgenera *Neobromus* and *Nevskiella* are monotypic and are found in North America and Central Asia, respectively. Subgenus *Ceratochloa* consists of 17 recognized species found in North and South America. Subgenus *Festucaria* is composed of approximately 60 recognized species which range from short-lived to long-lived perennials. This is the largest section and is distributed widely from western to eastern Eurasia and North and South America.

Several species of *Bromus* are of economic importance. The most important is *B. inermis* which is an important component of the forage throughout the United States corn belt and in western and central Canada (Molnar 1988). Other species of considerable forage value include *B. marginatus*, *B. carinatus*, and *B. catharticus* (Stebbins and Tobgy 1944). A number of other species are considered to be useful as forage in the early vegetative stages but are serious pests in rangelands after flowering (Crampton 1974). Some of the brome grasses have comparatively large and nutritious seeds, which makes them valuable for fattening stock and for hay. Finally, *Bromus* is related to other economically important grasses such as wheat, oats, barley and rye.



## Cross compatibilities and genomic relationships between the subgenera of *Bromus*.

The basic chromosome number of the genus is  $x = 7$ , upon which several polyploid series have been built (Stebbins and Crampton, 1960). The genomic relationships between the subgenera of *Bromus* (Fig. 1) have been deduced from chromosome pairing behavior in a few interspecific hybrids. No extensive attempt has been made to intercross species from the different subgenera of *Bromus* (Armstrong 1987). Intersubgeneric hybrids have been produced between species of *Ceratochloa* and *Neobromus* (Stebbins and Walters 1949), *Ceratochloa* and *Festucaria* (Stebbins 1947), *Ceratochloa* and *Bromus* (Knowles 1944), *Ceratochloa* and *Stenobromus* (Walters 1958), *Bromus* and *Stenobromus* (Knowles 1944) and *Bromus* and *Festucaria* (Armstrong 1977). These studies showed that there was no pairing between chromosomes coming from the different subgenera. This suggests that there are generally strong isolation barriers between the subgenera. Since these crosses involved species that differed considerably in chromosome size and DNA content which probably affected chromosome pairing (Armstrong 1987). However, it is probable that qualitative changes are also involved in genomic differentiation (Armstrong 1987).

Subgenus *Festucaria* includes both diploid and polyploid species. The diploid ( $2n = 14$ ) and tetraploid ( $2n = 28$ ) species are distributed primarily in the Americas while species with higher ploidy levels such as hexaploids, octoploids and decaploids have a Eurasian distribution (Armstrong 1981). Stebbins (1956) and Armstrong (1987) proposed a monophyletic origin for the *Festucaria* species, all of which contain different numbers of the L genome.

Subgenus *Ceratochloa* contains hexaploid and octoploid species, while diploid and tetraploid species are thought to be extinct. The *Ceratochloa* species fall into two morphologically distinct species complexes: the *B. catharticus* complex, which comprises hexaploids ( $2n = 42$ ) endemic to South America, and the *B. carinatus* complex, which are octoploids ( $2n = 56$ ) found mainly in North America (Stebbins 1981). The two complexes of subgenus *Ceratochloa* are assumed to combine

three ( $A_1B_1B_2$ ) or four ( $A_1B_1B_2L$ ) genomes (Stebbins, 1981). The hexaploid *B. catharticus* complex is considered to have evolved from presently extinct diploid and tetraploid species that contributed the  $A_1$ ,  $B_1$ , and  $B_2$  genomes. The *B. carinatus* complex is thought to have originated from hybridization between members of the hexaploid *B. catharticus* complex and diploid species of *Festucaria* (Stebbins and Tobgy 1944; Stebbins 1947, 1981). This species complex contains 42 small and 14 large chromosomes and was given the genomic formula  $A_1B_1B_2L$  (Stebbins and Tobgy 1944; Stebbins 1956). Cytogenetic studies have shown that the 14 large chromosomes were derived from diploid North American members of subgenus *Festucaria* (Stebbins and Tobgy 1944; Stebbins 1947). Stebbins and Tobgy (1944) suggested that different species of the diploid *Festucaria* have entered into the ancestry of the *B. carinatus* complex, favoring a partly polyphyletic origin for the group.

The genomic relationships proposed by Stebbins (1956, 1981) imply that subgenera *Festucaria* and *Ceratochloa* have different ancestry. Stebbins (1981) indicated that the relationships between the two subgenera are obscure, and their chromosomes so dissimilar, that a recent common origin is difficult to imagine. However, the smaller chromosomes found in polyploid *Festucaria* are similar in size to those found in the *Ceratochloa* and *Neobromus*. *Neobromus* is presumed to be distantly related to *Ceratochloa* since only one of their three genomes is reported to have a weak affinity to those of *Ceratochloa*. Cytogenetic studies also led Stebbins (1981) to conclude that the relationship between subgenera *Festucaria*, *Ceratochloa* and *Neobromus* is obscure and it is unlikely that they had a recent common origin. Since diploid and tetraploid species of *Ceratochloa* and *Neobromus* are extinct, tracing the direct origin of these subgenera is impossible. Stebbins (1981) has suggested that a thorough investigation of two genera, *Littledalea* and *Megalachne*, may provide clues to the origin of the *Ceratochloa*. With regard to the origin of *Neobromus*, three genera have been postulated to be related to the possible ancestor of the subgenus : *Pseudodanthonia*, *Metcalfia*, and *Sinochasea*. Subgenera *Bromus* and *Stenobromus* are thought to be derived from primitive members of *Festucaria* (Stebbins 1981). However, certain differences in morphological characteristics has led Stebbins (1981) to suggest that each subgenus originated from a different primitive species of

*Festucaria*. The genetic affinity between subgenera *Bromus* and *Stenobromus* and the other subgenera is unknown. Stebbins (1981) indicated that none of the contemporary subgenera of *Bromus* is either ancestral to the rest of the genus or even similar to the generalized species that were its actual ancestors.

In summary the relationships between the different subgenera of *Bromus* may either be described as distant, obscure or unknown.

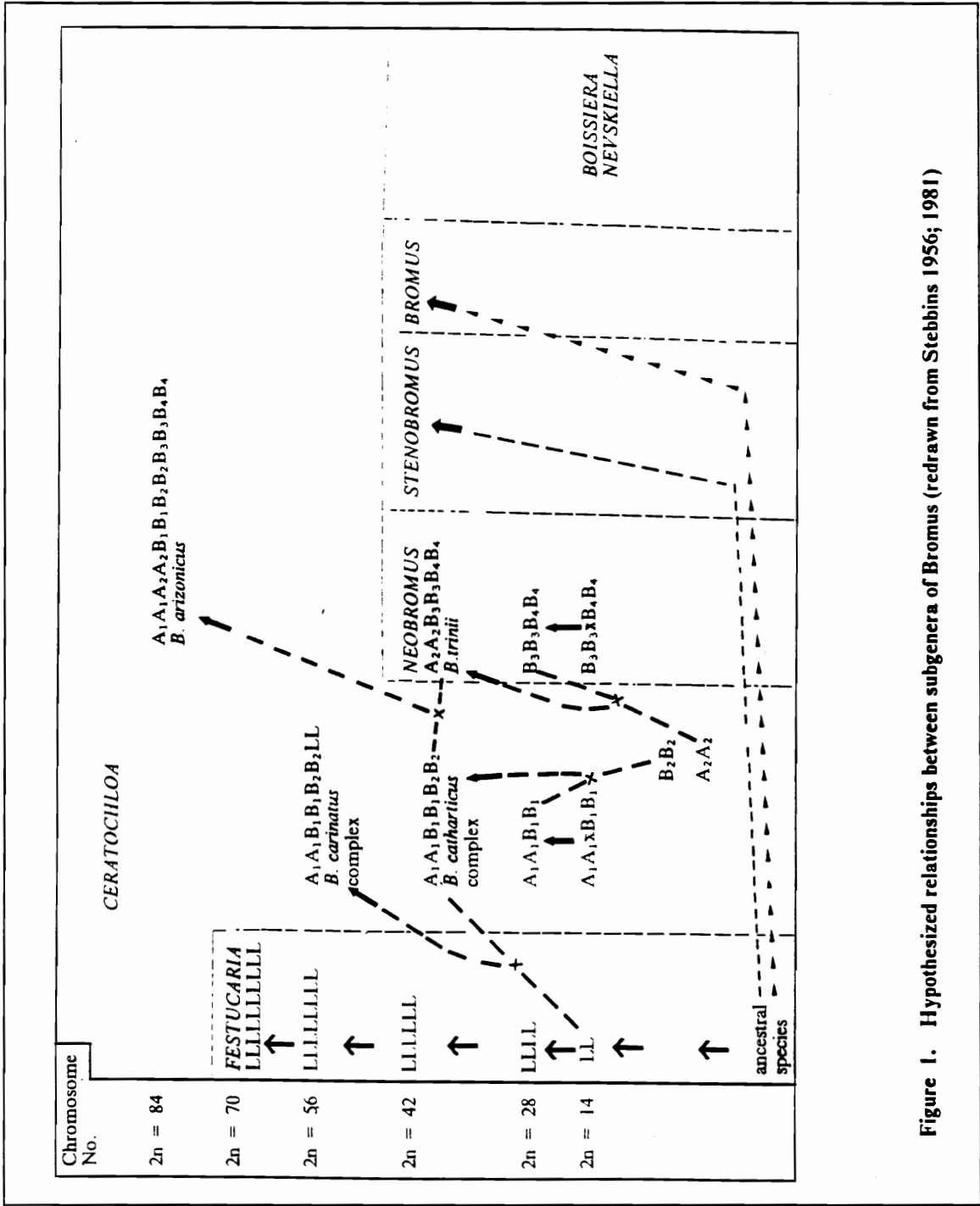


Figure 1. Hypothesized relationships between subgenera of Bromus (redrawn from Stebbins 1956; 1981)

# *Structure, evolution and the use of chloroplast DNA in systematics*

## **Introduction**

Chloroplasts are complex organelles capable of performing a multi-faceted physiological role in the plant cell. Convincing physical evidence for the presence of DNA in chloroplasts was confirmed in the early 1960s (Ris and Plaut 1962; Chun et al. 1963). Molecular techniques have now provided a wealth of information on the organization, gene content and gene structure of organellar genomes. These techniques have proved to be so powerful that the entire DNA sequencing of the chloroplast genomes of some plants has been completed. As detailed information accumulated 1) on restriction maps of chloroplast genomes in different species; 2) on the structure of specific chloroplast genes and 3) on the arrangement of these genes within the genome, it became possible to deduce evolutionary features of the DNA and exploit the organellar DNA variation in a phylogenetic sense.

## **General structure and origin of chloroplast DNA**

The structure of a typical chloroplast genome is shown in Fig. 2 which represents the physical and gene map of the tobacco cpDNA. The genome consists of a region present twice, each in opposite orientation known as the inverted repeat (IR) and approximately 25 kb long. The two copies of the IR are separated by a large single copy region which is about 80 kb long and a small single copy region about 20 kb in length. The typical chloroplast genome of land plants is densely packed with over 140 genes some of which are shown in Fig. 2. Chloroplast gene products function primarily

in photosynthesis and in transcription-translation. Both strands of the chloroplast genome are actively expressed. Many chloroplast genes are grouped functionally into polycistronic operons, such as those containing the four ribosomal genes in the inverted repeat.

The complete nucleotide sequences of chloroplast genomes are now available for *Marchantia polymorpha* (121,024 bp, Ohyama et al. 1986), for *Nicotiana tabacum* (155,844 bp, Shinozaki et al. 1986) and for rice (134,525 bp, Hiratsuka et al. 1989). Although these plants are taxonomically very distant from one another, their deduced chloroplast gene organization is remarkably similar (Ozeki et al. 1989). Other studies involving physical mapping, cloning and sequencing of chloroplast genes from a variety of higher plants show that there is a basic similarity in all chloroplast genomes. This implies that the chloroplast genomes in all land plants may have arisen from a unique ancestor. The endosymbiotic theory, which proposes that chloroplasts were derived from an ancestral photosynthetic prokaryote related to cyanobacteria, is currently the more favored hypothesis on the question of chloroplast origin. This event, which is thought to have been established 300-400 million years ago, is regarded as the single origin from which present day chloroplast genomes in plants have evolved, mainly by mutations in nucleotides and rearrangement of DNA, but rarely by changes in gene content (Ozeki et al. 1989).

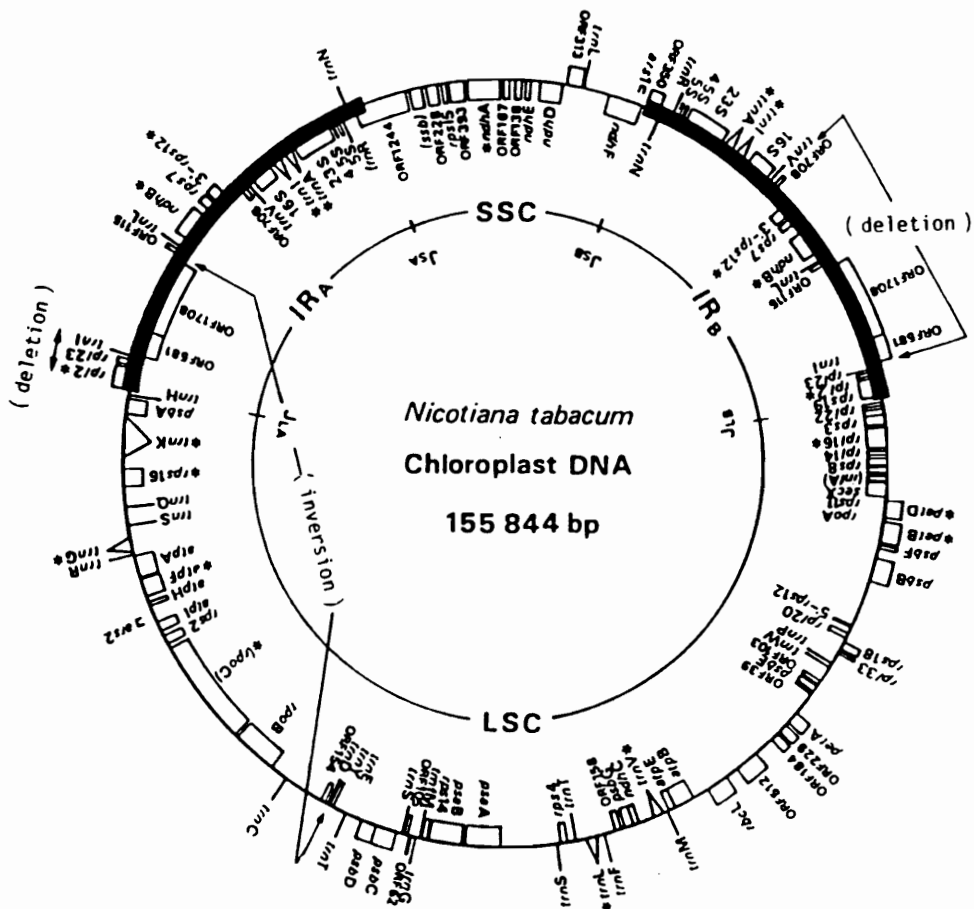


Figure 2. Physical and gene map of tobacco chloroplast DNA.: The large and small single copy regions are denoted as LSC and SSC. The inverted repeat regions are marked IR<sub>A</sub> and IR<sub>B</sub>. The gene symbols refer to Shinozaki et al. 1986. The sites of major inversions and deletions are indicated. (Map obtained from Ozeki et al. 1989)

## **Physico-chemical properties and structural organization of cpDNA**

Higher plant chloroplast DNAs have an average buoyant density of 1.697 g. cm<sup>3</sup> (Kung 1977; Bohnert et al. 1982; Kirk 1971). This constant density of higher plant cpDNA indicates that higher plants have a similar DNA base composition in the region of 37-38 % GC content (Kung 1977). This also suggests that the base composition of the cpDNA of higher plants is highly conserved. The IR regions are relatively G + C rich whereas the unique sequences are A + T rich (Herrmann and Possingham 1980).

One of the unique characteristics of cpDNA is the lack of modified bases such as methyl cytosine (Kirk 1976). This contrasts sharply with nuclear DNA in which about 25% of cytosines may be methylated (von Kalm et al. 1986). Cytosine methylation blocks the action of many but not all restriction enzymes (McClelland and Nelson 1985). The non-methylation of cpDNA has been used advantageously to visualize cpDNA present in total cellular DNA. Digestion of total cellular DNA with a methylation sensitive enzyme will produce more or less complete fractionation of cpDNA sequences and help to separate them from nuclear DNA sequences (Palmer 1986b).

Another unique property of cpDNA is the ease with which it can be renatured (Tewari and Wildman 1966; Kung and Williams 1969; ). This property of renaturation indicates the degree of simplicity and homogeneity of cpDNA, which is a much less complicated molecule than nuclear DNA, in both structural complexity and genetic content (Kung 1977).

Since cpDNA is present in many copies per cell, the unique sequence of the chloroplast genome is therefore amplified many times. This might represent a unique way of organelle gene amplification. In higher plants, cpDNA has no repeated sequences but exists in many copies. Kung (1977) therefore considers higher plant cpDNA to be polyploid and not redundant. Genetically, polyploidy is considered a conservative force in evolution.



## **Genome size and conformation**

Chloroplast DNA from most angiosperms is rather uniform in size and conformation. Although the total extent of size variation observed in angiosperms is 120-217 kb, in the great majority of plants examined cpDNA varies in size over a narrow range of 135-160 kb (Palmer 1985a, 1985b). The great variation in size is due to the presence or absence of the large IR sequence. Chloroplast DNA of higher plants is a double stranded circular molecule. The various types of plastids within a single plant such as chloroplasts, chromoplasts, etioplasts, proplastids, amyloplasts and elaioplasts are known to have an identical structure and sequence arrangement of their genomes (Palmer 1985a). Therefore the multiple circular chloroplast genomes of a single plant cell are homogeneous and indistinguishable from one another in all respects. The only exception is the presence of two genetically identical but physically distinct inversion isomers. A small proportion of these molecules exists as unicircular dimers or two interlocked monomers (Kolodner and Tewari 1979; Herrmann and Possingham 1980; Edelman 1981). Recent studies in spinach also show that the chloroplast genome exists in monomer, dimer, trimer, and tetramer forms (Deng et al. 1989). More recently, moving pictures and pulsed-field gel electrophoresis show linear DNA molecules in chloroplasts of pumpkin (Bendich and Smith 1990).

## **Repeated Sequences in chloroplast genomes**

Chloroplast chromosomes have a few repeated sequences. The most prominent of these is the large (10-76 kb) inverted duplication found in cpDNAs of most land plants. Smaller repeats of 200-1000 bp also occur in some plants. Although the IR can vary from 10 to 76 kb among angiosperms, in a great majority of species, it is rather constant being 22-26 kb in size. The IR regions of land plants also have several other features in common. These include their asymmetric positions (dividing the genome into small and large single copy regions of 12-28 kb and 80-100 kb), their locations relative

to the psbA genes and direction of transcription of the rRNA genes that are always towards the SSC. Three important genetic properties are associated with the inverted repeat region of chloroplast genomes. The first is concerted evolution which involves a gene conversion/copy-correction mechanism that maintains the sequence identity between the two repeat elements in a given genome. Concerted evolution occurs when members of a multigene family are more similar to each other than expected had they evolved independently from the time of the initial event that gave rise to the multigene family (Zimmer et al. 1988; Arnheim 1983). Therefore the two repeats are always found to be identical within an individual plant. All naturally occurring as well as induced mutations within the inverted repeat occur symmetrically in both segments. The IR is presumed to be responsible for intermolecular recombination within the inverted repeat. This results in the formation of head to head circular dimers found, for example, in spinach and lettuce, both of which contain the IR. Such structural features of the cpDNA are not present in plants such as the pea which lack the inverted repeat region (Kolodner and Tewari 1979). Intramolecular recombination between IRs is inferred from the observation that cpDNAs exist as an equimolar population of two molecules that differ only in the orientation of the single copy regions. The inverted repeat is absent in a single group of legumes in which one segment of the repeat is deleted. The loss of the repeat has consequently led to a reduction in genome size in this group to 120 kb, approximately 15 kb smaller than in the smallest inverted repeat-containing angiosperm. The inverted repeat is thought to increase the evolutionary stability of the chromosome (Palmer 1985a). The benefits, however, appear to be subtle, in as much as pea, broad bean, and the pine chloroplast DNAs are exceptions to this pattern and lack IRs (Koller and Delius 1980; Palmer and Thompson 1981; Strauss et al. 1988; Lidholm et al. 1988).

## **Cytological organization of chloroplast DNA**

Chloroplast DNA molecules are attached to fragments of membranes or proteinaceous structures following gentle lysis of the organelle (Birky 1988). Higher plants almost always contain multiple

copies of the plastid genome in each plastid (Possingham and Lawrence 1983). Plastid genomes are arranged into discrete structures within the plastid called nucleoids, each of which may contain between 1 to 25 copies of the genome (Possingham and Lawrence 1983). The number of copies of cpDNA per plastid can vary from very few to 200, with the normal range being around 20 to 80 molecules per plastid (Herrmann and Possingham 1980). This number can be influenced by physiological and developmental conditions. For example, during differentiation of proplastids into plastids in beet, there is a 25-fold increase in plastid volume that is accompanied by an increase from 10 to over 100 cpDNA molecules per plastid (Herrmann et al. 1974). In pea, Lamppa and Bendich (1979) estimated an average of 244 cpDNA molecules per chloroplast in young leaves, but 174 in fully green leaves. Although there is a decrease in cpDNA molecules per plastid, there is an increase in the number of cpDNA molecules per cell since the average number of plastids per cell increases from 24 to 64 during greening (Lamppa et al. 1980). A change in ploidy of plastid DNA and nucleoid number has also been reported in spinach. Scott and Possingham (1980) found that the approximately 20 chloroplasts from young leaves each had about 200 DNA molecules. In contrast, the approximately 150 chloroplasts from older leaves each contained about 30 DNA molecules in 10-15 nucleoids. This suggests that the replication of cpDNA takes place in young dividing, rather than in expanding, leaf cells in spinach. This phenomenon has also been reported in wheat (Boffey et al. 1979). The amount of cpDNA does not change much during the later stages of cellular development but remains rather constant per cell. Some studies (Butterfas 1973; 1983) have suggested that ploidy level and hence nuclear DNA content determines chloroplast number. Contrary to this hypothesis, Lamppa et al. (1980) found that an increase in chloroplast number in pea leaves is not paralleled by an increase in the amount of DNA per nucleus. Support for this statement was provided by Pyke and Leech (1987) who found that chloroplast number is closely related to cell size, irrespective of ploidy level in wheat mesophyll cells.

## The evolution of chloroplast DNA

The cpDNA of land plants has evolved very slowly in terms of size, gene content and nucleotide sequences. The chloroplast chromosomes vary from 120 to 217 kb, with most of the variation resulting from either an expansion or contraction of the inverted repeat so that the average size is only 110-150 kb. The number and location of chloroplast genes are also highly conserved. There are only two known differences among the 120 genes present in the cpDNAs of tobacco and *Marchantia*, which diverged about 400 million years ago (Palmer et al. 1988). The gene order found in tobacco is also found in most angiosperms and in at least one fern and one gymnosperm. It differs from *Marchantia* by a single inversion. Most of the changes in gene order found in a majority of cpDNAs of angiosperms are due to one or two simple inversions. The only known exceptions occur in some legumes and *Pelargonium*. The chloroplast genome is evolving quite slowly at the nucleotide sequence level (Palmer and Thompson 1982; Palmer and Zamir 1982; Shinozaki and Sugaira 1982; Palmer et al. 1983; Shinozaki et al. 1983; Whitfeld and Bottomley 1983; Gillham et al. 1985; Palmer 1985a, 1985b). The rate of silent substitutions in chloroplast genes is two or three times lower than in nuclear genes but three to four times higher than in plant mitochondrial genes. Transitions outnumber transversions in chloroplast genes relative to random expectations. This probably accounts for the low incidence of parallel and convergent restriction site mutations found at the interspecific level. Different portions of the chloroplast genome are known to evolve at different rates providing a range of characters which could be used to measure evolutionary distances and determine relationships.

## Length mutations, inversions and rearrangements

The processes of addition and deletion also play a role in chloroplast genome evolution. Most cpDNA length mutations in both coding and noncoding regions are small additions and deletions

of 1-10 bp in size. Larger length mutations of 50 to 1200 bp are commonly detected in restriction fragment analysis and are found to cluster in "hotspots" usually at the ends of the large single copy region. The ratio of length mutations to base substitutions in cpDNA approximates that in other genomes. Since the rate of cpDNA base substitutions is extremely low compared to other genomes, it is expected that length mutations also occur at a very low rate in cpDNA.

Inversions and other mutations that change the relative order of genes are extremely rare in cpDNA. Occasionally, a single inversion can change the gene order. For example, a large 50 kb inversion is found in all species from 12 genera and six tribes of the subfamily Papilionoideae of the Fabaceae (Palmer and Thompson 1982; Palmer et al. 1983; Spielmann et al. 1983). Other documented cases of large inversions in the chloroplast genome that feature the inverted repeat include the following: a 20 kb inversion within the large single copy region and characteristic of three genera of the Poaceae (Howe et al. 1983; Poulsen 1983; de Heij et al. 1983); a 30 kb inversion also located within the large single copy and characteristic of six tribes of the Asteraceae (Palmer et al. 1988). In a few cases, as in geranium and the pea, more extensive rearrangements are found (Palmer et al. 1988).

## **Constraints on the evolution of the chloroplast genome**

### ***Recombination of plastome DNAs***

Mutation is the ultimate source of genetic variability in all living organisms (Gillham et al. 1985). Recombination and independent assortment in meiosis are responsible for propagating new combinations of nuclear gene mutations. In plants with biparental inheritance of plastids, plastid fusion may be rare preventing recombination of mutations which have occurred in the genomes of different plastids. Several conditions must be met in order for recombination to occur between plastid DNAs: genetically different plastids must be introduced into the same cell, plastid fusion must occur and chloroplast DNAs must subsequently recombine (Sears 1983). Since chloroplasts

are inherited maternally in about two thirds of the plants thus far investigated, the opportunity for recombination and assortment does not arise (Gillham 1978; Kirk and Tilney-Bassett 1978; Sears 1980). In somatic cell hybrids having two different plastid types, no plastid recombinants have been observed. Only one parental chloroplast type or the other has been observed in plants derived from interspecific fusion products of *Nicotiana* (Chen et al. 1977; Maliga et al. 1982) and *Lycopersicon* (Schiller et al. 1982). In the *Nicotiana* fusion products, only one plant contained both parental cpDNAs, but rather than having mixed cells, this plant was a chimera (Uchimiya and Wildman 1979). Currently, recombinant chloroplast DNAs have not been observed in higher plants, enhancing the taxonomic value of the cpDNA molecule.

### ***Intermolecular recombination***

Chloroplast markers and chloroplast DNAs are known to recombine in the unicellular green alga *Chlamydomonas* (Gillham 1978; Lemieux et al. 1980). The chloroplast genetic system of *Chlamydomonas* is similar to those of higher plants in general physical size, gene content and organization. Therefore, it is conceivable that intermolecular recombination can occur in higher plant chloroplast DNAs. Recent observations have shown that intramolecular recombination can occur between the inverted repeats (Bohnert and Loffelhardt 1982; Palmer 1983). This suggests that the enzymes and mechanisms for recombination between chloroplast DNAs do exist. Thus, it is not known whether the failure to detect recombination is due to the absence of suitable experimental methods or to the failure of the plastids to fuse and exchange molecules.

### ***Transposable elements***

Transposable elements are known to cause mutations, deletions and inversions (Calos and Miller 1980). They also cause structural rearrangements such as co-integration of two circles into one, and the resolution of one circle into two (Birky 1988). Thus far, transposable elements have not been

identified in the plastome and their absence may contribute to the conservative nature of the chloroplast DNA (Sears 1983).

### ***Mutations of the chloroplast***

Several inherent traits of the genetic system of the chloroplast make the establishment of mutations difficult. The most important is the high degree of polyploidy. Most plant cells contain more than twenty chloroplasts and each chloroplast contains many copies of the chloroplast genomes. Expression and establishment of mutations will require a very long time. Even if a mutation becomes fixed, it may not be inherited if the cells are not part of the germ line (Sears 1983).

## **Methods of cpDNA comparison**

### ***Restriction fragment and restriction site analysis***

Three approaches are being used for measuring cpDNA variation in a phylogenetic context. The first technique is analysis of restriction endonuclease fragment variation. Restriction endonucleases are capable of cleaving DNA at specific sites. The target of most restriction endonucleases is usually a short sequence of DNA that is 4-6 bp long. The number of times a certain enzyme will cleave a DNA molecule depends on how many times the particular short sequence occurs. Certain of these short sequences with the specific base pairs are more common than others in cpDNAs so that the number of fragments obtained depends on the enzyme employed. Digestion of DNA from different taxa with various enzymes enables one to probe for sequence similarities and differences among taxa. If exactly the same sites for a particular enzyme occur in the DNA of different species, digestion will yield identical fragments. If differing fragments are obtained, it is assumed that a base substitution has occurred at a site resulting in either a gain or loss of restriction sites. Length

mutations are also frequently encountered in digestions of cpDNA and are detected as size differences among fragments without any concomitant change in fragment number.

Two methods are used to observe DNA fragment patterns after digestion with restriction enzymes and electrophoretic separation in agarose or polyacrylamide gels. Highly purified cpDNA that usually produces distinct fragment patterns on gels can be visualized by staining the gel in ethidium bromide and photographing under ultraviolet illumination. Another method involves transferring the DNA to a filter by Southern blotting and hybridizing the membrane with total cpDNA purified from one of the taxa under study (Palmer et al. 1985). This method is useful especially in cases where purified cpDNA is too limited to be visible by conventional ethidium bromide staining. Another advantage of this method is that it enables one to visualize cpDNA fragments from total cellular DNA preparations.

The DNA fragments migrate to specific places in the gel based on their size (length) and it is possible to determine the size of a fragment if known molecular weight markers are included in the same gel. Within this situation it is often possible to infer mutations by which two species differ by inspection. A particular fragment in one species may be replaced in a second species by two smaller fragments. If the combined sizes of the two smaller fragments are equal to the large fragment, it means that a mutation has occurred. The creation of a new restriction site will suggest that the change is from the larger to the smaller fragments while the loss of a site is indicative of a change in the opposite direction. The enzymes most suitable for comparisons of cpDNA are those that recognize GC-rich sequences and are 6 base cutters. These rare cleaving enzymes produce 10-30 fragments from the average 150 kb, AT-rich chloroplast genome. The fragment method applies best to situations of very limited divergence with few accumulated mutations whereby interpretation of fragmentation patterns can usually be done by inspection and addition/subtraction of fragment sizes (Palmer and Zamir 1982). However, since cpDNA evolves slowly, this approach has been useful at all levels of intra-specific variation and many levels of intrageneric variation.



When a large number of mutations has occurred, it is necessary to take additional steps, such as mapping of fragments using filter hybridization to establish the identities of the complex fragment patterns. In the construction of restriction site cleavage maps, cloned DNA fragments from one chloroplast genome are used to probe membranes onto which the restriction digest of a single enzyme of a number of DNAs has been blotted. Sequential probing of reusable filters with all of the cloned fragments of a reference genome can be used to produce a complete circular map of the chloroplast genome. Restriction site mapping has several advantages over the fragment method. Firstly, it allows comparison of more divergent cpDNAs that are difficult to analyze by inspection of fragment patterns. A single cpDNA probe will hybridize to a few fragments to enable sorting of the fragment variation in terms of discrete mutations. Another advantage of mapping is that it allows one to discriminate between restriction site mutations and length mutations.

### *DNA sequencing*

DNA sequencing allows one to compare the bases of a defined portion of a genome. This method of DNA analysis results in lower levels of parallelism and convergence (homoplasmy) than site mapping, where changes at any of six positions can cause a site loss (Palmer et al. 1988). Sequencing also overcomes the problem of excessive length mutations that make the alignment of restriction-site maps rather difficult. By sequencing a gene, one avoids the problem of length mutations and also gains resolution at a greater phylogenetic distance, since most genes are more conserved than the genome as a whole (Palmer et al. 1988).

Although few major studies of chloroplast gene sequences and phylogenetic relationships have been published (Doebley et al. 1990; Gianassi et al. 1990), the chloroplast gene *rbcL*, encoding the large subunit of ribulose-1,5-bisphosphate carboxylase, has emerged as the preferred gene for examining higher level phylogenetic relationships (Soltis et al. 1990). Sequencing is the most labor intensive, time consuming and expensive of the three methods of cpDNA comparison (Palmer 1986a). Consequently, sequencing seems to be more appropriate at higher taxonomic levels such as the

family and above where restriction site analysis is inadequate for phylogenetic inference, owing to excessive homoplasy and length mutations (Palmer et al. 1988).

## **Sequence evolution of chloroplast DNA**

Nucleotide substitutions occur at a very slow rate in cpDNA. The most detailed study of the pattern and dynamics of nucleotide substitutions of the chloroplast genome is provided by Zurawski et al. (1984) and Zurawski and Clegg (1984), who compared the *rbcL*, *atpB*, and *atpE* genes and flanking regions in barley and maize. In the case of synonymous substitutions, an estimate of per site, per year of nucleotide substitution is approximately  $1 \times 10^{-9}$ . This compares with estimates of  $2-3 \times 10^{-9}$  synonymous substitutions/site /year in animal nuclear genes (Kimura, 1983) and  $5 \times 10^{-9}$  substitutions/site/year for pseudogenes (Li 1983). These rates are consistent with the conservative rate of evolution of the chloroplast genes. An interesting evolutionary feature of the chloroplast genome is that both protein-coding and noncoding regions evolve at the same rate (Clegg et al. 1986).

## **Phylogenetic utility of chloroplast DNA in comparison with other genomes**

Several properties make chloroplast DNA molecules especially well-suited as a useful tool in phylogenetic and evolutionary studies. First, cpDNA is abundant in a typical leaf cell (Whitfield and Bottomley 1983; Possingham and Lawrence 1983) making it relatively easy to isolate cpDNA in pure form in many plants (Herrmann 1982; Palmer 1986a). In contrast, mtDNA is much less abundant in plants and its isolation and purification is often quite difficult. Although plant nuclear DNA is easily isolated, it is much larger than organelle genomes, making it relatively difficult to isolate and clone specific genes. An exception in this regard are the ribosomal DNA genes that are generally present in thousands of very similar or identical copies making them the easiest plant

sequences to isolate and compare (Palmer 1987). Second, small size of the chloroplast genome makes it possible to compare the entire pattern of restriction fragments produced by many restriction enzymes on a single agarose gel. When these DNA fragments are transferred to a membrane, restriction site maps could be constructed generating data for phylogenetic analysis. The small size of the genome also permits the identification and isolation of genes for sequencing studies. Third, many evolutionary processes such as gene duplication and deletion, widespread concerted evolution and pseudogene formation, which are very common in nuclear genes, are not associated with chloroplast DNA. The large IR repeat is the only significant repeat family in the chloroplast genome. Although the inverted repeat does undergo concerted evolution, it is very rapid and no sequence differences have been observed at the interspecific level (Palmer 1986). Chloroplast DNA evolves primarily by point mutations and is easily studied by either restriction-enzyme techniques or DNA sequencing. Fourth, the slow rate of cpDNA evolution and the rare genomic rearrangements mean that cpDNA molecules can be compared easily by the analysis of restriction fragments. Finally, restriction fragment mapping of cpDNA provides data that are divisible into multiple character states, which can be easily coded into a series of single evolutionary steps and are scorable for all taxa (Schilling and Jansen 1989).

## **Applications of chloroplast DNA in systematic studies**

Chloroplast DNA data have been used at various levels in systematics ranging from intraspecific to higher order relationships. However, most studies involving cpDNA comparisons have focused on closely related plants, usually congeneric species (Palmer et al. 1988). There are many excellent reviews (Palmer 1985a, 1985b, 1986a, 1986b, 1987; Palmer et al. 1988; Gianassi and Crawford 1986) on the utility of cpDNA in systematics. Consequently, in this review, a synopsis of some studies at the different levels of taxonomy will be considered.

### ***Intraspecific relationships***

The very low rate of structural and sequence level changes limits the utility of cpDNA as a source of variation for assessing intraspecific relationships (Palmer 1987). Relatively little cpDNA variation was observed in two extensive intraspecific studies (Wagner et al. 1987). For example, in *Lupinus texensis*, Banks and Birky (1985) found only two restriction site mutations and one deletion in 100 plants representing three populations. Other studies have shown that species from the same population usually have identical chloroplast genomes. In spite of these limitations, intraspecific differences have been quite important, especially regarding the origins of several crop plants (Palmer et al. 1983; Clegg et al. 1984; Doebley et al. 1987). Although intraspecific variation of cpDNA is limited, it is believed that by sampling a greater percentage of the nucleotides, many micro-evolutionary questions could be addressed (Palmer et al. 1988). This could be achieved by using more restriction enzymes, especially ones that cut frequently, accompanied by utilizing electrophoretic techniques, such as polyacrylamide gels to resolve the fragments and hybridizing blots made from these gels repeatedly with cloned chloroplast DNA fragments. It is hoped that, in the long term, development of technical strategies will allow the accumulation of sufficient numbers of cpDNA mutations to make the molecule useful below the species level.

### ***Interspecific relationships***

In recent years, there has been an explosion in research on cpDNA restriction site variation to assess phylogenetic relationships at the interspecific level. The availability of cloned cpDNA fragments and modified techniques, such as the rapid isolation of total DNA from a small quantity of plant material, have enabled an increasing number of laboratories to engage in such research. The most important feature of cpDNA data compared to most data from morphology and chemistry is the low levels of homoplasy, which ranged from 0% in *Pisum* (Palmer et al. 1985), to 2.5% in *Lycopersicon-Solanum* (Palmer and Zamir 1982), 3.3% in *Zea* (Doebley et al. 1987), 3.8% in

*Brassica* (Palmer et al. 1983), 4.8% in *Clarkia* section *Peripetasma* (Sytsma and Gottlieb 1986), 4.9% in *Cucumis* (Perl-Treves and Galun 1985). The average level of homoplasy in these studies is 3.9%. While many of the cpDNA studies have provided results that are concordant with those derived by other taxonomic approaches, a number of new and unexpected findings was also obtained. The studies of both Erickson et al. (1983) and Palmer et al. (1983) in *Brassica* may be cited as an example. These studies provided considerable insight into *Brassica* evolution and the two groups of investigators reached very similar conclusions by applying techniques of comparative restriction analysis. Besides deducing the parentages of amphidiploid species, the cpDNA data enabled both groups to make conclusions about the timing of the hybridization events.

### ***Familial relationships***

Chloroplast DNA has now been used to address systematics questions in the Asteraceae, Orchidaceae, Fabaceae, Solanaceae and the Poaceae (Palmer et al. 1988). The approaches involved at this level include an assessment of genome rearrangements, comparative restriction site mapping and a comparative study of *rbcL* sequences. For example, in the Fabaceae, the three subfamilies are characterized by having legume specific markers such as a 50 kb inversion and the loss of the *rpl22* gene (gene for ribosomal protein) from the chloroplast genome. In the Poaceae, the different subfamilies are characterized by cpDNA inversions. These inversions are lacking in four other monocot families that have been mapped (Palmer et al. 1988). Wider sampling of the Poaceae is needed to ascertain whether these inversions represent a phyletic marker for the family (Giannasi and Crawford 1986).

### ***Higher order relationships***

Higher order relationships can be explored primarily by DNA sequencing and rare and unusual rearrangements. The best documented case is the loss of the *rpl2* (gene coding for 50S protein of

ribosomes) intron ,an event that unites the Caryophyllales (Centrospermae) (Palmer et al. 1988). Other rearrangements which may indicate specific branchings include the loss of the large inverted repeat in two genera of the Pinaceae (Strauss et al. 1988) and a 30 kb inversion that distinguishes vascular plants from the single liverwort and moss examined to date (Ohyama et al. 1986; Calie and Hughes 1987). Chloroplast DNA has been and will increasingly be employed for addressing a variety of questions in plant phylogeny (Curtis and Clegg 1984; Palmer 1985b). Presently, cpDNA has been most useful at the interspecific level, perhaps because this is where it has been most widely studied. An increasing number of studies is now indicating that cpDNA will be used to determine or solve higher level systematic questions in the future.

## **Chloroplast DNA inheritance**

Chloroplasts are self-replicating organelles and arise only by growth and division of pre-existing chloroplasts (Birky 1978). The genetic system of the chloroplast is thus autonomous and obeys its own unique rules of inheritance. There is an extreme diversity of modes and mechanisms of plastid inheritance in most phyla of plants (Kirk and Tilney-Basset 1978; Hagemann 1979; Sears 1980, 1983; Whatley 1982, 1983). Most angiosperms exhibit maternal inheritance of the plastids, with approximately one third having some degree of biparental inheritance (Kirk and Tilney-Bassett 1978). Even in cases where inheritance is biparental, the maternal contribution is much greater than the paternal.

Biparental plastid inheritance has commonly been noted when green and white sectorized tissue appeared in progeny following crosses between a normal green plant and one having a plastome-encoded chlorophyll deficiency. For example, *Oenothera* (Kirk and Tilney-Bassett 1978; Sears 1980; Chiu et al. 1988) and *Pelargonium* (Kirk and Tilney-Bassett 1978; Sears 1980; Tilney-Bassett 1978) are two genera exhibiting biparental plastid inheritance that have been studied in this way.

In contrast to the angiosperms, plastid transmission in the gymnosperms may be either strictly or largely paternal. Plastid mutants have been used to indicate that plastids were inherited paternally 90-99% of the time in *Cryptomeria* (Ohba et al. 1971), providing the first genetic evidence for predominantly paternal transmission of plastids. Recently, several reports using cpDNA restriction fragment length polymorphisms have documented high levels of paternal inheritance in *Pinus* (Wagner et al. 1987), *Larix* (Szmidski et al. 1987), *Pseudotsuga* (Neale and Sederoff 1988), and *Picea* (Stine et al. 1989).

There is no general mechanism at the cellular level for the phenomenon of uniparental inheritance. The reasons often cited for elimination of plastids from one parent, usually the paternal, are exclusion from the male gamete during spermatogenesis, loss from the motile sperm, exclusion during fertilization or degradation within the zygote (Hagemann 1979; Sears 1980; Whatley 1982; Connett 1987). However, fine ultrastructural studies have not established "exclusion" as the only mechanism for maternal inheritance. Connett (1987) states that any mechanism proposed to account for maternal inheritance would have to be able to account for the continuum of variation that typifies plastid inheritance. There may be many mechanisms explaining maternal inheritance, which may be derived from each other or which may arise independently in different taxa in response to the same or differing selective forces and histories (Sears 1980).

Since cpDNA is predominantly maternally inherited, it offers particular advantages over biparentally inherited characters for studying the origin and parentage of hybrid and polyploid species that are very common among plants. Important insights into the origin, specific parentage and timing of the hybridization event of polyploid crop plants such as wheat (Ogihara and Tsunewaki 1982; Tsunewaki and Ogihara 1983; Bowman et al. 1983), tobacco (Kung et al. 1982), *Brassica* (Palmer et al. 1983; Erickson et al. 1983), potato (Hosaka and Hanneman 1988; Hosaka et al. 1988) have been provided by cpDNA studies. Since evolution in *Bromus* is based on hybridization and polyploidy, cpDNA may be a useful taxonomic character for the genus.

A cpDNA phylogeny will represent, in most angiosperms, a maternal phylogeny. One of the objectives of the present study was to compare this maternal phylogeny with data obtained from characters that are biparentally inherited. Consequently, the evolution of the ribosomal DNA genes was examined in a phylogenetic sense.

## ***Structure and evolution of ribosomal DNA***

### **Introduction**

The plant nuclear genome is composed of segments of DNA that are represented once or several times (unique sequence) per haploid genome up to sequences that are present a million times (highly repetitive) (Giannasi and Crawford 1986). Repetitive DNA may consist of identical or nearly identical sequences occurring in tandem arrays or various complex arrangements of different repeated sequences (Flavell 1980). One example of tandem arrays of repetitive sequences is represented by the major ribosomal DNA genes which have perhaps the greatest potential in systematic studies. Among the grasses, ribosomal DNAs has been used in systematic and population studies in wheat (Appels and Dvorak 1982; McIntyre et al. 1988), barley (Saghai-Maroo et al. 1984), maize and its ancestors (Zimmer et al. 1988), sorghum, sugarcane and maize (Springer et al. 1989), *Hordeum* (Molnar and Fedak 1989) and rice (Cordesse et al. 1990; Sano and Sano 1990).



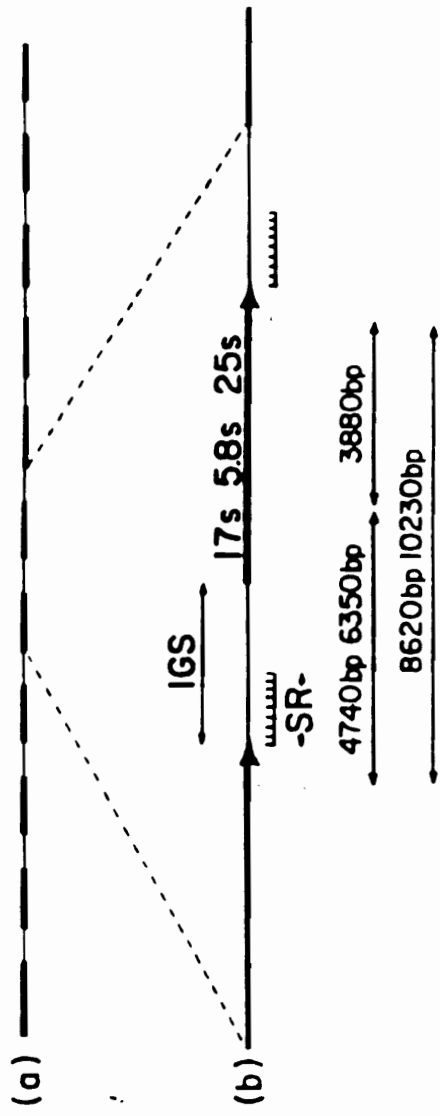
## Physical and genetic description of ribosomal DNA

Ribosomal DNA (rDNA) is the unit of DNA sequences that directs the synthesis of ribosomal RNA. Each segment of rDNA is composed of a gene or coding region and a spacer which separates one gene from the next (Long and Dawid 1980). A cluster of rRNA genes is identified cytologically as the nucleolar organiser region (NOR). One or several clusters of rRNA genes are found per haploid chromosome set. The rDNAs are highly reiterated in higher plants and a cell typically contains 500 to 40,000 copies per diploid genome (Appels and Honeycutt 1986; Rogers and Bendich 1987), comprising from 0.1 up to 10% of the total nuclear DNA of plants (Hemleben et al. 1986). The rDNA repeating units differ in size from about 8 kb (soybean, flax, radish ) to about 14-17 kb (*Trillium, Paris*) (Hemleben et al. 1986). The number of copies of rDNA varies within a species by as much as four-fold (Cullis and Davies 1975; Long and Dawid 1980). This variation in copy number is probably the result of unequal crossing over (Szostak and Wu 1980). The rDNA repeat units within a plant are highly homogeneous although some heterogeneity may exist among copies of rDNA (Jorgensen and Cluster 1988). This implies that, while several types of repeat unit length classes may be present in a single plant, several hundreds of repeat units are identical. This homogeneity is presumably the result of concerted evolution. It is thought that gene conversion, unequal crossing over, or a combination of these are responsible for concerted evolution of ribosomal genes (Schaal and Learn 1988). In species where rDNA is found to occupy two or more genetic loci, the repeat units are found to be quite homogeneous within each locus and homogeneity is greater within loci than between them (Appels and Dvorak 1982; Dvorak 1990).

The physical structure of plant rDNA is similar to that in other higher eukaryotes and consists of a number of regions that vary in functional constraints and evolutionary rate. The organization of a rDNA repeat unit is illustrated in Fig. 3. Each rDNA repeat contains a transcription unit from which the rRNA precursor is transcribed and a so called non-transcribed or intergenic spacer between the transcription units of adjacent repeats. The three rRNA coding regions lie in the order 5', 17S, 5.8S, 25S, 3' and are transcribed as a single large precursor, which is subsequently processed

to the mature rRNA forms. Several hundred base pairs called the internal transcribed spacers separate the 5.8S coding region from both the 17S and 25S regions. The intergenic spacer (IGS) that separates the coding region ranges in length from 1-8 kb in plants and contain a series of subrepeating elements. These subrepeats are generally 100 to 200 bp and this length varies only slightly within species. The number of these subrepeating elements within a given rDNA repeat is variable, and thus the overall length of the IGS is variable within and between populations.

The rDNA arrays of a single plant are often heterogeneous with respect to length, nucleotide sequence and/or base modification (Jorgensen and Cluster 1988). A fourth mode of rDNA variation involves variability in the copy number of rDNA per haploid genome. Since this is a quantitative character and is rarely measured, its taxonomic use is limited.



**Figure 3.** Schematic representation of ribosomal DNA repeat structure.: Line a represents the tandem array of ribosomal RNA genes found in each nucleolar organizer. Line b expands two complete repeat units and shows the tandem subrepeats. 17S, 5.8S, and 25S refer to ribosomal RNA genes. IGS and SR refer to intergenic spacer and subrepeat, respectively. (Taken from Saghai-Marooof et al. 1984)

## Modes and tempos of variation in different rDNA regions

### *Base modifications*

The most common type of base modification observed in rDNA genes is base methylation. DNA methylation is supposed to play a role in regulating gene expression in eukaryotes (Razin and Riggs 1980; Ehrlich and Wang 1981). Although plant DNA is considered to be heavily methylated, there is very little information concerning its function. The primary modified base found in eukaryotes is 5-methylcytosine (5-mC) (Shapiro 1976). Plant DNA contains 5-mC at C-A, C-T, and C-C as well as C-G sites and the extent of methylation at these sequences is variable (Gruenbaum et al. 1981). The predominant site of cytosine methylation is the CG dinucleotide but the CNG trinucleotide is also a site of significant methylation (Gruenbaum et al. 1981). In several grasses, 25-30% of the cytosine residues are methylated (Bedbrook et al. 1978). The *BamHI* site in the 25S gene is regarded as the most common evolutionarily conservative type of base modification (Jorgensen and Cluster 1988). This site is modified in about half of the rDNA repeats in legumes (Jorgensen et al. 1988) and in a number of other plants. Siegel and Kolacz (1983) have suggested that this methylation is due to the CCG sequence of which the *BamHI* recognition site (GGATCC) is a part. Modification of restriction sites for other enzymes also seems to exist in plants. Little information is available whether this modification is due to (a) the CG, CNG system, (b) a completely different system, or (c) sequences adjacent to the site (Jorgensen and Cluster 1988). It is known that adenine modification also prevents cleavage by certain restriction enzymes, but these have not been studied in detail.

It is important to recognize the limitations inherent in the use of restriction enzyme analysis of plant rDNA for phylogenetic investigations. Since plant nuclear DNA is methylated at most CG dinucleotides and CXG trinucleotides, many restriction enzymes that cleave sequences containing CG or CXG do not cleave if these sequences are methylated (Jorgensen and Cluster 1988).

## *Single base pair substitutions*

**1. Coding regions:** The coding regions of the ribosomal genes are expected to vary the least. Although this is true, limited variability is possible because of a range of functional constraints (Schaal and Learn 1988). Most studies of rDNA variation have analysed restriction endonuclease sites and little variation has been reported for sites within the coding regions. For example, Jorgensen et al. (1982) compared restriction site maps of cloned repeats from pea, wheat and pumpkin to estimate the number of nucleotide changes. They found that the 18S genes of pea and wheat were different at 3 out of 60 bp, while the genes of wheat and pumpkin differed at 5 out of 9 bp, and the genes of pea and pumpkin at 2 of 8 bp. The 5' end of the 25S gene showed no site conservation in comparisons of these species. The rest of the 25S genes showed great similarity among species of pea and pumpkin differing at three of ten sites, pea and wheat at eight of twelve, and wheat and pumpkin at five of ten. Jorgensen et al. (1982) found substantially less sequence divergence in the rDNA genes of several legumes. Only two cleavage site mutations were detected in a survey of 19 cleavage sites (114 bp). It is hopeful that direct sequence analysis would be much more sensitive for detecting variation in the coding regions.

**2. The IGS region:** Sequencing studies (Appels and Dvorak 1982; Yakura et al. 1984; Lassner and Dvorak 1986; McMullen et al. 1986; Toloczyki and Feix 1986) and other genetic analyses (Appels and Dvorak 1982; Rogers et al. 1986) indicated that the subrepeat and non subrepeat regions of the IGS differ in function and evolve at different rates. In the IGS subrepeat segments, DNA sequence and restriction analysis indicated that the subrepeat sequences vary within and among individuals of a species (Appels and Dvorak 1982; Jorgensen et al. 1987). This variation in single base pair substitutions in the IGS is of interest primarily within species. Since this variability is very difficult to detect with restriction enzymes, it is of limited phylogenetic utility. The IGS subrepeats of wheat and maize have been shown to have a certain degree of sequence similarity (Appels and Dvorak 1982; McMullen et al. 1986; Toloczyki and Feix 1986). Therefore, the subrepeats are thought to have a functional role in plants. The subrepeat sequences are now thought to act as transcription

promoters and enhancers in *Xenopus laevis* and are positively correlated with nucleolar dominance in frogs and wheat (Reeder 1984). The nonsubrepeat segment of the IGS region usually reveals many cleavage site variants. Such variants are said to have some utility in assessing phylogenetic relationships and are useful in genetic studies of interfertile populations and species (Zimmer et al. 1988).

### ***Length Variation***

**1. Coding Regions:** Most studies of the rDNA genes have in the past involved restriction enzyme analysis and agarose gel electrophoresis. This method is not sensitive enough to detect very small changes in length. In a study involving eight restriction enzymes, no length variation was found in the coding regions of *Pisum sativum*, *Vicia sativa*, and *Phaseolus vulgaris* (Jorgensen and Cluster 1988). However, sequencing of the 5.8S coding region of pea, broad bean, and lupine showed that the 164 bp 5.8S gene differs in length between pea and broad bean by a single base pair and between pea and lupine by two adjacent base pairs (Tanaka et al. 1980; Rafalski et al. 1983). Length variation of this sort is also expected to be found in the 18S and 25S genes by DNA sequencing comparisons, but not by restriction fragment comparisons.

**2. IGS region:** The most variable region of the rDNA repeat unit is the subrepeat containing region. Length variants of restriction fragments within this region almost always differ by a multiple of the length of the subrepeat. For example, 14 distinct fragment lengths were observed in a sample of 12 pea lines, each length differing from the others by a multiple of 180 bp (Jorgensen et al. 1987). This large number of variants results in a large number of IGS phenotypes observed within and among populations. The part of the IGS region without subrepeats is the next most length-variable region. Besides restriction site differences, small length differences are typically found in this region. Therefore, caution should be exercised in the interpretation of the molecular basis of mutations in this region in the absence of direct DNA sequence information (Jorgensen and Cluster 1988).

## Utility of ribosomal DNA in systematics

The attractiveness of rDNA for systematics is due to a number of characteristics leading to flexibility of experimental design and technical ease of analysis (Doyle 1987). The repeated nature of the rDNA family makes it most amenable for systematic studies. The thousand or more genes occur in one, or at most, a relatively few, major forms. The individual rDNA units do not evolve independently but undergo concerted evolution. This phenomenon leads to species-specific patterns and, when combined with the high copy number, can be easily visualized by DNA hybridization techniques. This is because, after digestion with restriction endonucleases and subsequent probing of transferred DNA fragments with rDNA hybridization probes, each band can be easily detected since it contains multiple copies of a particular fragment. This in turn reduces the amount of DNA that must be digested and electrophoresed, but also decreases the rigor with which transfers need be done, the specific activity of the probe required, and the amount of time needed to see results (Doyle 1987). The different regions of the eukaryotic rDNA diverge at very different rates. The coding regions evolve very slowly and are useful for examining higher taxonomic level. The IGS region diverges more rapidly and is of utility for looking at relationships at lower levels, such as within and between species (Giannasi and Crawford 1986).

However, Appels and Honeycutt (1986) have indicated that restriction enzyme analyses per se of the rDNA unit do not provide useful information on the relationships between species. The reason for this is that, in such analyses, lengths of DNA segments are measured and a single mutation can lead to loss of a restriction site and a dramatic change in length of the DNA segment being measured. In addition, within the rDNA system, length variation within a species can be just as extensive as the variation observed between species. Therefore, restriction endonuclease analysis of the rDNA units has to be approached cautiously if the data are to be used for phylogenetic purposes.

## *Construction of phylogenetic trees from molecular data*

The study of evolution has become increasingly analytical since molecular techniques were introduced (Nei 1987). Various mathematical and statistical theories were introduced for measuring the extent of DNA polymorphism within and between populations. With these methods, it is now possible to compare the extent of genetic variation between any pair of species. Another important statistical development was the theory of estimation of the number of nucleotide substitutions from observed sequence data or restriction site maps (Nei 1987). A related concept was the estimation of genetic distance between populations. Genetic distance is the extent of gene differences (genomic difference) between populations or species that is measured by some numerical quantity (Nei 1987). Many different kinds of molecular data are now being used to construct phylogenetic trees. The numerical taxonomists were the first to develop various methods for constructing trees from morphological characters. Some of these methods are being used directly with molecular data while various new methods are being invented especially for these data. Currently, there are many unsolved problems and controversies in this area.

A phylogenetic tree illustrates the evolutionary relationships among a group of organisms (Nei 1987; Li and Graur 1990). A phylogenetic tree is a graph of nodes and branches, in which only one branch connects any two adjacent nodes. The nodes represent the taxonomic units, and the branches define the relationships among the units in terms of descent and ancestry (Li and Graur 1990). The branching pattern of a tree is called the topology. The branch length usually indicates the number of changes that have occurred in that branch. Phylogenetic trees can be either rooted or unrooted. In a rooted tree, there is a particular node called the root, from which a unique path leads to any other node. The root represents the common ancestor of all the taxonomic units in a tree (Li and Graur 1990). An unrooted tree only specifies the relationships among the taxonomic units and does not define the evolutionary path.



The numerous tree making methods in the literature can be classified into two types: distance matrix methods and maximum parsimony methods. In the distance matrix method, evolutionary distances are computed for all pairs of taxa, and a phylogenetic tree is constructed by using an algorithm based on some functional relationships among the distance values (Li and Graur 1990). The evolutionary distance is usually the number of nucleotide or amino acid substitutions separating two taxonomic units. In maximum parsimony methods, the nucleotide or amino acid sequences of ancestral species are inferred from those of extant species, and a tree is produced by minimizing the number of evolutionary changes for the entire tree (Nei 1987). Parsimony methods operate by selecting trees that minimize the total tree length (Swofford and Olsen 1990).

The mathematical theory used in phylogenetic tree construction depends on a number of assumptions.

1. Causes of gain/loss of restriction sites. A basic assumption of RFLP analysis is that changes in fragment pattern are due to sequence rearrangements, the addition or deletion of DNA, or base substitutions within cleavage sites (Upholt 1977). However, because the activity of most restriction enzymes is sensitive to methylation, variation in the state of methylation can mimic the gain/loss of restriction sites (Dowling et al 1990). Methylation is usually a problem in the case of nuclear DNA but does not appear to be a problem for RFLP analysis of cpDNA since such modification of bases has not been observed in cpDNA.
2. Causes of changes in fragment migration. Another general assumption is that changes in the mobility of DNA fragments are due to differences in molecular weight. However, there are reports that the conformation of DNA may also affect migration patterns (Singh et al. 1987; Springer et al. 1989). The writer has also observed such anomalous migration of total DNA after digestion with different restriction endonucleases.
3. Identity of shared fragments or cleavage sites. It is also assumed that if two or more closely related samples share a particular sized fragment, they also share flanking cleavage sites

(Dowling et al. 1990). The likelihood of convergence, that there are two samples with similar sized fragments but produced by different cleavage sites, increases as the samples become more divergent (Dowling et al. 1990)

## **Indirect Estimation of the number of nucleotide substitutions**

Further assumptions are necessary to estimate the number of nucleotide substitutions per site between DNA sequences from restriction fragment or restriction site data. These assume that the four nucleotides are equally frequent and that they are randomly distributed within the DNA sequence of interest (Li and Graur 1990). If it is assumed that all differences in fragment patterns are caused by gain/loss of cleavage sites, then the proportion of shared fragments ( $F$ ) can be used to estimate the amount of sequence divergence ( $p$ ). The mathematical details of these principles are found in Upholt (1977), Nei and Li (1979) and Li and Graur (1990). A comparison of mapped cleavage sites is thought to eliminate problems of convergent fragment lengths, although the sites themselves may be convergent (Dowling et al. 1990). The probability of convergent site losses is far greater than that of convergent site gains because a site loss can be caused by any point mutation with the cleavage site, whereas a site gain requires a specific base substitution at a particular base pair (Templeton 1983; DeBry and Slade 1985; Li 1986).

Considering the assumptions and the other inequalities associated with DNA as a tool for the study of molecular evolution, the writer acknowledges and consequently urges extreme caution in the interpretation of DNA data for the construction of phylogenetic trees. The writer also feels that DNA data should be regarded as just another tool in biosystematics and not the panacea that accompanied the era of DNA systematics.

## References

- Antonov AS (1986) Molecular analysis of plant DNA genomes: conserved and diverged DNA sequences. In: Dutta SK (ed) DNA systematics II Plants. CRC Press, Boca Raton, Florida, pp 171-184
- Appels R, Dvorak J (1982) The wheat ribosomal DNA spacer region: its structure and variation in populations and among species. *Theor Appl Genet* 63:337-348
- Appels R, Honeycutt RL (1986) rDNA evolution over a billion years. In: Dutta SK (ed) DNA systematics II Plants. CRC Press, Boca Raton, Florida, pp 81-135
- Armstrong KC (1977) Hybrids of the annual *Bromus arvensis* with perennial *Bromus inermis*. *Pflanzenzuchtung* 78:244-252
- Armstrong KC (1981) The evolution of *Bromus inermis* and the related species of *Bromus* section *Pnigma*. *Bot Jahrb Syst* 102:426-443
- Armstrong KC (1987) Chromosome evolution in *Bromus*. In: Tsuchiya T, Gupta TK (eds) Chromosome engineering in plant genetics and breeding. Elsevier Science Publ., Amsterdam (in press)
- Arnheim N (1983) Concerted evolution of multigene families. In Nei M, Koehn RK (Eds) Evolution of genes and proteins. Sinauer, Sunderland, Massachusetts pp 38-61
- Banks JA, Birky Jr CW (1985) Chloroplast DNA diversity is low in a wild plant, *Lupinus texensis*. *Proc Natl Acad Sci USA* 82:6950-6954
- Bedbrook J, Gerlach WL, Thompson RD, Jones J, Flavell RB (1978) Molecular cloning of higher plant DNA. In: Rubenstein I, Gengenbach B, Phillips RL (eds) Genetic improvement of crops. University of Minnesota Press, Minneapolis, pp 93-114
- Bendich AJ, Smith SB (1990) Moving pictures and pulsed-field gel electrophoresis show linear DNA molecules from chloroplasts and mitochondria. *Curr Genet* 17:421-425
- Birky Jr CW (1978) Transmission genetics of mitochondria and chloroplasts. *Ann Rev Genet* 12:471-512
- Birky Jr CW (1988) Evolution and variation in plant chloroplast and mitochondrial genes. In: Gottlieb LD, Jain SK (eds) Plant evolutionary biology. Chapman and Hall, London, pp 23-53
- Boffey SM, Ellis JR, Seldon G, Leech RM (1979) Chloroplast division and DNA synthesis in light-grown wheat leaves. *Plant Physiol* 64:502-505
- Bohnert HJ, Crouse EJ, Schmitt JM (1982) Organization and expression of plastid genomes. In: Parthier B, Boulter D (eds) Nucleic acids and proteins in plants. Springer-Verlag, Berlin, pp 473-530
- Bohnert HJ, Löffelhardt W (1982) Cyanelle DNA from *Cyanophora paradoxa* exists in two forms due to intramolecular recombination. *FEBS (Fed Eur Biochem Soc) Lett* 150:403-406

- Bowman CM, Bonnard G, Dyer TA (1983) Chloroplast DNA variation between species of *Triticum* and *Aegilops*. Location of the variation on the chloroplast genome and its relevance to the inheritance and classification of the cytoplasm. *Theor Appl Genet* 65:247-262
- Butterfass T (1973) Control of plastid division by means of nuclear DNA amount. *Protoplasma* 76:167-195
- Butterfass T (1983) A nucleotypic control of chloroplast reproduction. *Protoplasma* 118:71-74
- Calie PJ, Hughes KW (1987) An efficient protocol for the isolation and purification of chloroplast DNA from moss gametophyte tissue. *Pl Mol Biol Rep* 4:206-212
- Calos MP, Miller JH (1980) Transposable elements. *Cell* 20:579-595
- Chen K, Wildman SG, Smith HH (1977) Chloroplast DNA distribution in parasexual hybrids as shown by polypeptide composition of fraction I protein. *Proc Natl Acad Sci USA* 74:5109-5112
- Chiu W-L, Stubbe W, Sears BB (1988) Plastid inheritance in *Oenothera*: organelle genome modifies the extent of biparental plastid transmission. *Curr Genet* 13:181-189
- Chun EHL, Vaughan MH, Rich A (1963) The isolation and characterization of DNA associated with chloroplast preparations. *J Mol Biol* 7:130-141
- Clayton WD (1981) Evolution and distribution of grasses. *Ann Missouri Bot Garden* 68:5-14
- Clegg MT, Rawson JRY, Thomas K (1984) Chloroplast DNA variation in pearl millet and related species. *Genetics* 106:449-461
- Clegg MT, Ritland K, Zurawski G (1986) Processes of chloroplast DNA evolution. In: Karlin S, Nevo E (eds) *Evolutionary processes and theory*. Academic Press, New York, pp 275-294
- Connett MB (1987) Mechanisms of maternal inheritance of plastids and mitochondria: developmental and ultrastructural evidence. *Pl Mol Biol Rep* 4:193-205
- Cordesse F, Second G, Delseny M (1990) Ribosomal gene spacer length variability in cultivated and wild rice species. *Theor Appl Genet* 79:81-88
- Crampton B (1974) *Grasses in California*. University of California Press, Berkeley.
- Cullis C, Davies DR (1975) Ribosomal DNA amounts in *Pisum sativum*. *Genetics* 81:485-492
- Curtis SE, Clegg MT (1984) Molecular evolution of chloroplast DNA sequences. *Mol Biol Evol* 1:291-301
- DeBry RW, Slade NA (1985) Cladistic analysis of restriction endonuclease cleavage maps within a maximum-likelihood framework. *Syst Zool* 34:21-34
- De Heij HT, Lustig H, Moeskops DJM, Bovenberg WA, Bisanz C, Groot GSP (1983) Chloroplast DNAs of *Spinacia*, *Petunia* and *Spirodela* have similar gene organization. *Curr Genet* 7:1-6
- Deng X-W, Wing RA, Gruissem W (1989) The chloroplast genome exists in multimeric forms. *Proc Natl Acad Sci USA* 86:4156-4160

- Dewey DR (1982) Genomic and phylogenetic relationships among North American perennial *Triticeae*. In: Estes JR, Tyrl RJ, Brunken JN (eds) Grasses and grasslands. University of Oklahoma Press, Norman, pp 55-58
- Doebley J, Renfroe W, Blanton A (1987) Restriction site variation in the *Zea* chloroplast genome. *Genetics* 117:139-147
- Doebley J, Durbin M, Golenberg EM, Clegg MT, Ma DP (1990) Evolutionary analysis of the large subunit of carboxylase (rbcL) nucleotide sequence among the grasses (Gramineae) *Evolution* 44:1097-1108
- Dowling TE, Moritz C, Palmer JD (1990) Nucleic acids II: restriction site analysis In: Hillis DM, Moritz C (eds) *Molecular systematics*. Sinauer, Sunderland, Massachusetts, pp 250-317
- Doyle JJ (1987) Variation at the DNA level: uses and potential in legume systematics. In: Stirton CH (ed) *Advances in legume systematics, Part 3*. Royal Botanic Gardens, Kew, pp 1-30
- Dvorak J 1990 Evolution of multigene families: the ribosomal RNA loci of wheat and related species. In: Brown ADH, Clegg MT, Kahler AL, Weir BS (eds) *Plant Population genetics, breeding, and genetic resources*. Sinauer, Sunderland, Massachusetts, pp 83-97
- Dvorak J, Appels R (1982) Chromosome and nucleotide sequence differentiation in genomes of polyploid *Triticum* species. *Theor Appl Genet* 63:349-360
- Edelman M (1981) Nucleic acids of chloroplasts and mitochondria. In: Marcus A (ed), *The biochemistry of plants, vol 6, Proteins and nucleic acids*. Academic Press, New York, pp 249-301
- Ehrlich M, Wang RYH (1981) 5-Methylcytosine in eukaryotic DNA. *Science* 212:1350-1357
- Erickson LR, Straus NA, Beversdorf WD (1983) Restriction patterns reveal origins of chloroplast genomes in *Brassica* amphidiploids. *Theor Appl Genet* 65:201-206
- Flavell R (1980) The molecular characterization and organization of plant chromosomal DNA sequences. *Ann Rev Pl Physiol* 31:569-596
- Gianassi DE, Crawford DJ (1986) Biochemical systematics In: Hecht MK, Wallace B, Prance GT (eds) *Evolutionary biology Vol 20*. Plenum Press, New York, pp 25-248
- Giannasi DE, Zurawski G, Clegg MT (1990) *Syst Bot* (in press)
- Gillham NW (1978) *Organelle heredity*. Raven Press, New York.
- Gillham NW, Boynton JE, Harris EH (1985) Evolution of plastid DNA In: Cavalier-Smith T (ed) *DNA and evolution: natural selection and genome size*. Wiley, New York, pp 299-351
- Gould FW, Shaw RB (1983) *Grass systematics*. 2nd ed. McGraw-Hill Book Company, New York
- Gruenbaum Y, Naveh-Many T, Cedar H, Razin A (1981) Sequence specificity of methylation in higher plant DNA. *Nature* 292:860-862
- Hagemann R (1979) Genetics and molecular biology of plastids in higher plants. *Stadler Symp* 11:91-105

- Hemleben V, Ganai M, Gerstner J, Schiebel K, Torres RA (1986) Organization and length heterogeneity of plant ribosomal RNA genes. In: Kahl G (ed) The architecture of eukaryotic genes. VHC, Weinheim, pp 371-383
- Hennig W (1966) Phylogenetic systematics. University of Illinois Press, Urbana and Chicago
- Herrman RG (1982) The preparation of circular DNA from plastids. In: Edelman M, Hallich R, Chua N-H (eds) Methods in chloroplast molecular biology. Elsevier, Amsterdam, pp 259-280
- Herrman RG, Possingham JV (1980) Plastid DNA - The plastome. In: Reinert J (ed) Results and problems in cell differentiation, vol 10, chloroplasts. Springer-Verlag, Berlin, pp 45-96
- Herrman RG, Kowallik KV, Bohnert HJ (1974) Structural and functional aspects of the plastome. I. The organization of the plastome. Portugalia Acta Biol Ser A 14:91-110
- Hiratsuka J, Shimada H, Whittier RF, Ishibashi T, Sakamoto M, Mori M, Kondo C, Hongi Y, Sun C-R, Meng B-Y, Li Y-Q, Kanno A, Nishizawa Y, Hirai A, Shinozaki K, Sugiura M (1989) The complete sequence of the rice (*Oryza sativa*) chloroplast genome. Mol Gen Genet 217:185-194
- Hosaka K, Hanneman RE Jr. (1988) The origin of the cultivated tetraploid potato based on chloroplast DNA. Theor Appl Genet 76:172-176
- Hosaka K, de Zoeten GA, Hanneman RE Jr. (1988) Cultivated potato chloroplast DNA differs from the wild type by one deletion - Evidence and implications. Theor Appl Genet 75:741-745
- Howe CJ, Bowman CJ, Dyer TA, Gray JC (1983) The genes for the alpha and proton-translation subunits of wheat chloroplast ATP synthase are close together on the same strand of chloroplast DNA. Mol Gen Genet 190:51-55
- Jackson RC (1984) Chromosome pairing in species and hybrids. In: WF Grant (ed) Plant biosystematics. Academic Press, Toronto pp 67-86
- Jorgensen RA, Cluster PD (1988) Modes and tempos in the evolution of nuclear ribosomal DNA: new characters for evolutionary studies and new markers for genetic and population studies. Ann Missouri Bot Gard 75:1238-1247
- Jorgensen RA, Cuellar RE, Thompson WF (1982) Modes and tempos in the evolution of nuclear encoded ribosomal RNA genes in legumes. Carnegie Inst Washington Yearbook 81:98-101
- Jorgensen RA, Cuellar RE, Thompson WF, Kavanaugh TA (1987) Structure and variation in ribosomal RNA genes of pea. Pl Mol Biol 8:3-12
- Kahler AL, Krzakowa M, Allard RW (1981) Isozyme phenotypes in five species of *Bromus* sect. *Genea*. Bot Jahrb Syst 102:401-409
- Kaul MLH, Murthy TGK (1985) Mutant genes affecting higher plant meiosis. Theor Appl Genet 70:449-466
- Kimura M (1983) The neutral theory of molecular evolution. In: Nei M, Koehn RK (eds) Evolution of genes and proteins. Sinauer, Sunderland, Massachusetts, pp 208-233
- Kirk JTO (1971) Will the real chloroplast please stand up. In: Boardman NK, Linnane AW, Smillie RM (eds) Autonomy and biogenesis of mitochondria and chloroplasts. Elsevier North-Holland, Amsterdam, pp 267-276

- Kirk JTO (1976) Chloroplast nucleic acids. In: Fasman GD (ed) Handbook of biochemistry and molecular biology, vol II. CRC Press, Cleveland, pp 356-362
- Kirk JTO, Tillney-Bassett RAE (1978) The plastids: their chemistry, structure, growth, and inheritance. 2nd ed. Elsevier, Amsterdam
- Knowles PF (1944) Interspecific hybridization of *Bromus*. Genetics 29:128-140
- Koller B, Delius H (1980) *Vicia faba* chloroplast DNA has only one set of ribosomal genes as shown by partial denaturation mapping and R-loop analysis. Mol Gen Genet 178:261-269
- Kolodner R, Tewari KK (1979) Inverted repeats in chloroplast DNA from higher plants. Proc Natl Acad Sci USA 76:41-45
- Kolodner R, Tewari KK (1975) The molecular size and conformation of the chloroplast DNA from higher plants. Biochim. Biophys. Acta 402:372-390.
- Kung S (1977) Expression of chloroplast genomes in higher plants. Ann Rev Pl Physiol 28:401-437
- Kung S, Williams JP (1969) Chloroplast DNA from broad bean. Biochim Biophys Acta 195:434-445
- Kung SD, Zhu YS, Shen GF (1982) *Nicotiana* chloroplast genome III. Chloroplast DNA evolution. Theor Appl Genet 61:73-79
- Lamppa GK, Bendich AJ (1979) Changes in chloroplast DNA levels during development of pea (*Pisum sativum*). Pl Physiol 64:126-130
- Lamppa GK, Elliot LV, Bendich AJ (1980) Changes in chloroplast number during pea leaf development. Planta 148:437-443
- Lassner M, Dvorak J (1986) Preferential homogenization between adjacent and alternate subrepeats in wheat rDNA. Nucleic Acid Res 14:5499-5512
- Lemieux C, Turmel M, Lee RW (1980) Characterization of chloroplast DNA in *Chlamydomonas eugametos* and *C. moewusii* and its inheritance in hybrid progeny. Curr Genet 2:139-147
- Li W-H (1983) Evolution of duplicate genes and pseudogenes. In: Nei M, Koehn RK (eds) Evolution of genes and proteins. Sinauer, Sunderland, Massachusetts, pp 14-37
- Li W-H (1986) Evolutionary change of restriction cleavage sites and phylogenetic inference. Genetics 113:187-213
- Li W-H, Graur D (1990) Fundamentals of molecular evolution. Sinauer, Sunderland, Massachusetts
- Lidholm J, Szmidi AE, Hallgren J-E, Gustafsson P (1988) The chloroplast genome of conifers lack one of the rRNA-encoding inverted repeats. Mol Gen Genet 212:6-10
- Long EO, Dawid IB (1980) Repeated genes in eukaryotes. Ann Rev Biochem 49:727-764
- Maliga P, Loiz H, Lazar G, Nagy F (1982) Cytoplasmic protoplast fusion for interspecific chloroplast transfer in *Nicotiana*. Mol Gen Genet 185:211-215
- McIntyre CL, Clarke BC, Appels R (1988) DNA sequence analysis of the ribosomal spacer regions in the *Triticeae*. Pl Syst Evol 160:91-104

- McClelland M, Nelson M (1985) The effect of site specific methylation on restriction endonuclease digestion. *Nucleic Acid Res* 13 Suppl. r201-r207
- McMullen MD, Hunter DB, Phillips RL, Rubenstein I (1986) The structure of the maize ribosomal DNA spacer region. *Nucleic Acid Res* 14:4953-4968
- Molnar SJ (1988) Smooth bromegrass (*Bromus inermis* Leyss) In: Bajaj YPS (ed) *Biotechnology in agriculture and forestry*. Vol 6. Springer-Verlag, Berlin, pp 428-443
- Molnar SJ, Fedak G (1989) Polymorphism in ribosomal DNA repeat units of 12 *Hordeum* species. *Genome* 32:1124-1127
- Neale DB, Sederoff RR (1988) Inheritance and evolution of conifer organelle genomes. In: Hanover JW, Keathley DE (eds) *Genetic manipulation of woody plants*. Plenum Press, New York, pp 251-264
- Nei M (1987) *Molecular evolutionary genetics*. Columbia University Press, New York.
- Nei M, Li W-H (1979) Mathematical model for studying genetic variation in terms of restriction endonucleases. *Proc Natl Acad Sci USA* 76:5269-5273
- Obha K, Iwakawa M, Okada Y, Murai M (1971) Paternal transmission of a plastid anomaly in some reciprocal crosses of sugi, *Cryptomeria japonica* D Don *Silvae Genet* 20:101-107
- Ogihara Y, Tsunewaki K (1982) Molecular basis of the gene diversity of the cytoplasm in *Triticum* and *Aegilops*. I. Diversity of the chloroplast genome and its lineage revealed by the restriction pattern of ctDNAs. *Japan J Genet* 57:371-396
- Ohyama K, Fukazawa H, Kohchi T, Shirai H, Sano T, Sano S, Umesono K, Shiki Y, Takeuchi M, Chang Z, Aota D, Inokuchi H, Ozeki H (1986) Chloroplast gene organization deduced from complete sequence of liverwort *Marchantia polymorpha*. *Nature* 322:572
- Ozeki, H, Umesono K, Inokuchi H, Kohchi H, Ohyama K (1989) The chloroplast genome of plants: a unique origin. *Genome* 31:169-174
- Palmer J (1983) Chloroplast DNA exists in two orientations. *Nature* 301:92-93
- Palmer JD (1985a) Comparative organization of chloroplast genomes. *Ann Rev Genet* 19:325-354
- Palmer JD (1985b) Evolution of chloroplast and mitochondrial DNA in plants and algae. In: MacIntyre RJ (ed) *Monographs in evolutionary biology: molecular evolutionary genetics*. Plenum, New York, pp 131-240
- Palmer JD (1986a) Chloroplast DNA and phylogenetic relationships. In: S. K. Dutta (ed) *DNA systematics*. CRC Press, Inc. Boca Raton, Florida, pp 63-80
- Palmer JD (1986b) Isolation and structural analysis of chloroplast DNA. *Methods Enzymol* 118:167-186
- Palmer JD (1987) Chloroplast DNA evolution and biosystematic uses of chloroplast DNA variation. *Am Nat* 130, Suppl, pp S6-S29
- Palmer J, Thompson WF (1981) Chloroplast DNA rearrangements are more frequent when a large inverted repeat sequence is lost. *Cell* 29:537-550



- Palmer JD, Zamir D (1982) Chloroplast DNA evolution and phylogenetic relationships in *Lycopersicon*. Proc Natl Acad Sci USA 79:5006-5010
- Palmer JD, Jorgensen RA, Thompson WF (1985) Chloroplast DNA variation and evolution in *Pisum*: patterns of change and phylogenetic analysis. Genetics 109:195-213
- Palmer JD, Shields CR, Cohen DB, Orton TJ (1983) Chloroplast DNA evolution and origin of amphidiploid *Brassica*. Theor Appl Genet 65:181-189
- Palmer JD, Jansen RK, Michaels HJ, Chase M, Manhart J (1988) Chloroplast DNA variation and plant phylogeny. Ann Missouri Bot Gard 75:1180-1206
- Perl-Treves R, Galun E (1985) The *Cucumis* plastome: physical map, intrageneric variation and phylogenetic relationships. Theor Appl Genet 71:417-429
- Possingham JV, Lawrence ME (1983) Controls to plastid division. Int Rev Cytol 84:1-56
- Poulsen CR (1983) The barley chloroplast genome: physical structure and transcriptional activity in vivo. Carlsberg Res Commun 48:57-80
- Pyke KA, Leech RM (1987) The control of chloroplast number in wheat mesophyll cells. Planta 170:416-420
- Rafalski JA, Wiewjorowski M, Soll D (1983) Organization of ribosomal DNA in yellow lupine (*Lupinus luteus*) and sequence of the 5.8S RNA gene. FEBS (Fed Eur Biochem Soc) Lett 152:241-244
- Razin A, Riggs AD (1980) DNA methylation and gene function. Science 210:604-610
- Reeder RH (1984) Enhancers and ribosomal gene spacers. Cell 38:349-351
- Ris H, Plaut W (1962) The ultrastructure of DNA-containing areas in the chloroplasts of *Chlamydomonas*. J Cell Biol 13:383-391
- Rogers SO, Bendich AJ (1987) Ribosomal RNA genes in plants: variability in copy number and in the intergenic spacer. Pl Mol Biol 9:509-520
- Rogers SO, Honda S, Bendich A (1986) Variation in the ribosomal genes among individuals of *Vicia faba*. Pl Mol Biol 6:339-345
- Saghai-Marooif MA, Soliman KM, Jorgensen RA, Allard RW (1984) Ribosomal DNA spacer-length polymorphisms in barley: Mendelian inheritance, chromosomal location, and population dynamics. Proc Natl Acad Sci USA 81:8014-8018
- Sano Y, Sano R (1990) Variation of the intergenic spacer region of ribosomal DNA in cultivated and wild rice species. Genome 33:209-218
- Schaal BA, Learn GH (1988) Ribosomal DNA variation within and among plant populations. Ann Missouri Bot Gard 75:1207-1216
- Schiller G, Herrman RG, Melchers G (1982) Restriction endonuclease analysis of plastid DNA from tomato, potato and some of their somatic hybrids. Mol Gen Genet 186:453-459
- Schilling EE, Jansen RK (1989) Restriction fragment analysis of chloroplast DNA and the systematics of *Viguiera* and related genera (Asteraceae: *Heliantheae*). Am J Bot 76:1769-1778

- Scott NS, Possingham JV (1980) Chloroplast DNA in expanding spinach leaves. *J Exp Bot* 31:1081-1092
- Sears BB (1980) Elimination of plastids during spermatogenesis and fertilization in the plant kingdom. *Plasmid* 4:233-255
- Sears BB (1983) Genetics and evolution of the chloroplast. *Stadler Symp* 15:119-139
- Shapiro HS (1976) *CRC Handbook. Biochem Mol Biol* 2:259
- Shear CL (1900) A revision of the North American species of *Bromus* occurring north of Mexico. USDA Bull No. 23, Government Printing Office, Washington
- Shinozaki K, Sugiura M (1982) The nucleotide sequence of the tobacco chloroplast gene for the large subunit of ribulose-1,5- bisphosphate carboxylase/oxygenase. *Gene* 20:91-102
- Shinozaki K, Yamada C, Takahata N, Sugiura M (1983) Molecular cloning and sequence analysis of the cyanobacterial gene for the large subunit of ribulose-1,5-bisphosphate carboxylase/oxygenase. *Proc Natl Acad Sci USA* 80:4050-4054
- Shinozaki K, Ohme M, Tanaka M, Wakasugi T, Hayashida N, Matsubayashi T, Zaita N, Chunwongse J, Obokata J, Yamaguchi-Shinozaki K, Ohto C, Torazawa K, Meng BY, Sugita M, Deno H, Kamogashira T, Yamada K, Kusuda J, Takaiwa F, Kato A, Tohdoh N, Shimada H, Sugiura M (1986) The complete nucleotide sequence of the tobacco chloroplast genome: Its gene organization and expression. *EMBO (Eur Mol Biol Organ) J* 5:2043-2049
- Siegel A, Kolacz K (1983) Heterogeneity of pumpkin ribosomal DNA. *Pl Physiol* 72:166-171
- Singh G, Neckelmann N, Wallace DC (1987) Conformational mutations in human mitochondrial DNA. *Nature* 329:270-272
- Smith PM (1972) Serology and species relationships in annual bromes (*Bromus* L. sect. *Bromus*). *Ann Bot* 36:1-30
- Smith P (1970) Taxonomy and nomenclature of the brome grass (*Bromus* L. sl.). *Notes Roy Bot Gard Edinburgh* 30:361-376
- Soderstrom TR, Beaman JH (1968) The genus *Bromus* (Gramineae) in Mexico and Central America. *Publ Museum Michigan State University* 3:469-519
- Soltis DE, Soltis PS, Clegg MT, Durbin M (1990) rbcL sequence divergence and phylogenetic relationships in Saxifragaceae sensu lato. *Proc Natl Acad Sci USA* 87:4640-4644
- Springer PS, Zimmer EA, Bennetzen JL (1989) Genomic organization of the ribosomal DNA of sorghum and its close relatives. *Theor Appl Genet* 77:844-850
- Stebbins GL (1947) The origin of the complex of *Bromus carinatus* and its phytogeographic implications. *Contr Gray Herb* 165:42-55
- Stebbins GL (1956) Cytogenetics and evolution in the grass family. *Am J Bot* 43:890-905
- Stebbins GL (1981) Chromosomes and evolution in the genus *Bromus* (Gramineae). *Bot Jahrb Syst* 102:359-379

- Stebbins GJ, Crampton B (1960) A suggested revision of the grass genera of temperate North America. In: Recent advances in botany, IX International Botanical Congress, Montreal 1959, I:133-145. Toronto University Press
- Stebbins GL, Tobgy HH (1944) The cytogenetics of hybrids in *Bromus*. 1. Hybrids within the section "*Ceratochloa*". Am J Bot 31:1- 11
- Stebbins GL, Walters MS (1949) The evolutionary significance of two synthetic allopolyploid species of *Bromus*. Portugaliae Acta Biol, Ser A Vol R. B. Goldschmidt:106-136
- Stine M, Sears BB, Keathley DE (1989) Inheritance of plastids in interspecific hybrids of blue spruce and white spruce. Theor Appl Genet 78:768-774
- Strauss SH, Palmer JD, Howe GT, Doerksen AH (1988) Chloroplast DNA of two conifers lack a large inverted repeat and are extensively rearranged. Proc Natl Acad Sci USA 85:3898
- Swofford DL, Olsen GJ (1990) Phylogeny reconstruction In: Hillis DM, Moritz C (eds) Molecular systematics. Sinauer, Sunderland, Massachusetts, pp 411-501
- Sytsma KJ, Gottlieb LD (1986) Chloroplast DNA evolution and phylogenetic relationships in *Clarkia* section *Peripetasma* (Onagraceae). Evolution 40:1248-1261
- Szmidt AE, Alden T, Hallgren J-E (1987) Paternal inheritance of chloroplast DNA in *Larix* Pl Mol Biol 9:59-64
- Szostak JW, Wu R (1980) Unequal crossing-over in the ribosomal DNA of *Saccharomyces cerevisiae*. Nature 284:426-430
- Tanaka Y, Dyer T, Brownlee GG (1980) An improved direct RNA sequence method: its applications to *Vicia faba* 5.8S ribosomal RNA. Nucleic Acid Res 8:1259-1271
- Templeton AR (1983) Phylogenetic inference from restriction endonuclease cleavage site maps with particular reference to the humans and apes. Evolution 37:221-244
- Tewari KK, Wildman SG (1966) Chloroplast DNA from tobacco leaves. Science 153:1269-1271
- Tillney-Bassett RAE (1978) The inheritance and genetic behavior of plastids. In: Kirk JTO, Tilney-Bassett RAE (eds) The plastids. Elsevier/North-Holland, Amsterdam, pp 251-524
- Toloczki C, Feix G (1986) Occurrence of homologous repeat units in the external spacer region of nuclear maize rRNA gene unit. Nucleic Acid Res 14:4969-4986
- Troitsky AV, Bobrova VK (1986) 23S rRNA-derived small ribosomal RNAs: their structure and evolution with reference to plant phylogeny. In: Dutta SK (ed) DNA systematics II. Plants CRC Press, Boca Raton, Florida, pp 137-170
- Tsvelev NN (1976) Grasses of the Soviet Union [Zlaki SSSR] Part 1. Nauka Publ. Leningrad, pp 298-339 [Translated from Russian]
- Upholt WB (1977) Estimation of DNA sequence divergence from comparison of restriction endonuclease digests. Nucleic Acid Res 4:1257-1265
- von Kalm I, Vize PD, Smyth DR (1986) An undermethylated region in the spacer of ribosomal RNA genes of *Lilium henryi*. Pl Mol Biol 6:33-39

- Wagner DB, Furnier GR, Saghai-Maroo MA, Williams SM, Dancik BP, Allard RW (1987) Chloroplast DNA polymorphisms in lodgepole and jack pines and their hybrids. *Proc Natl Acad Sci USA* 84:2097-2100
- Wagnon KH (1952) A revision of the genus *Bromus*, section *Bromopsis*, of North America. *Brittonia* 7:415-480
- Walters MS (1958) Aberrant chromosome movement and spindle formation in meiosis of *Bromus* hybrids: an interpretation of spindle organization. *Am J Bot* 45:271-289
- Whatley JM (1982) Ultrastructure of plastid inheritance: green algae to angiosperms. *Biol Rev* 57:527-569
- Whatley JM (1983) Plastids - past, present and future. *Int Rev Cytol Suppl* 14:329-374
- Whitfield PR, Bottomley W (1983) Organization and structure of chloroplast genes. *Ann Rev Pl Physiol* 34:279-310
- Yakura K, Kato A, Tanifuji S (1984) Length heterogeneity of the large spacer of *Vicia faba* rDNA is due to the differing numbers of a 326 bp repetitive sequence element. *Mol Gen Genet* 193:400-405
- Zimmer EA, Jupe ER, Walbot V (1988) Ribosomal gene structure, variation, and its inheritance in maize and its ancestors. *Genetics* 120:1125-1136
- Zurawski G, Clegg MT (1984) The barley chloroplast DNA *atpBE*, *trnM2*, and *trnVI* loci. *Nucleic Acids Res* 12:2549-2559
- Zurawski G, Clegg MT, Brown AHD (1984) The nature of nucleotide sequence divergence between barley and maize chloroplast DNA. *Genetics* 106:735-749

# *Chapter 1: Chloroplast DNA variation in diploid and polyploid species of Bromus (Poaceae) subgenera Festucaria and Ceratochloa*

## *Summary*

Chloroplast DNA restriction endonuclease patterns were used to examine phylogenetic relationships between *Bromus* subgenera *Festucaria* and *Ceratochloa*. *Festucaria* is considered monophyletic based on the L genome, while *Ceratochloa* encompasses two species complexes: the *B. catharticus* complex which evolved by combining three different genomes, and the *B. carinatus* complex which is thought to have originated from hybridization between polyploid species of *B. catharticus* and diploid members of *Festucaria*. All species of subgenus *Ceratochloa* (hexaploids and octoploids) were identical in chloroplast DNA sequences. Similarly, polyploid species of subgenus *Festucaria*, except for *B. auleticus*, were identical in cpDNA sequences. In contrast, diploid species of subgenus *Festucaria* showed various degrees of nucleotide sequence divergence.

Species of subgenus *Ceratochloa* appeared monophyletic and phylogenetically closely related to the diploid *B. anomalus* and *B. auleticus* of subgenus *Festucaria*. The remaining diploid and polyploid species of subgenus *Festucaria* appeared in a distinct grouping. The study suggests that the *B. catharticus* complex must have been the maternal parent in the proposed hybrid origin of *B. carinatus* complex. Although there is no direct evidence for the paternal parent of the latter complex, the cpDNA study shows the complex to be phylogenetically very related to the diploid *B. anomalus* of subgenus *Festucaria*.

Key words: Chloroplast DNA - Phylogeny - *Bromus* - Poaceae - Grasses

## ***Introduction***

*Bromus* L. (brome-grasses, Poaceae) is a genus of about 100 diploid and polyploid species that includes important forage, range and weedy species (Gould and Shaw 1983). Evolution in the genus is based on polyploidy and hybridization within and/or between the different subgenera, which has given rise to species with complex genomic constitutions (Stebbins 1981). This proposed pattern of evolution has generated questions regarding the phylogenetic relatedness of the different sections of the genus. Stebbins (1956, 1981) presented one of the more comprehensive treatments of *Bromus*. He divided the genus into seven distantly related subgenera (*Festucaria*, *Ceratochloa*, *Neobromus*, *Stenobromus*, *Bromus*, *Nevskiella*, and *Boissiera*), and proposed an evolutionary scheme based primarily on information from gross morphology, chromosome size, and analyses of meiotic chromosome pairing in a few interspecific hybrids. The effectiveness of this information in resolving phylogenies is sometimes limited by factors such as parallel and convergent evolution in morphology, high degree of phenotypic plasticity (Clayton 1981), and difficulties of hybridizing species from the different subgenera. Other limitations include problems associated with

interpreting chromosome pairing in hybrids, particularly those of polyploid origin, due to asynaptic or desynaptic mutations or re-diploidization of polyploids (Dewey 1982; Jackson 1984; Kaul and Murthy 1985).

In this study, chloroplast DNA (cpDNA) variation is used to assess the phylogenetic relationships between polyploid species of subgenus *Ceratochloa* and species of subgenus *Festucaria*. Chloroplast DNA, being evolutionarily highly conserved, has been very useful in inferring phylogenetic relationships in polyploid complexes (Tsunewaki and Ogihara 1983; Perl-Treves and Galun 1985; Ichikawa et al. 1986; Kishima et al. 1987; Hilu 1988; Hosaka and Hanneman 1988; Hosaka et al. 1988; Soltis and Soltis 1989; Lumerat et al. 1989). Chloroplast DNA is mostly maternally inherited in flowering plants (Kirk and Tilney-Basset 1978; Sears 1980), and consequently, the phylogenies resulting from this study will be discussed in that frame.

### ***Proposed genomic relationship between Festucaria and Ceratochloa***

Subgenera *Festucaria* Grenier & Godron (*Pnigma* and *Bromopsis* of Dumortier) and *Ceratochloa* Beauv. contain approximately 60 and 16 species, respectively (Smith 1970). In each subgenus, there are valuable pasture species such as *B. inermis*, *B. carinatus* and *B. catharticus* (Stebbins and Tobgy 1944). Subgenus *Festucaria* includes both diploid and polyploid species. The diploid ( $2n = 14$ ) and tetraploid species are distributed primarily in the Americas while species with higher ploidy levels such as hexaploids, octoploids and decaploids have a Eurasian distribution (Armstrong 1981). Subgenus *Ceratochloa*, on the other hand, consists entirely of higher polyploid species. The *Ceratochloa* species fall into two morphologically distinct complexes: the *B. catharticus* complex, which is hexaploid ( $2n = 42$ ) endemic to South America, and the *B. carinatus* complex, which is octoploid found mainly in North America (Stebbins 1981).

Stebbins (1956) and Armstrong (1987) proposed a monophyletic origin for the *Festucaria* species, all of which contain different numbers of the L genome. The two complexes of subgenus

*Ceratochloa* are assumed to combine three ( $A_1B_1B_2$ ) or four ( $A_1B_1B_2L$ ) genomes (Stebbins 1981). The hexaploid *B. catharticus* complex is considered to have evolved from presently extinct diploid and tetraploid species that contributed the  $A_1$ ,  $B_1$ , and  $B_2$  genomes. The *B. carinatus* complex is thought to have originated from hybridization between members of the hexaploid *B. catharticus* complex and diploid species of *Festucaria* (Stebbins and Tobgy 1944; Stebbins 1947, 1981). Therefore, the *B. carinatus* complex is assumed to contain the  $A_1$ ,  $B_1$ ,  $B_2$  and L genomes. Stebbins and Tobgy (1944) suggested that different species of the diploid *Festucaria* have entered into the ancestry of the *B. carinatus* complex, favoring a partly polyphyletic origin for the group.

The genomic relationships proposed by Stebbins (1956, 1981) also imply that subgenera *Festucaria* and *Ceratochloa* have different ancestry. Stebbins (1981) indicated that the relationships between the two subgenera are obscure, and their chromosomes so dissimilar that a recent common origin is difficult to imagine.

## ***Materials and methods***

### ***Plant material***

The two subgenera were represented in this study by fifteen diploid and polyploid species. The species used, their chromosome numbers, and sources of seed material are listed in Table 2. Plants were grown from seeds under greenhouse conditions. In most cases, seeds were sown in 15 cm pots and leaves were repeatedly harvested when the plants were three to four weeks old. In the case of *B. inermis* and *B. carinatus*, large amounts of seed were sown in 28 X 53 cm flats. All plants were destarched in the dark for one or two days before leaf harvests. Leaf material was either used immediately for cpDNA extraction, or freeze-dried in liquid nitrogen for storage in a -70° C freezer.



## *cpDNA extraction, restriction enzyme digestion and electrophoresis*

Chloroplast DNA was isolated using the method of Kemble (1987) with minor modification. For each extraction, 25 g of leaf material was ground in a mortar with a pestle using liquid nitrogen. The powder was then resuspended in 400 to 500 ml of the isolation buffer. This method of grinding resulted in little or no shear of the cpDNA in comparison with the grinding of leaves by homogenizing with a Waring blender. Chloroplast DNA was digested with the restriction enzymes *AvaII*, *BamHI*, *BglII*, *EcoRI*, *HindIII*, *KpnI* and *Sall* according to the supplier's instructions. The DNA fragment digests were resolved on 0.5% to 1% agarose gels depending on the number of fragments generated by the enzyme. Gels were stained with ethidium bromide and photographed under ultraviolet light. Lambda DNA-*HindIII* fragments (Bethesda Research Laboratories) were used as size standards.

## *Analysis of data*

The cpDNA restriction endonuclease patterns of the various species were scored for fragment length differences. The data obtained from using the different enzymes were pooled and a genetic distance matrix was then computed using Nei and Li (1979) equations 21 and 20 which calculate the  $F$  ( $F = 2n_{xy} / [n_x + n_y]$ ) and Sigma values ( $F \approx P^4 / 3 - 2P$ ), respectively. These algorithms are put forth to estimate genetic distances from fragment length differences instead of restriction sites. The genetic distance matrix (Sigma values) were then analyzed by the Unweighted Pair-group Method (UPGMA) using the NT-SYSPc package of computer programs developed by F. J. Rohlf (version 1.50, 1989; Exeter Publishing, Ltd, New York). The genetic distance matrix was also analyzed by the Fitch and Margoliash (1967) cladistic method for genetic distances using the PHYLIP 3.1 computer program developed by Joseph Felsenstein. In the latter analysis, the Jumble option was used for entering the species in a random order.

## Results

The enzymes *EcoRI*, *BamHI*, *HindIII*, *AvaI*, *KpnI*, *BglI* and *Sall* revealed mutational events that produced differences in restriction fragment sizes between at least two species. A representative restriction fragment pattern of the species is presented in Fig. 4 for the endonuclease *HindIII*. The restriction endonucleases *BglI*, *KpnI* and *Sall* generated an average of 11 fragments while the others produced an average of 25 fragments per species. Of the total 2093 fragments produced by all the restriction enzymes, 1760 fragments (84%) were shared by all the fifteen species of *Bromus*. The percent shared fragments (*F* values) and genetic distances calculated from these data are presented in Table 3.

When the genetic distance matrix was subjected to UPGMA analysis, three distinct clusters of species were apparent (Fig. 5a). One cluster included the diploid *B. porteri*, octaploids *B. inermis* and *B. pumpellianus*, and decaploid *B. biebersteinii*, all members of subgenus *Festucaria*. The second cluster included the diploid *B. anomalus* and hexaploid *B. auleticus* of subgenus *Festucaria*, as well as all members of subgenus *Ceratochloa* (the hexaploids *B. catharticus*, *B. valdivianus*, *B. stamineus*, *B. coloratus*, and *B. brevis* of the *B. catharticus* complex, and the octaploids *B. carinatus*, *B. sitchensis* and *B. breviaristatus* of *B. carinatus* complex). The third one included the North American *B. ciliatus*, a diploid species of subgenus *Festucaria*. The cophenetic correlation between the grouping generated by the UPGMA and the genetic matrices was very high ( $r = 0.953$ ), indicating a very low level of distortion.

The network obtained from using Fitch and Margoliash distance method is shown in Fig. 5b. Species of subgenus *Ceratochloa* and *B. anomalus* (subgenus *Festucaria*) appeared monophyletic, and phylogenetically closely related to *B. auleticus*. *Bromus pumpellianus*, *B. inermis* and *B. biebersteinii*, which were identical in the cpDNA nucleotide sequences, appeared in the same clade

with *B. ciliatus*; all species are members of subgenus *Festucaria*. *Bromus porteri* was more distant from the *B. pumpellianus* clade, which is incongruent with the UPGMA results (Fig. 5a).

## ***Discussion***

One of the most striking features of this study is the lack of cpDNA sequence divergence among most polyploid species within each subgenus. All members of subgenus *Ceratochloa* (both hexaploids and octaploids) were identical in cpDNA fragment patterns. Similarly, polyploid species of subgenus *Festucaria* with the exception of *B. auleticus* revealed no cpDNA nucleotide sequence differences. The cpDNA of these species was digested with restriction endonucleases such as *EcoRI*, *BamHI* and *HindIII* that produced a large number of fragments, and thus were useful in revealing mutational events between closely related taxa. In contrast, the restriction endonucleases did reveal various numbers of mutational events in the diploid species.

The cpDNA results could imply that the two groups of polyploid species with identical restriction endonuclease site sequences had monophyletic plastome origins, and that they have not accumulated resolvable mutational events since their divergence because of the slow rate of evolution of the chloroplast genome. Stebbins (1981) suggested that 1) the archaic species of *Bromus* originated in Eurasia in the Miocene, 2) the polyploid species of *Ceratochloa* differentiated in Eurasia and their hexaploids spread to North America during the Pliocene, and 3) the octoploid species of *Ceratochloa* evolved in North America during the Pleistocene and Recent. Therefore, the lack of mutational differences among cpDNAs of the polyploids is possible if one considers a maternal monophyletic origin, Pliocene differentiation and the estimated synonymous rate of nucleotide substitution for cpDNA of 1.1 nucleotide substitution/site/ $10^{-9}$  (Zurawski et al. 1984), and the 0.7% sample of the cpDNA genomes surveyed in this study. The rates of synonymous nucleotide substitutions tend to be higher than the rates of non-synonymous substitution (Clegg

et al. 1984; Birky 1988), and the overall rate of cpDNA nucleotide substitution was estimated to be  $0.3-0.8 \times 10^{-9}$  (Birky 1988). Therefore, if those estimates are used, then the monophyletic plastome origin and the Pliocene differentiation becomes even more likely.

The literature on cpDNA restriction endonuclease analysis does not include any studies involving complex polyploid species such as in *Bromus*. However, it appears that diploid species generally show more variability in their restriction fragment patterns than their related polyploids. This situation was particularly true in *Triticum* and *Aegilops* (Bowman et al. 1983) and for groups of species in *Cucumis* (Perl-Treves and Galun 1985), *Beta* (Kishima et al. 1987), and *Dactylis* (Lumaret et al. 1989). In the case of *Triticum* and *Aegilops*, Tsunewaki (1989) has indicated that plasmon diversity decreases with the increase of ploidy level. While this pattern of the plastome is true in this study, the situation in *Bromus* subgenus *Ceratochloa* is an extreme in that nine species representing the full geographic distribution of the subgenus (both North and South America) were identical with respect to restriction sites of seven enzymes.

Members of subgenus *Ceratochloa* grouped together by virtue of their identical restriction site sequences (Fig. 4). However, species of subgenus *Festucaria* appeared in three clusters and clades (Figs. 5a,b). The cpDNA results suggest that the Eurasian species of subgenus *Festucaria* (*B. inermis*, *B. pumpellianus* and *B. biebersteinii*) are related to the North American diploids *B. porteri* (UPGMA, Fig. 2a) and *B. ciliatus* (Fitch and Margoliash method, Fig. 5b). On the other hand, the North American diploid species *B. anomalus* and the South American hexaploid *B. auleticus* show high affinities to members of *Ceratochloa*. The disparity between the UPGMA and the Fitch and Margoliash methods of analysis is in terms of the position of *B. ciliatus* and is not a significant one. *Bromus ciliatus* had higher F values and smaller genetic distance with the *B. pumpellianus* group and with *B. porteri* than with the other species of *Festucaria* and subgenus *Ceratochloa* (Table 2).

The presence of the species of *Festucaria* in three groups raises the question of the cohesiveness of the subgenus. Dumortier (1823, cited by Wagnon 1952) divided the species of what is now called *Festucaria* into sections *Bromopsis* and *Pnigma*. Armstrong (1983) indicated that the smaller chromosome species would fall under *Pnigma* while the larger chromosome species would fall under *Bromopsis*. This cpDNA study does not reflect that division since species with large chromosomes (*B. porteri*, *B. ciliatus*, *B. auleticus*, and *B. anomalus*) fall in two different lineages (Fig. 5). Armstrong (1983) also indicated that the results from cross compatibilities do not provide sufficient information for the subdivision of the section. A further study of the remaining subgenera of *Bromus* is needed before a decision can be made on the integrity of *Festucaria* as a subgenus.

In *Festucaria*, octoploids *Bromus inermis* and *B. pumpellianus* are considered subspecies since they intercross easily and their F<sub>1</sub> progeny are fertile (Elliot 1949a, 1949b; Nielsen et al. 1962). However, hybrids between their tetraploid cytotypes show some degree of disruption in chromosome pairing, but fertility was restored to 50% in amphiploids (Armstrong 1982, 1987). Based on the lower fertility at the tetraploid level, Armstrong (1987) suggested the treatment of *B. inermis* and *B. pumpellianus* as two different species. He indicated that the evolution of *B. inermis* and *B. pumpellianus* has probably involved autopolyploidy, allopolyploidy, and F<sub>1</sub> hybridization and genetic introgression between different populations and ploidy levels of the two species (Armstrong 1985). The cpDNA study emphasizes the strong genetic affinity between these two species. The cpDNA study also suggests that the decaploid *B. biebersteinii* shares at least a maternal genomic ancestor with *B. inermis* and *B. pumpellianus*.

The lack of cpDNA sequence variation in subgenus *Ceratochloa* presents a case where evolution in the chloroplast genome lags behind changes in the nuclear genome. Various degrees of intersterility barriers have evolved between species of the two complexes and among species of each complex. The wide distribution of the *B. carinatus* complex from Alaska to Mexico has given rise to morphologically intermediate populations. Hybrids formed between these populations range from being fertile to completely sterile, depending on the morphological similarity of the parent

populations (Stebbins 1981). The reproductive isolation was more pronounced in the *B. catharticus* complex where interspecific hybrids are either completely sterile as to seed set or weakly fertile (Stebbins 1949; Hall 1955). The two complexes are maintained as distinct entities since they are morphologically quite distinct and differ in the presence of the L genome in the *B. carinatus* group (Stebbins 1981). The disparity in the evolution of the chloroplast and nuclear genomes in these high polyploid complexes is in contrast with the coevolutionary changes between these genomes reported by Kung et al. (1982) and Timothy et al. (1979) in tobacco and maize.

The identical cpDNA nucleotide sequences of the two complexes of *Ceratochloa* indicates that the *B. catharticus* complex may have been the maternal parent in the proposed hybrid origin of the *B. carinatus* complex. Although there is no direct evidence for the paternal parent of the latter complex, the cpDNA study shows the complex to be phylogenetically related to the diploid *B. anomalus* of subgenus *Festucaria* (Fig. 5). This relationship provides indirect evidence that a diploid species of *Festucaria* might have been involved in the parentage of the *B. carinatus* complex.

The cpDNA divergence values between subgenera *Festucaria* and *Ceratochloa* are within the range of those found in other studies at the intrageneric level. The genetic distances between species in this study ranged from 0.0000 to 0.0082. The p values calculated for various monocot and dicot species belonging to the same genus ranged between 0.0017 to 0.0156 (Palmer and Zamir 1982; Clegg et al. 1984; Sytsma and Gottlieb 1986; Doebley et al. 1987). However, the genetic distance value of 0.0006 found between *B. anomalus* and the *B. catharticus* - *carinatus* complexes is very low for species belonging to taxa that are considered to be two separate genera (Tsvelev 1976) or even two subgenera (Stebbins 1981). These genetic distance values do not support Stebbins' statement that the relationships between subgenera *Festucaria* and *Ceratochloa* are obscure, and their chromosomes so dissimilar, that a recent common origin is difficult to imagine.

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## *References*

- Armstrong KC (1981) The evolution of *Bromus inermis* and related species of *Bromus* sect. *Pnigma*. Bot Jahrb Syst 102:427-443
- Armstrong KC (1982) Hybrids between the tetraploids of *Bromus inermis* and *Bromus pumpellianus*. Can J Bot 60:476-482
- Armstrong KC (1983) The relationship between some Eurasian and American species of *Bromus* section *Pnigma* as determined by the karyotypes of some F<sub>1</sub> hybrids. Can J Bot 61:700-707
- Armstrong KC (1985) Chromosome pairing in octoploid F<sub>1</sub> and octoploid amphiploid hybrids between *Bromus inermis* and *B. pumpellianus*. Can J Genet Cytol 27:538-541
- Armstrong KC (1987) Chromosome evolution in *Bromus*. In: Tsuchiya T, Gupta TK (eds) Chromosome engineering in plant genetics and breeding. Elsevier Science Publ. Amsterdam (in press)
- Birky CW (1988) Evolution and variation in plant chloroplast and mitochondrial genomes. In: Gottlieb LD, Jain SK (eds) Plant evolutionary biology. Chapman and Hall, London, pp 23-53
- Bowman CM, Bonnard G, Dyer TA (1983) Chloroplast DNA variation between species of *Triticum* and *Aegilops*. Location of the variation on the chloroplast genome and its relevance to the inheritance and classification of the cytoplasm. Theor Appl Genet 65:247-262
- Clayton WD (1981) Evolution and distribution of grasses. Ann Missouri Bot Garden 68:5-14
- Clegg MT, Rawson JRY, Thomas K (1984) Chloroplast DNA variation in pearl millet and related species. Genetics 106:449-461

- Dewey DR (1982) Genomic and phylogenetic relationships among North American perennial *Triticeae*. In: Estes JR, Tyrl RJ, Brunken JN (eds) Grasses and Grasslands. University of Oklahoma Press, Norman, Oklahoma, pp 55-58
- Doebley J, Renfroe W, Blanton A (1987) Restriction site variation in the *Zea* chloroplast genome. *Genetics* 117:139-147
- Elliot FC (1949a) The cytology and fertility relations of *Bromus inermis* and some of its relatives. *Agron J* 41:298-303
- Elliot FG (1949b) *Bromus inermis* and *B. pumpellianus* in North America. *Evolution* 3:142-149
- Fitch WM, Margoliash E (1967) Construction of phylogenetic trees. *Science* 155:279-284
- Gould FW, Shaw RB (1983) Grass Systematics. 2nd ed. McGraw-Hill Book Company, New York
- Hall BM (1955) Genetic analysis of interspecific hybrids in the genus *Bromus*, section *Ceratochloa*. *Genetics* 40:175-192
- Hilu KW (1988) Identification of the "A" genome of finger millet using chloroplast DNA. *Genetics* 118:163-167
- Hosaka K, Hanneman RE Jr. (1988) The origin of the cultivated tetraploid potato based on chloroplast DNA. *Theor Appl Genet* 76:172-176
- Hosaka K, de Zoeten GA, Hanneman RE Jr. (1988) Cultivated potato chloroplast DNA differs from the wild type by one deletion - evidence and implications. *Theor Appl Genet* 75:741-745
- Ichikawa H, Hirai A, Katayama T (1986) Genetic analysis of *Oryza* species by molecular markers for chloroplast genomes. *Theor Appl Genet* 72:353-358
- Jackson RC (1984) Chromosome pairing in species and hybrids. In: Grant WF (ed) Plant biosystematics. Academic Press, Toronto, pp 67-86
- Kaul MLH, Murthy TGK (1985) Mutant genes affecting higher plant meiosis. *Theor Appl Genet* 70:449-466
- Kemble RJ (1987) A rapid, single leaf, nucleic acid assay for determining the cytoplasmic organelle complement of rapeseed and related *Brassica* species. *Theor Appl Genet* 73:364-370
- Kirk JTO, Tilney-Bassett RAE (1978) The plastids. Elsevier/North-Holland, Amsterdam
- Kishima Y, Mikami T, Hirai A, Sugiura M, Kinoshita T (1987) *Beta* chloroplast genomes: analysis of fraction 1 protein and chloroplast DNA variation. *Theor Appl Genet* 73:330-336
- Kung SD, Zhu YS, Shen GF (1982) *Nicotiana* chloroplast genome III. Chloroplast DNA evolution. *Theor Appl Genet* 61:73-79
- Lumaret R, Bowman CM, Dyer TA (1989) Autopolyploidy in *Dactylis glomerata* L. : further evidence from studies of chloroplast DNA variation. *Theor Appl Genet* 78:393-399
- Nei M, Li W-H (1979) Mathematical model for studying genetic variation in terms of restriction endonucleases. *Proc Natl Acad Sci USA* 76:5269-5273



- Nielsen EL, Drolsom PN and Jalal SM (1962) Analysis of F<sub>2</sub> progeny from *Bromus* species hybrids. *Crop Sci* 2:459-462
- Palmer JD, Zamir D (1982) Chloroplast DNA evolution and phylogenetic relationships in *Lycopersicon*. *Proc Natl Acad Sci USA* 79:5006-5010
- Perl-Treves R, Galun E (1985) The *Cucumis* plastome: physical map, intrageneric variation and phylogenetic relationships. *Theor Appl Genet* 71:417-429
- Rohlf FJ (1989) Numerical taxonomy and the multivariate analysis system. Exeter Publishing, Ltd. New York
- Sears BB (1980) Elimination of plastids during spermatogenesis and fertilization in the plant kingdom. *Plasmid* 4:233-255
- Smith P (1970) Taxonomy and nomenclature of the brome grass (*Bromus* L. s.l.). *Notes Roy Bot Gard Edinburgh* 30:361-376
- Soltis DE, Soltis PS (1989) Allopolyploid speciation in *Tragopogon*: insights from chloroplast DNA. *Am J Bot* 76:1119-1124
- Stebbins GL (1947) The origin of the complex of *Bromus carinatus* and its phylogeographic implications. *Contr Gray Herb* 165:42-55
- Stebbins GL (1949) The evolutionary significance of natural and artificial polyploids in the family Gramineae. *Hereditas, Suppl.*:461-485
- Stebbins GL (1956) Cytogenetics and evolution in the grass family. *Am J Bot* 43:890-905
- Stebbins GL (1981) Chromosomes and evolution in the genus *Bromus* (Gramineae). *Bot Jahrb Syst* 102:359-379
- Stebbins GL, Tobgy HH (1944) The cytogenetics of hybrids in *Bromus*. 1. Hybrids within the section "*Ceratochloa*". *Am J Bot* 31:1-11
- Sytsma KJ, Gottlieb LD (1986) Chloroplast DNA evolution and phylogenetic relationships in *Clarkia* section *Peripetasma* (Onagraceae). *Evolution* 40:1248-1261
- Timothy DH, Levings CS III, Pring DR, Conde MF, Kermicle JL (1979) Organelle DNA variation and systematic relationships in the genus *Zea*: teosinte. *Proc Natl Acad Sci USA* 76:4220- 4224
- Tsunewaki K (1989) Plasmon diversity in *Triticum* and *Aegilops* and its implication in wheat evolution. *Genome* 31:143-154
- Tsunewaki K and Ogihara Y (1983) The molecular basis of genetic diversity among cytoplasm of *Triticum* and *Aegilops* species: II. On the origin of polyploid wheat cytoplasm as suggested by chloroplast restriction fragments. *Genetics* 104:155-171.
- Tsvelev NN (1976) Grasses of the Soviet Union Part 1. Nauka Publ., Leningrad, pp 298-339 [Translated from Russian]
- Wagon KH (1952) A revision of the genus *Bromus*, section Bromopsis, of North America. *Brittonia* 7:415-480

Zurawski G, Clegg MT, Brown AHD (1984) The nature of nucleotide sequence divergence between barley and maize chloroplast DNA. *Genetics* 106:735-749

Table 2. List of Bromus species, their chromosome numbers and sources of seed material used in this study

Species	Chromosome No.	Accession No.	Source of material
Subgenus <i>Festucaria</i>			
<i>B. porteri</i> (Coult.) Nash	14*	74-34	K. Armstrong, Canada
<i>B. ciliatus</i> L.	14*	387908	P.I. Station, Pullman
<i>B. anomalus</i> Rupr.	14*	232200	P.I. Station, Pullman
<i>B. auleticus</i> Trin. ex Nees	42*	162779	P.I. Station, Pullman
<i>B. inermis</i> Leyss.	56*		Sharp Bros. Seed Co.
<i>B. pumpellianus</i> Scribn.	56	70-140	K. Armstrong, Canada
<i>B. biebersteinii</i> Roem. et Schultz	70	372614	P.I. Station, Pullman
Subgenus <i>Ceratochloa</i>			
<i>B. catharticus</i> Vahl.	42*		Daehnfeldt Inc, Oregon
<i>B. coloratus</i> Steud.	42	202696	P. I. Station, Pullman
<i>B. stamineus</i> Desv.	42	251107	P. I. Station, Pullman
<i>B. brevis</i>	42	384465	P.I. Station, Pullman
<i>B. valdivianus</i> Phil.	42	368864	P.I. Station, Pullman
<i>B. carinatus</i> H.& A.	56*		Sharp Bros. Seed Co.
<i>B. sitchensis</i> Trin.	56	202534	P.I. Station, Pullman
<i>B. breviaristatus</i> Buckl.	56	392355	P.I. Station, Pullman

\* Chromosomes numbers confirmed by squashing technique

Table 3. Chloroplast DNA data matrix for 15 species of *Bromus* showing proportion of shared fragments (F values; upper right) and estimates of cpDNA nucleotide sequence divergence ( $\sigma \times 100$ ); lower left) following Nei and Li (1979)

	1	2	3	4	5	6	7	8
1. <i>B. porteri</i>	-	0.912	0.902	0.927	0.966	0.966	0.966	0.901
2. <i>B. anomalus</i>	0.516	-	0.859	0.984	0.931	0.931	0.931	0.988
3. <i>B. ciliatus</i>	0.576	0.852	-	0.866	0.921	0.921	0.921	0.863
4. <i>B. auleticus</i>	0.422	0.086	0.802	-	0.946	0.946	0.946	0.972
5. <i>B. pumpellianus</i>	0.192	0.397	0.458	0.306	-	1.000	1.000	0.920
6. <i>B. biebersteinii</i>	0.192	0.397	0.458	0.306	0.000	-	1.000	0.920
7. <i>B. inermis</i>	0.192	0.397	0.458	0.306	0.000	0.000	-	0.920
8. <i>B. catharticus</i> and <i>B. carinatus</i> complexes*	0.585	0.064	0.824	0.152	0.465	0.465	0.465	-

\* species belonging to these two complexes are listed in Table 2.

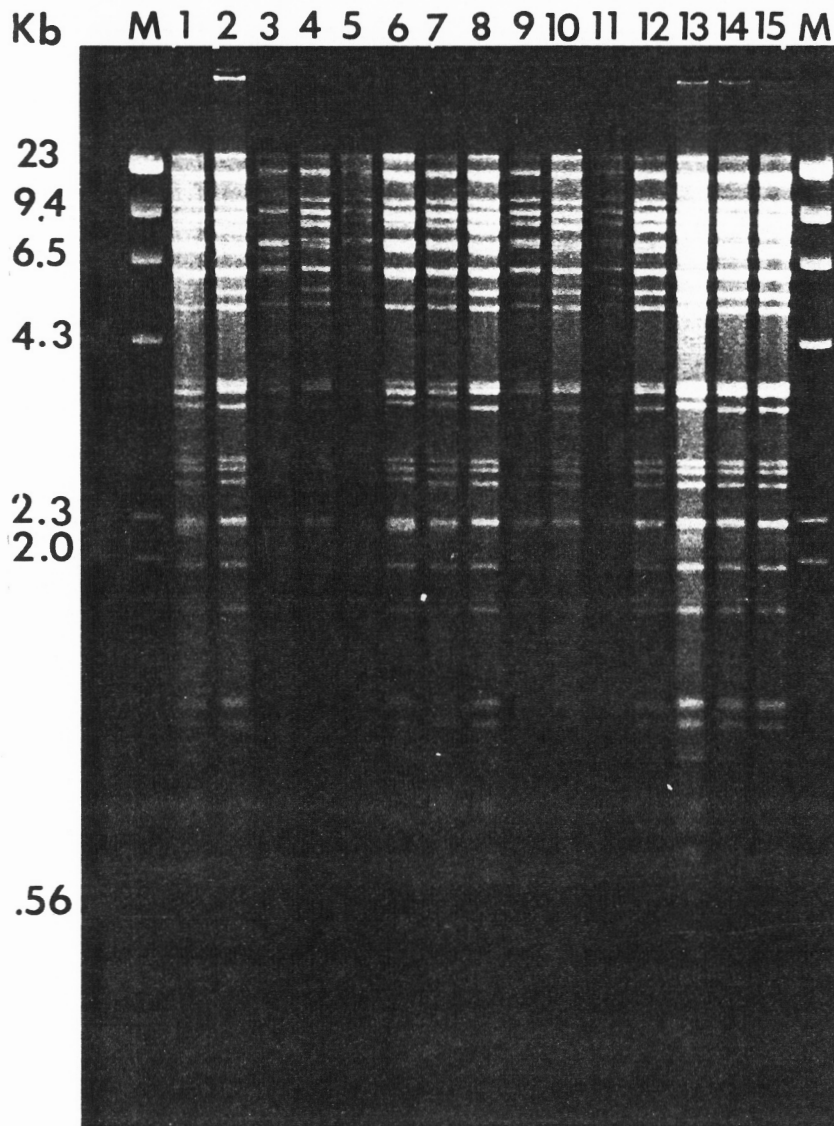


Figure 4. Hind III endonuclease restriction fragment patterns of cpDNAs from the fifteen species of Bromus.: *B. porteri* (1), *B. anomalus* (2), *B. ciliatus* (3), *B. auleticus* (4), *B. pumpellianus* (5), *B. biebersteinii* (6), *B. inermis* (7), *B. carinatus* (8), *B. breviaristatus* (9), *B. sichensis* (10), *B. catharticus* (11), *B. brevis* (12), *B. valdivianus* (13), *B. coloratus* (14) and *B. stamineus* (15). M = Lambda DNA- Hind III restriction fragments used as size markers

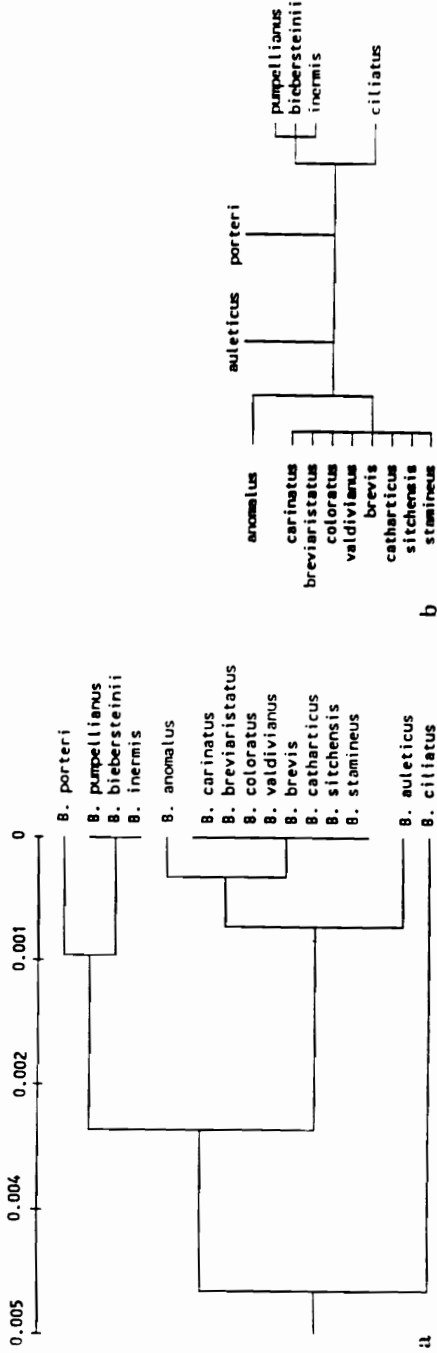


Figure 5. Phenogram and cladogram of the fifteen species of *Bromus* based on a genetic distance matrix calculated with Nei and Li's genetic distance measures. a) Phenogram based on the Unweighted pair-group method (UPGMA), b) cladogram based on the genetic distance method of Fitch and Margoliash (1967)

## *Chapter 2: Chloroplast DNA restriction site analysis in the genus Bromus L. (Poaceae)*

### *Summary*

Chloroplast DNA (cpDNA) restriction site variation was examined in 38 species of *Bromus*, using 10 restriction endonucleases, and filter hybridization experiments. Mutations were scored relative to rye, leading to a total of 38 phylogenetically informative restriction sites. Cladistic analysis indicates two major lines of evolution within the genus. One clade includes the perennial species of subgenera *Festucaria*, *Neobromus* and *Ceratochloa*, and the other comprises the annuals of *Stenobromus* and *Bromus*. The cpDNA trees indicate greater genetic relationships among the subgenera of *Bromus* when compared to results from morphology and cytogenetics. The cpDNA cladograms indicate that *Stenobromus* and *Bromus* are well separated from the other subgenera of the genus. The cladograms show that the annual species appear to be evolutionarily advanced in the genus. The cpDNA results were not able to differentiate species of subgenera *Stenobromus* and *Bromus*. The cpDNA results suggest that additional characters are needed to provide further

information on the taxonomy and evolution of the genus or perhaps a taxonomic re-evaluation of *Bromus* is warranted.

Key words: Chloroplast DNA - restriction analysis - phylogeny - *Bromus* - grass

## *Introduction*

*Bromus* L is comprised of approximately 130 annual and perennial species with a very wide geographical distribution. The genus has been divided variously into sections (Smith 1972), subgenera (Stebbins 1981) or even genera (Tsvelev 1976). The phylogenetic relationships between the various intrageneric divisions have not been investigated in detail. Earlier systematic investigations within the genus have depended on morphological, serological and cytogenetical evidence. These approaches led Stebbins (1981) to divide the genus into seven distantly related subgenera (*Festucaria*, *Ceratochloa*, *Neobromus*, *Stenobromus*, *Bromus*, *Nevskiella*, and *Boissiera*). The largest subgenus, *Festucaria*, with about 60 species, is highly developed in Eurasia and North and South America (Armstrong 1981). The inter-relationships of species in the subgenus are poorly known (Stebbins 1956; Armstrong 1987). Species with chromosome numbers ranging from diploid  $2n = 14$  to octoploid and decaploid indicate that highly developed polyploid complexes exist in this subgenus. The species of *Festucaria* are thought to have a common ancestry and possess different numbers of a common genome designated LL (Stebbins 1956; 1981). Subgenus *Ceratochloa* consists entirely of polyploids found in North and South America. *Ceratochloa* contains 7 hexaploid species endemic to South America and 10 octoploids distributed mainly in North America. There are no extant diploid and tetraploid species in *Ceratochloa*. Stebbins (1956) has indicated that cytological evidence shows that *Ceratochloa* has not descended from either diploids or tetraploids of *Festucaria* but has originated from now extinct diploid species. Two species complexes, based



on chromosome complements, certain morphological characters and geographic distribution, are recognized in *Ceratochloa* (Stebbins 1947). These are the octoploid *B. carinatus* complex of North America and the hexaploid *B. catharticus* complex of South America. The octoploid members of *Ceratochloa* need special mention. These species contain 42 small and 14 large chromosomes (Stebbins and Tobgy 1944). It has been suggested that the 14 large chromosomes were derived from diploid members of subgenus *Festucaria* such as *B. anomalus* and *B. ciliatus*, favoring a partly polyphyletic origin of the *B. carinatus* complex (Stebbins and Tobgy 1944). *Neobromus* is monotypic with the hexaploid *B. trinii* distributed in South America. It is thought that one genome of *Neobromus* has a weak affinity with a corresponding genome of *Ceratochloa* (Stebbins 1981). The two subgenera *Stenobromus* and *Bromus* contain about 50 diploid and tetraploid species and are concentrated chiefly in the Mediterranean area and the Near East. They are considered evolutionarily the most advanced taxa (Armstrong 1987). *Stenobromus* and *Bromus* are distantly related to *Festucaria* and are thought to have arisen from similar ancestral species. The main source of information concerning relationships between species and subgenera of *Bromus* has been the analysis of meiotic pairing in artificially produced inter- and intrageneric hybrids (Armstrong 1973, 1977, 1982, 1983, 1984b, 1985b; Knowles 1944; Stebbins 1947; Stebbins and Tobgy 1944; Walters 1952). While genome analysis has been used to identify groups of related species, analysis of experimentally produced hybrids is incapable of resolving relationships among taxa whose genomes show little or no affinity (Doyle et al. 1990) This is especially true in *Bromus* since chromosomes of the different subgenera are nearly or completely non-homologous and pairing affinities between species of different subgenera are weak or nonexistent (Stebbins 1981). The assessment of genome differentiation based on genome analysis may be further complicated in *Bromus* because 1) a comparatively wide range in chromosome sizes exists within and between subgenera, 2) of the difficulty of crossing species belonging to the different subgenera, and 3) there is some indication that chromosome pairing may be genetically controlled (Armstrong 1987, 1984). The use of genome analysis as a systematic tool has declined in recent years as exceptions to the correlation between pairing and genome homology have been discovered (Doyle et al. 1990).

Recent systematic investigations utilizing chloroplast DNA (cpDNA) data have enabled a reassessment of evolutionary relationships between *Festucaria* and *Ceratochloa* (Pillay and Hilu 1990). In the present study, cpDNA restriction site changes were used to evaluate the phylogenetic relationships among the *Bromus* subgenera *Festucaria*, *Ceratochloa*, *Neobromus*, *Stenobromus*, and *Bromus*. Chloroplast DNA has proved to be very useful for phylogenetic purposes at various taxonomic levels (reviewed by Palmer et al. 1988). The conservative rates of both structural change and nucleotide substitution of the chloroplast genome are the primary features exploited in phylogenetic studies (Palmer 1987). We have chosen the systematic scheme proposed by Stebbins (1956, 1981) as the framework for examining evolutionary relationships in the genus.

## ***Materials and methods***

The 38 species of *Bromus*, the outgroups oats and rye used in this study, and their sources are listed in Table 4. The sample represents the various ploidy levels and geographical range of the genus. *Nevskiella* and *Boissiera* are not represented in this study because of unavailability of seed material and thus will not be discussed. Chloroplast DNA isolation, digestion with restriction endonucleases, and electrophoretic separation of DNA fragments on agarose gels are described in Pillay and Hilu (1990). The following restriction enzymes were used: *AvaII*, *BamHI*, *BglI*, *DraI*, *EcoRI*, *HindIII*, *KpnI*, *Sall*, *XbaI*, and *XhoI*. The DNA fragments were nicked for 5 minutes under UV and transferred to nylon membranes (Zetaprobe from Bio-Rad) under high alkaline conditions (0.4M NaOH, 1M NaCl) (Reed and Mann 1985). The membranes were baked at 65° C for at least 2 hours.

### ***<sup>32</sup>P radiolabelling***

Plasmids containing the DNA probes were labelled by nick translation using the method of Rigby et al.(1977) with 50  $\mu$ Ci of <sup>32</sup>P  $\alpha$ -dCTP and 1  $\mu$ g of DNA per reaction. Unincorporated <sup>32</sup>P  $\alpha$ -dCTP was removed by centrifugation (Maniatis et al. 1982). The DNA probes were a library of 10 PstI cpDNA clones of barley (Fig. 6) provided by Dr. A. Day (University of Geneva) and Dr. THN Ellis (John Innes Institute). The probes cover about 98% of the chloroplast genome of *Bromus*.

### ***Hybridization with nick-translated probes***

The cpDNAs bound to the membranes were probed sequentially with the 10 PstI clones to resolve restriction site differences. Hybridization was carried out in sealed pouches (Dazey Corporation) at 65° C for 12 to 20 hours in 3X SSC, 20mM NaH<sub>2</sub>PO<sub>4</sub> pH 7, 7% SDS, 10X Denhardtts, denatured salmon sperm at 100  $\mu$ g/ml. After hybridization, the membranes were washed three times in 2X SSC/0.1% SDS at 65° C for 15 minutes with gentle agitation. A single wash in 1X SSC/0.5%SDS followed by another in 0.5X SSC/0.5% SDS were also used especially where gross cross hybridization was suspected. Autoradiograms were produced by exposing the hybridized nylon membranes to Kodak XAR-5 X-ray film in cassettes containing intensifying screens at -70° C from a few to several hours depending on the observed intensities. The Zetaprobe were stripped of probe by washing in a large volume of 0.1X SSC/0.5% SDS at 95° C before reprobing.

### ***Interpretation of Autoradiograms***

The molecular weight markers used in this study were *HindIII* digests of lambda DNA and the one kilobase marker (BRL). These were used to estimate the molecular weight of unknown fragments using a nonlinear regression analysis computer program. The estimated sizes of fragments were

used to infer phylogenetically informative mutations. Only those fragment pattern differences that appeared to result from at least a single point mutation, and that were shared by at least two species were used in the analysis. Restriction fragment length mutations ranging from 500-1500 bp were observed with multiple enzymes, but were not included in the phylogenetic analysis because restriction fragment comparison does not provide sufficient evidence to determine whether such changes are homologous. Mutations that appeared to be of more complex origin were scored but not used in the analyses. Such complex mutations were visible especially with the enzymes *DraI*, and *EcoRI*. Apparent restriction site gains, which resulted in a larger fragment being replaced by smaller ones, were scored as a 1; site losses were scored as 0 (Fig. 7, Table 6). In some cases, because of the proximity and sequence homology of some probes, more than one probe often hybridized to fragment patterns that resulted from a single mutation. Therefore, autoradiograms were extensively cross-checked to ensure that each mutation was used as a single character.

### *Analysis of data*

The character state matrix was subjected to parsimony analysis with HENNIG-86 (Farris 1988), and Wagner parsimony method using PHYLIP (phylogeny inference package, version 2.7) developed by J. Felsenstein (1987). The tree was rooted with rye as the outgroup species. The direction of the evolutionary change of restriction sites was determined by comparison with rye. Restriction patterns that were shared with the outgroup species were designated as the ancestral states.

## Results

The 100 probe/enzyme combinations, yielded 38 phylogenetically informative restriction sites listed in Table 5. The analysis from Hennig-86 produced 11 most parsimonious trees of 82 steps. The consistency index was 0.46 and the homoplasy 54%. Autapomorphies were not included in these calculations. The cpDNA phylogeny (Fig. 9) produced with Hennig-86 indicates that there are three distinctive lineages within *Bromus*. The basal group contains the diploid *B. porteri* of *Festucaria*. The second clade includes all the short- to long-lived perennials of *Festucaria*, *Ceratochloa* and *Neobromus*. Within this lineage there are two groups. One group includes *B. rigidus* of *Stenobromus* and all the Eurasian species of *Festucaria*. The other group includes the diploid *B. anomalus* and hexaploids *B. auleticus* and *B. runnsorensis* of *Festucaria*, *B. trinii* of *Neobromus* and all the species of *Ceratochloa*. The third lineage includes *B. ciliatus* of *Festucaria* and the remaining species of *Stenobromus* and subgenus *Bromus*. There is very little resolution of relationships in this lineage due to the paucity of cpDNA variation. The mixed parsimony algorithm of PHYLIP produced 63 trees with 82 steps. The topologies of the trees produced with PHYLIP and Hennig-86 were essentially the same. There were two notable differences. With PHYLIP, *B. rigidus* of *Stenobromus* formed an independent lineage, while *B. riparius*, *B. erectus* and *B. variegatus* grouped as a single clade independent of the other species of *Festucaria*. For this discussion, the tree (Fig. 9) representing most closely the strict consensus tree from Hennig-86 will be used.

## *Discussion*

### *Systematic implications of cpDNA phylogeny*

The phylogenetic analysis of the cpDNA restriction site variation provides a detailed evolutionary history of *Bromus*. Several evolutionary and systematic problems are raised by this cpDNA phylogeny when it is compared with existing systematic results from morphological, cytogenetical and serological characteristics.

The cpDNA phylogeny distinguishes the short- to long-lived perennials of subgenera *Festucaria*, *Ceratochloa* and *Neobromus* from the annual and biennial species of subgenera *Stenobromus* and *Bromus*. The position of *B. porteri* of *Festucaria* as the basal clade from which *Festucaria*, *Neobromus* and *Ceratochloa* have descended suggests a close genetic relationship among these subgenera. This result supports our previous studies (Pillay and Hilu 1990) that also showed the close genealogical relationship between *Festucaria* and *Ceratochloa*. In addition, this study indicates that subgenus *Neobromus* is also closely related to *Festucaria* and *Ceratochloa*. Stebbins (1981) had indicated that there is a weak relationship between one of the genomes of subgenera *Neobromus* and *Ceratochloa*. The cpDNA cladogram therefore does not support Stebbins' hypothesis that the chromosomes of these three subgenera are so dissimilar that it is unlikely they had a recent common origin.

The cpDNA restriction site data also suggest strong genetic affinity between subgenera *Stenobromus* and *Bromus* and show that *Stenobromus* and *Bromus* are distantly related to *Festucaria*, *Ceratochloa* and *Neobromus*. The cpDNA results suggest two main divisions of the genus *Bromus* (Fig. 9) and do not provide strong support for previous divisions of the genus *Bromus* into well defined sections (Smith 1972), subgenera (Stebbins 1981) or even genera (Tsvelev 1976). However, an important consideration has to be made when comparing results from cpDNA data with morphological,

biochemical and cytogenetic data. The cpDNA phylogeny represents a maternally inherited lineage while the systematic relationships proposed by Stebbins (1956, 1981) were derived from biparentally inherited characters.

The position of *Stenobromus* and *Bromus* supports the notion that these subgenera are the most evolutionarily advanced (Armstrong 1987). Stebbins (1981) has indicated that *Neobromus*, *Ceratochloa* and *Festucaria* differentiated in the Pliocene while the differentiation of *Stenobromus* and *Bromus* occurred in the Pleistocene and Recent. The cpDNA phylogeny seems to support such a pattern of origin for the different subgenera of *Bromus*.

The cpDNA restriction site data do not discriminate between species of *Stenobromus* and *Bromus* since they were not resolved in separate clades. Species of *Stenobromus* and *Bromus* are distinguishable on morphological and cytological considerations as belonging to distinct taxa (Kahler et al. 1981; Stebbins 1981). Smith (1972) has indicated that subgenus *Bromus* is genetically isolated from the other subgenera of *Bromus*. The cpDNA phylogeny, however, suggests a very high genetic affinity among species of these two complexes. This result is explicable if the low rate of evolution of the chloroplast genomes is considered together with the proposed recent origin of *Stenobromus* and *Bromus* (Stebbins 1981). The species of these two subgenera have not accumulated sufficient resolvable mutations to be distinguished as belonging to different subgenera.

The cpDNA results may be used to provide an answer to the questionable origin of *Bromus* and radiation of subgeneric groups. The cpDNA tree indicates that a single clade contains *B. anomalus*, *B. auleticus*, *B. trinii*, *B. runssorensis* and all species of *Ceratochloa*. These species are representatives of three continents and three subgenera. *Bromus auleticus* and *B. runssorensis* of *Festucaria* are found in South America and Africa, respectively. *Bromus trinii* of *Neobromus* is endemic to South America and so are all the species of *Ceratochloa*. This clade is closely related to the Eurasian species of *Festucaria*. If the similarity of the chloroplast genomes of these species indicates that they had a common origin, the cpDNA results could support Stebbins' proposed Eurasian origin for the genus *Bromus* and its subsequent radiation to the other continents.

## *cpDNA and species relationships in the different subgenera of Bromus*

**Festucaria** The distinct clade comprising the Eurasian species of *Festucaria* supports the nuclear genomic relationships observed between some of these species. It is known that *B. inermis*, *B. pumpellianus*, *B. riparius*, *B. ramosus*, *B. benekeii*, the tetraploid of *B. erectus* and the diploid of *B. variegatus* contain a similar genome (Armstrong 1973, 1984b, 1987). The cpDNA restriction site data therefore support shared maternal ancestry between the Eurasian species of *Festucaria*. The cpDNA results suggest a close genetic relationship between *B. ramosus* and *B. benekeii*. The *B. ramosus* complex is known to contain a unique nuclear genome (Armstrong 1987). *Bromus ramosus* and *B. benekeii* are the only Eurasian species of *Festucaria* with pinhead satellites (Armstrong 1982). *Bromus ramosus* is reported to have diploid, tetraploid and hexaploid cytotypes while *B. benekeii* is a tetraploid (Smith 1980). Stebbins (1981) has regarded the tetraploid *B. ramosus* as being conspecific with *B. benekeii* (Stebbins 1981). This study shows that the *B. ramosus* and *B. benekeii* do not have identical chloroplast genomes and are therefore unlikely to be conspecific. *Bromus benekeii* is apparently different from *B. ramosus* by its smooth or puberulent upper sheaths, more numerous lower panicle branches, and smaller spikelets (Rozhevits and Shishkin 1934).

Armstrong (1982), considering morphological and cytological characteristics, has suggested that the *B. ramosus* complex may be related to the North American diploids and tetraploids of *Festucaria*. This study shows that *B. porteri* is the only North American diploid that is remotely associated with the *B. ramosus* complex. However, in this regard it has been shown that the cpDNA genomes of diploid species in *Bromus* are generally more variable than those of polyploid species (Pillay and Hilu 1990). Therefore cpDNA may not be an ideal indicator of the proposed relationships between these groups of plants. The small satellited species of the Eurasia *Festucaria* are also considered to have played a role in the evolution of the *B. inermis*-*B. pumpellianus* complex (Armstrong 1981). The grouping of *B. pumpellianus*, *B. benekeii* and *B. ramosus* in one clade may support this idea. However, there is a considerable difference in size between the chromosomes of the small satellited



species and the higher polyploids (Armstrong 1982). Consequently, Armstrong (1982) suggested that the evolution of the large chromosome species of *Festucaria* occurred before the buildup of the polyploid complexes in the smaller chromosome species. If the assumption that the larger chromosome species of *Festucaria* evolved from the smaller chromosome species (Stebbins 1981) is correct, then the high similarity of the chloroplast genomes suggests that these species probably had a common origin.

**Ceratochloa** In a previous study (Pillay and Hilu 1990), it was shown that both hexaploid and octoploid members of *Ceratochloa* had identical cpDNA fragment patterns. In this study the cpDNA of an additional species, *B. marginatus*, was included in the sample. Furthermore, the cpDNAs were digested with three additional restriction endonucleases. These enzymes were *DraI*, *XbaI* and *XhoI* and produced a total of 81 new restriction sites. The chloroplast genomes of the nine species of *Ceratochloa* remained remarkably identical. We have already provided a possible explanation for the identical nature of these chloroplast genomes (Pillay and Hilu 1990).

This study also supports previous results that indicated a close relationship between *B. anomalus* and *B. auleticus* of *Festucaria* and the species of *Ceratochloa*. In addition this study shows that *B. runssorensis*, a hexaploid member of *Festucaria*, is even more closely related to *Ceratochloa*.

**Bromus** Previous studies on species relationships within subgenus *Bromus* are not extensive. Information on a few interspecific hybrids have been reported (Knowles 1944; Jahn 1959, cited by Armstrong 1987). Smith (1972) has reviewed the taxonomic history of the subgenus and proposed a classification based on morphology, serology, cytogenetics and geography. In that scheme (Table 6 of Smith 1972), a number of species pertinent to this study, such as *B. japonicus*, *B. adoensis*, *B. alopecuros* (*B. alopecuroides*) have been placed in separate groups reflecting the morphological and serological distinctness of these species. The chloroplast DNA results also reflect such a division of the species in this subgenus since a number of species are placed in different lineages. The distinctness of the cpDNA of species in this group is probably due to the fact that five of the ten

species examined were diploids. Knowles (1944) has shown that *B. mollis* and *B. racemosus* contain identical nuclear genomes. The clustering of these tetraploid species in a single clade suggests that they also possess very similar chloroplast genomes. The chloroplast DNA results place *B. japonicus* close to *B. mollis* and *B. racemosus*. Since nothing is currently known about the relationship of *B. japonicus* to either *B. mollis* or *B. racemosus*, this study suggests that the three species are cytoplasmically closely related. The cpDNA results do not support Smith's (1972) grouping of *B. racemosus*, *B. commutatus*, *B. secalinus* and *B. arduennensis* (*B. bromoideus*). *Bromus racemosus* and *B. commutatus* are morphologically very similar. The cpDNA results indicate that a single restriction site mutation separates *B. racemosus* from *B. commutatus*. *Bromus racemosus* and *B. commutatus* or closely related species are thought to be ancestors of *B. secalinus*. However, hybridization experiments (Knowles 1944) have suggested that *B. secalinus* is not closely related to either *B. racemosus* or *B. commutatus*. The cpDNA results also reflect such a relationship. This study suggests that the chloroplast genomes of *B. secalinus* and *B. alopecuros* are very similar. However, the rDNA coding sequences of these two species differ in the arrangement of the *BstEII* restriction sites. Furthermore, Smith (1972) has placed these two species in quite different categories. Further work is required to clarify the inconsistency of chloroplast and nuclear DNA results concerning the relationships between *B. secalinus* and *B. alopecuros*. Similarly this study places *B. arduennensis* (*B. bromoideus*) very close to *B. adoensis* and *B. popovii*. *Bromus popovii* is not included in Smith's (1972) classification but *B. arduennensis* and *B. adoensis* are placed in two very different groups. The relationship of *B. popovii* to *B. arduennensis* and *B. adoensis* is not known. *Bromus popovii* and *B. anatolicus* have similar rDNA coding sequences. The close grouping of their chloroplast genomes suggests that *B. anatolicus* and *B. popovii* may also be related maternally.

**Stenobromus** Nothing is known about either the nuclear genomic relationships or the number of genomes present in subgenus *Stenobromus*. *Bromus tectorum* and *B. sterilis* were identical in cpDNA restriction sites. These diploid species are morphologically distinct with *B. sterilis* having longer spikelets and longer awns (Mohlenbrock 1972). The cpDNA results suggest that these

species were recently derived from common maternal ancestry and their chloroplast genomes have not gained any resolvable mutations. *Bromus madritensis* and *B. rubens* are the only other species of this subgenus that show cpDNA affinity. These species resemble each other in general habit and various morphological features and are distinguished only by the number and length of spikelets and the length of the rachilla (Yavin 1969). Bor (1968) also emphasized the close morphological relationship between *B. rubens* and *B. madritensis* since depauperate plants of these two species are hard to separate. Since both species are tetraploids, the similarity of their cpDNAs suggests a common ancestry.

The anomalous grouping of *B. rigidus* of *Stenobromus* with species of *Festucaria* needs further attention since *B. rigidus* is morphologically very different. In this regard the trees generated with Wagner parsimony of PHYLIP always placed *B. rigidus* in an independent lineage. Similarly the grouping of *B. ciliatus* of *Festucaria* with *Stenobromus* and *Bromus* was not expected and is probably the result of homoplasy. Alternatively, the cpDNA data are supportive of Stebbins' (1981) hypothesis that *Festucaria*, *Stenobromus* and *Bromus* have descended from similar ancestral species. These subgenera, although very distinct in chromosome morphology, do share a few morphological characteristics such as the length and nervation of glumes and lemmas (Stebbins 1981).

### ***Neobromus***

This monotypic subgenus represented by *B. trinii*, groups closely with *B. auleticus* and *B. runsorensis* of *Festucaria* and the *Ceratochloa* species. The hexaploid *B. auleticus* is the only South American member of *Festucaria*. Morphological differences between these two species are too great to suggest common ancestry. An alternative explanation is that the species did have a recent common ancestry but morphological divergence was greater. This explanation is plausible considering the slow rate of cpDNA evolution. The similarity of chloroplast genomes in

*Neobromus* and *Ceratochloa* supports Stebbins' (1981) hypothesis that these subgenera contain a homoeologous genome.

The cladistic analysis of restriction mutations for cpDNA was useful for constructing a phylogeny of *Bromus* even though the relationships of some groups of closely related species were not satisfactorily resolved. The cpDNA phylogeny suggests a much closer relationship among the different intrageneric divisions of *Bromus* than relationships portrayed by morphological and cytogenetical evidence. This study suggest that additional characters are needed to provide further information on the taxonomy and evolution of this genus. Perhaps the taxonomy of the genus might need a serious re-evaluation.

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## *References*

- Armstrong KC (1973) Chromosome pairing in hexaploid hybrids from *Bromus erectus* x *B. inermis* (2n = 56). *Can J Genet Cytol* 15:427-436
- Armstrong KC (1977) Hybrids of the annual *Bromus arvensis* with perennial *B. inermis* and *B. erectus*. *Can J Genet Cytol* 21:65-71
- Armstrong KC (1981) The evolution of *Bromus inermis* and related species of *Bromus* sect. *Prigma*. *Bot Jahrb Syst* 102:427-443
- Armstrong KC (1982) Hybrids between the tetraploids of *Bromus inermis* and *Bromus pumpellianus*. *Can J Bot* 60:476-482

- Armstrong KC (1983) The relationship between some Eurasian and American species of *Bromus* section *Prigma* as determined by the karyotypes of some F<sub>1</sub> hybrids. *Can J Bot* 61:700-707
- Armstrong KC (1984a) Chromosome pairing affinities between Old and New World species of *Bromus* section *Prigma*. *Can J Bot* 62:581- 585
- Armstrong KC (1984b) The genomic relationship of the diploid *Bromus variegatus* to *Bromus inermis*. *Can J Genet Cytol* 26(4):469- 474
- Armstrong KC (1985a) Chromosome pairing in octoploid F<sub>1</sub> and octoploid amphiploid hybrids between *Bromus inermis* and *B. pumpehianus*. *Can J Genet Cytol* 27:538-541
- Armstrong KC (1985b) Chromosome pairing failure in an intersectional amphiploid of *Bromus altissimus* x *B. arvensis*. *Can J Genet Cytol* 27:705-709
- Armstrong KC (1987) Chromosome evolution in *Bromus*. In: Tsuchiya T, Gupta TK (eds) *Chromosome engineering in plant genetics and breeding*. Elsevier Science Publ., Amsterdam (in press)
- Bor NL (1968) Gramineae In: Townsend CC, Guest E, Al-Rawi A (eds) *Flora of Iraq*, Vol 9. Published by Ministry of Agriculture, Baghdad
- Clayton WD (1981) Evolution and distribution of grasses. *Ann Missouri Bot Garden* 68:5-14
- Clegg MT, Rawson JRY, Thomas K (1984) Chloroplast DNA variation in pearl millet and related species. *Genetics* 106:449-461
- Day A, Ellis THN (1985) Deleted forms of plastid DNA in albino plants from cereal anther culture. *Curr Genet* 9:671-678
- Doyle JJ, Doyle JL, Brown ADH (1990) A chloroplast-DNA phylogeny of the wild perennial relatives of soybean (*Glycine* subgenus *Glycine*): congruence with morphological and crossing groups. *Evolution* 44:371-389
- Farris JS (1988) Hennig-86 ver 1.5 (computer software and manual) [published by author]
- Felsenstein J (1987) PHYLIP ver 3.0 (computer software and manual) [published by author]
- Kahler AL, Krzakowa M, Allard RW (1981) Isozyme phenotypes in five species of *Bromus* sect. *Genea*. *Bot Jahrb Syst* 102:401-409.
- Maniatis T, Fritsch EF, Sambrook J (1982) *Molecular Cloning. A Laboratory Manual*. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York
- Mohlenbrock RH (1972) *Grasses - Illustrated Flora of Illinois. Grasses. Bromus to Paspalum* Southern Illinois University Press, Carbondale
- Knowles PF (1944) Interspecific hybridization of *Bromus*. *Genetics* 29:128-140
- Palmer JD (1987) Chloroplast DNA evolution and biosystematic uses of chloroplast DNA variation. *Am Natur Suppl.* 130:S6-S29
- Palmer JD, Jansen RK, Michaels HJ, Chase MW, Manhart JR (1988) Chloroplast DNA variation and plant phylogeny. *Ann Missouri Bot Gard* 75:1180-1206

- Pillay M, Hilu KW (1990) Chloroplast DNA variation in diploid and polyploid species of *Bromus* (Poaceae) subgenera *Festucaria* and *Ceratochloa*. *Theor Appl Genet* 80:326-332
- Reed KC, Mann DA (1985) Rapid alkaline transfer of DNA from agarose gels to nylon membranes. *Nucleic Acid Res* 13:6207-7221
- Rigby PWJ, Dieckmann M, Rhodes C, Berg P (1977) Labelling deoxyribonucleic acid to high specific activity in vitro by nick translation with DNA polymerase I. *J Mol Biol* 113:237-251
- Rozhevits RYu, Shishkin BK (1934) *Flora of the USSR, vol II*. Izdatel'stvo Akademii Nauk SSSR, Leningrad
- Smith PM (1972) Serology and species relationships in annual Bromes (*Bromus* L. sect. *Bromus*) *Ann Bot* 36:1-30
- Smith PM (1980) *Bromus* L. In: Tutin TG, Heywood VH, Burges NA, Moore DM, Valentine DH, Walters SM, Webb DA (eds) *Flora Europaea*. Cambridge University Press, Cambridge, pp 182-189
- Stebbins GL (1947) The origin of the complex of *Bromus carinatus* and its phytogeographic implications. *Contr Gray Herb* 165:42-55
- Stebbins GL (1956) Cytogenetics and evolution in the grass family. *Am J Bot* 43:890-905
- Stebbins GL (1981) Chromosomes and evolution in the genus *Bromus* (Gramineae). *Bot Jahrb Syst* 102:359-379
- Stebbins GL, Tobgy HH (1944) The cytogenetics of hybrids in *Bromus*. 1. Hybrids within the section "*Ceratochloa*". *Am J Bot* 31:1-11
- Walters MS (1952) Spontaneous chromosome breakage and atypical chromosome movement in meiosis of the hybrid *Bromus marginatus* x *B. pseudolaevipes*. *Genetics* 37:8-25
- Tsvelev NN (1976) *Grasses of the Soviet Union Part 1*. Nauka Publ., Leningrad [Translated from Russian] pp 298-339
- Yavin ZO (1969) Cytotaxonomy of the genus *Bromus* of Palestine. *Israel J Bot* 18:195-216

**Table 4. List of Bromus species, their chromosome numbers and sources of seed material used in this study.**

Species	Chromosome No.	Accession No.	Source of material
<b>Subgenus <i>Festucaria</i></b>			
1 <i>B. anomalus</i> Rupr.	14*	232200	P.I. Station, Pullman
2 <i>B. ciliatus</i> L.	14*	387908	P.I. Station, Pullman
3 <i>B. porteri</i> (Coult.) Nash	14*	74-34	K. Armstrong, Canada
4 <i>B. benekenii</i> (Lange) Trimen	28	440181	P.I. Station, Pullman
5 <i>B. ramosus</i> Huds.	28	253299	P.I. Station, Pullman
6 <i>B. auleticus</i> Trin. ex Nees	42*	162779	P.I. Station, Pullman
7 <i>B. erectus</i> Huds.	42	383616	P.I. Station, Pullman
8 <i>B. runssorensis</i> K. Schum.	42	273869	P.I. Station, Pullman
9 <i>B. variegatus</i> M. Bieb.	42	325233	P.I. Station, Pullman
10 <i>B. inermis</i> Leyss.	56*		Sharp Bros. Seed Co.
11 <i>B. pumpellianus</i> Scribn.	56	70-140	K. Armstrong, Canada
12 <i>B. biebersteinii</i> Roem. et Schult.	70	372614	P.I. Station, Pullman
13 <i>B. riparius</i> Rehm.	70	440214	P.I. Station, Pullman
<b>Subgenus <i>Ceratochloa</i></b>			
14 <i>B. brevis</i>	42	384465	P.I. Station, Pullman
15 <i>B. catharticus</i> Vahl.	42*		Daehnfeldt Inc., Oregon
16 <i>B. coloratus</i> Steud.	42	202696	P.I. Station, Pullman
17 <i>B. stamineus</i> Desv.	42	238251	P.I. Station, Pullman
18 <i>B. valdivianus</i> Phil.	42	368864	P.I. Station, Pullman
19 <i>B. breviaristatus</i> Buckl.	56	392355	P.I. Station, Pullman
20 <i>B. carinatus</i> H.& A.	56*		Sharp Bros Seed, Kansas
21 <i>B. marginatus</i> Nees.	56		Jacklin Seed Co., Idaho
22 <i>B. sitchensis</i> Trin.	56	202534	P.I. Station, Pullman
<b>Subgenus <i>Stenobromus</i></b>			
23 <i>B. madritensis</i> L.	14	442459	P.I. Station, Pullman
24 <i>B. sterilis</i> L.	14	442456	P.I. Station, Pullman
25 <i>B. tectorum</i> L.	14	483443	P.I. Station, Pullman
26 <i>B. rubens</i> L.	28	487414	P.I. Station, Pullman
27 <i>B. rigidus</i> Roth	42	442462	P.I. Station, Pullman
<b>Subgenus <i>Bromus</i></b>			
28 <i>B. alopecuroides</i> Poir.	14	202674	P.I. Station, Pullman
29 <i>B. arduennensis</i> (Lej.) Crep.	14	442454	P.I. Station, Pullman
30 <i>B. anatolicus</i> Boiss. et Heldr.	14	204861	P.I. Station, Pullman
31 <i>B. japonicus</i> Thunb.	14	442461	P.I. Station, Pullman
32 <i>B. popovii</i> Drob.	14	283198	P.I. Station, Pullman
33 <i>B. adoensis</i> Hochst.	28	202531	P.I. Station, Pullman
34 <i>B. commutatus</i> Schrader	28	205287	P.I. Station, Pullman
35 <i>B. secalinus</i> L.	28	442463	P.I. Station, Pullman
36 <i>B. mollis</i> L.	28	469232	P.I. Station, Pullman
37 <i>B. racemosus</i> L.	28	362114	P.I. Station, Pullman
<b>Subgenus <i>Neobromus</i></b>			
38 <i>B. trinii</i> Desv.	42	202154	P.I. Station, Pullman

\* Chromosomes numbers confirmed by squashing technique.

**Table 5. Chloroplast DNA restriction site mutations used in phylogenetic analyses of 38 species of Bromus.**

Mutations are listed with ancestral states listed first, followed by the derived state. Ancestral state was determined by comparison with the outgroup. Taxa sharing mutations are listed by the numbers given in table 4.

No.	Enzyme	Probe	Mutation	Mutated species
1	<i>AvaII</i>	P2	1.6 + 0.6 = 2.2	7, 8, 14-22
2	<i>AvaII</i>	P3	2.7 = 1.6 + 1.1	1, 2, 23-25, 28-30, 32-37
3	<i>AvaII</i>	P4	6.1 = 3.4 + 2.7	6, 12, 13
4	<i>AvaII</i>	P4	6.1 = 3.1 + 3.0	1, 2, 23, 24
5	<i>AvaII</i>	P4	3(x2) + 1.5 = 7.9	30, 31, 33, 34
6	<i>AvaII</i>	P6/P9	2.8 = 1.7 + 1.1	4, 5, 6, 9-13, 27
7	<i>BamHI</i>	P2	6.2 = 4.8 + 1.4	2, 23-26, 28-37
8	<i>BamHI</i>	P4	3.9 = 2.8 + 1.1	2, 23, 24, 28-37
9	<i>BamHI</i>	P5	3.7 + 6.5 = 10.1	3, 8, 14-22, 38
10	<i>BglI</i>	P1/P3	41.3 = 31 + 10.3	4, 5, 9-11, 27
11	<i>BglI</i>	P2	12.5 + 3.8 = 16.3	29, 32
12	<i>BglI</i>	P4	17.1 + 6.2 = 23.3	1-5, 7, 8, 9-11, 13, 14-38
13	<i>DraI</i>	P1	5.0 = 2.6 + 2.4	6, 24, 25, 29, 30, 32, 33, 38
14	<i>DraI</i>	P1	2.6 = 1.6 + 1.0	24, 25, 29, 30, 32, 33
15	<i>DraI</i>	P2	2.3 = 1.7 + 0.6	7, 9, 27
16	<i>DraI</i>	P3	2.8 = 1.8 + 1.0	24, 25, 32
17	<i>DraI</i>	P3	2.8 = 1.4 + 1.4	14-22, 38
18	<i>DraI</i>	P3	2.9 = 2.0 + 0.9	23, 26, 27
19	<i>DraI</i>	P6/P9/P10	5.0 = 2.6 + 2.4	7, 9
20	<i>DraI</i>	P6/P10	1.5 + 1.0 = 2.5	1, 8, 14-22
21	<i>EcoRI</i>	P1	4.5 = 2.5 + 2.0	6, 12, 13, 27
22	<i>EcoRI</i>	P1	4.5 = 2.9 + 1.6	2, 23-26, 28-37
23	<i>EcoRI</i>	P1/P3	4.1 = 3.3 + 0.7	12, 14-22, 38
24	<i>EcoRI</i>	P2	3.8 = 2.3 + 1.5	6, 12, 13, 27
25	<i>EcoRI</i>	P4/P7	1.4 = 1.1 + 0.3	7, 14-22
26	<i>EcoRI</i>	P7	6.3 = 5.8 + 0.5	7, 16-22
27	<i>EcoRI</i>	P7	6.7 = 5.8 + 0.9	7, 14-22
28	<i>EcoRI</i>	P8	15.7 = 14.0 + 1.7	2, 23-26, 28-37
29	<i>HindIII</i>	P2/P3	8.1 = 6.6 + 1.5	2, 8, 23-26, 28-37
30	<i>HindIII</i>	P4	10.2 = 7.3 + 2.9	2, 6, 8, 12, 3, 23-26, 38
31	<i>HindIII</i>	P5/P7	6.1 = 5.7 + 0.4	7, 14-22, 38
32	<i>KpnI</i>	P4	6.4 + 10.2 = 17	3, 7, 8, 14-22, 38
29	<i>XbaI</i>	P1	7.5 = 5.2 + 2.3	2, 7, 14-22, 38
33	<i>XbaI</i>	P6/P8/P9/P10	23.7 = 16.2 + 7.5	2, 4, 5, 6, 8, 9-13
34	<i>XbaI</i>	P7	16.2 = 10.5 + 5.7	28-37
35	<i>XhoI</i>	P1	6.2 = 3.9 + 2.3	3, 4, 8, 6, 7, 12-22, 27, 38.
36	<i>XhoI</i>	P4	20.3 = 15.5 + 4.8	2, 23, 24, 30, 33, 34
37	<i>XhoI</i>	P5/P7	20 = 11.7 + 8.9	3, 8, 7, 14-22, 38
38	<i>XhoI</i>	P8/P9	17.4 + 6.4 = 23.8	3, 6-8, 12, 14-22



Table 6. Table showing presence (1) or absence (0) of apomorphic restriction sites (RS) among species of *Bromus* and the outgroup species, rye.

Names of taxa are arranged in order as listed in Table 4. RS arranged by enzyme.

	1	2	3	4	5	6	7	8	9	0	1	2	3	4	5	6	7	8	9	0	1	2	3	4	5	6	7	8	9	0	1	2	3	4	5	6	7	8													
	*					*					*					*						*					*					*				*															
rye	0	0	1	0	0	0	0	0	0	0	0	0	0	0	1	1	1	0	0	1	0	1	1	1	1	0	0	1	1	0	0	1	1	0	1	1	1	1													
<i>anomalous</i>	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	1	0	0	0	0	0	0	0	0	0	0	0	1	0	1	1	1	0	0	1	0	0	0	0											
<i>ciliatus</i>	0	1	0	0	0	1	1	1	0	1	0	1	1	1	0	1	0	0	1	0	0	0	1	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0											
<i>porteri</i>	0	1	0	0	0	1	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0											
<i>benekenii</i>	0	0	0	0	1	0	0	0	0	1	0	0	0	0	0	1	1	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0										
<i>ramosus</i>	0	0	0	0	1	0	0	0	0	1	0	0	0	0	0	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0										
<i>auleticus</i>	0	0	0	1	0	0	1	1	0	1	0	0	0	0	0	1	1	0	0	0	0	0	0	0	0	0	0	1	0	1	1	1	0	0	0	1	0	0	0	0											
<i>erectus</i>	1	0	0	0	1	0	0	1	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	1	0	1	0	1	0	1	0	0	1	1	0	0	0	0									
<i>runssorensis</i>	0	0	0	1	0	0	0	0	1	0	0	1	0	0	0	1	0	0	0	1	0	0	0	0	0	0	1	1	0	1	1	1	0	0	1	0	0	0	0	0	0										
<i>variegatus</i>	1	0	0	0	1	0	0	1	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	1	0	0	0	1	0	1	0	0	1	1	0	0	0	0	0									
<i>inermis</i> \$	0	0	0	0	1	0	0	0	0	1	0	0	0	0	0	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0								
<i>riparius</i>	1	0	0	0	1	0	0	1	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	1	0	0	1	0	1	0	0	0	0	0	0	0	0	0	0	0								
<i>brevis</i> #	0	0	0	1	0	0	0	1	0	0	1	0	0	1	1	0	0	0	1	1	0	0	0	0	1	1	1	0	1	1	0	0	1	0	0	1	0	0	0	0	0	0	0	0							
<i>madritensis</i>	0	0	0	0	0	1	1	0	0	0	0	1	0	0	0	1	0	0	0	1	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0						
<i>sterilis</i> &	0	1	0	0	0	1	1	1	0	0	0	0	1	1	0	1	0	0	1	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0					
<i>rubens</i>	0	0	0	0	0	1	1	1	0	0	0	0	0	0	1	0	1	0	0	1	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0				
<i>rigidus</i>	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	1	1	0	0	0	1	1	0	0	0	1	1	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0				
<i>alopeurus</i>	0	0	0	0	0	1	1	1	0	0	1	0	1	1	0	1	0	1	0	1	1	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0			
<i>arduennensis</i>	0	0	1	0	0	1	1	1	0	0	1	0	1	1	0	1	0	0	0	1	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0			
<i>anatolicus</i>	0	0	1	0	0	0	1	1	0	0	1	0	1	1	0	1	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0			
<i>japonicus</i>	0	0	0	0	0	1	1	1	0	0	1	0	1	1	0	1	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0		
<i>popovii</i>	0	0	1	0	0	1	1	1	0	0	1	0	1	1	0	1	0	0	0	1	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0		
<i>adoensis</i>	0	0	1	0	0	1	1	1	0	0	1	0	1	1	0	1	0	0	0	1	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0		
<i>commutatatus</i>	0	0	0	0	0	1	1	1	0	0	1	0	0	1	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	
<i>secalinus</i>	0	0	0	0	0	1	1	1	0	0	1	0	1	0	1	0	1	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	
<i>mollis</i>	0	0	0	0	0	1	1	1	0	0	1	0	1	0	1	0	1	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	
<i>racemosus</i>	0	0	0	0	0	1	1	1	0	0	1	0	1	0	1	0	1	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
<i>trinii</i>	0	0	0	1	0	0	0	1	1	0	0	1	0	0	1	1	0	0	0	0	0	0	0	0	0	0	1	0	1	0	0	0	1	1	0	0	0	1	0	0	0	1	0	0	0	0	0	0	0	0	0

\* marks first character for each enzyme RS set as follows *AvaII*, *HindIII*, *XbaI*, *BamHI*, *BglI*, *EcoRI*, *XhoI*, *KpnI*, *DraI*  
 \$ same as *B. pumpellianus* and *B. biebersteinii*  
 # same in all species of the *Ceratochloa*  
 & same as *B. tectorum*

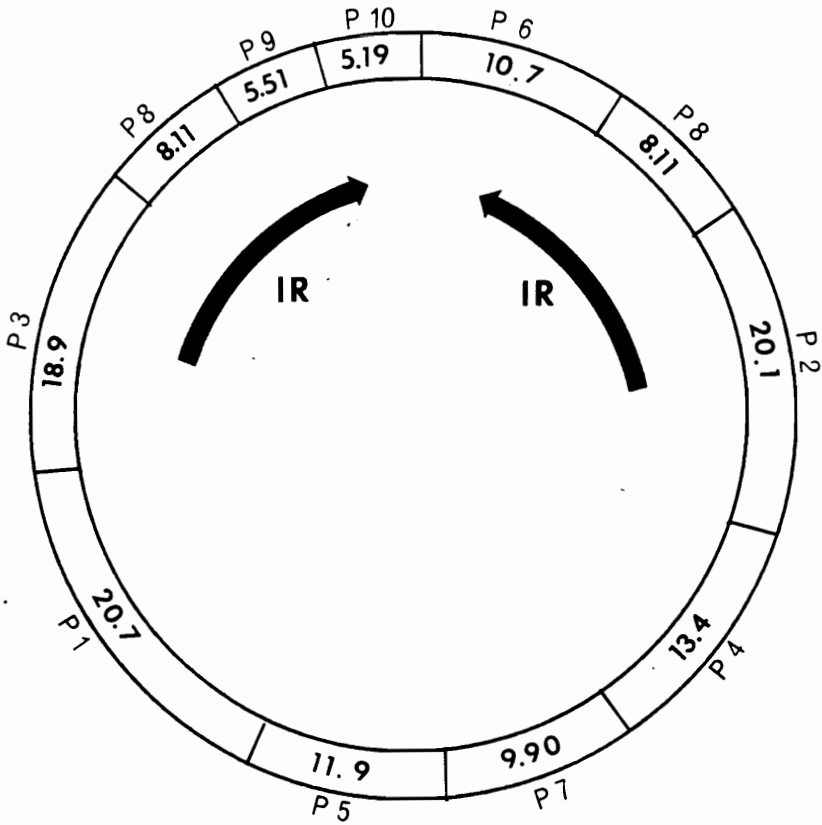


Figure 6. Location of heterologous chloroplast DNA clones used against *Bromus* DNAs. Clones represent Pst I fragments from barley.

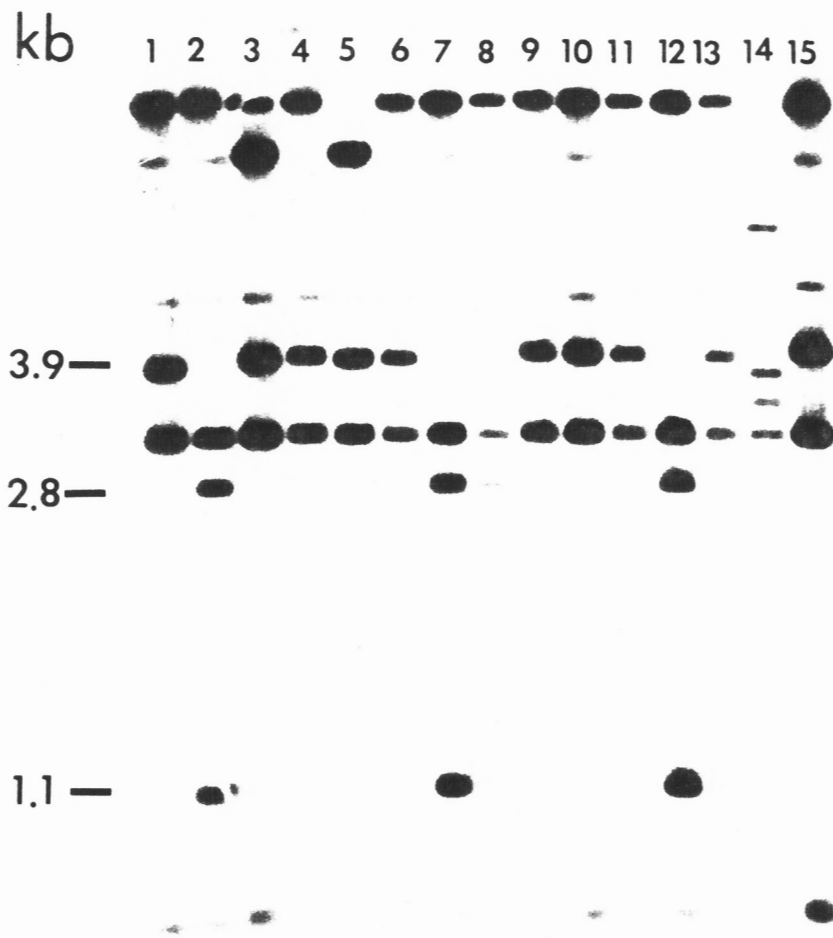


Figure 7. Typical autoradiogram showing hybridization analysis of cpDNA restriction site mutation in *Bromus*. Species and lanes : *B. porteri* (1), *B. ciliatus* (2)\*, *B. auleticus* (3), *B. inermis* (4), *B. carinatus* (5), *B. trinii* (6), *B. tectorum* (7)\*, *B. sterilis* (8)\*, *B. rubens* (9), *B. madritensis* (10), *B. rigidus* (11), *B. adoensis* (12)\*, *B. commutatus* (13), oats (14), and rye (15). Numbers at left indicate fragment sizes in kb. \* indicates restriction site gain ( $3.9 = 2.8 + 1.1$ ).

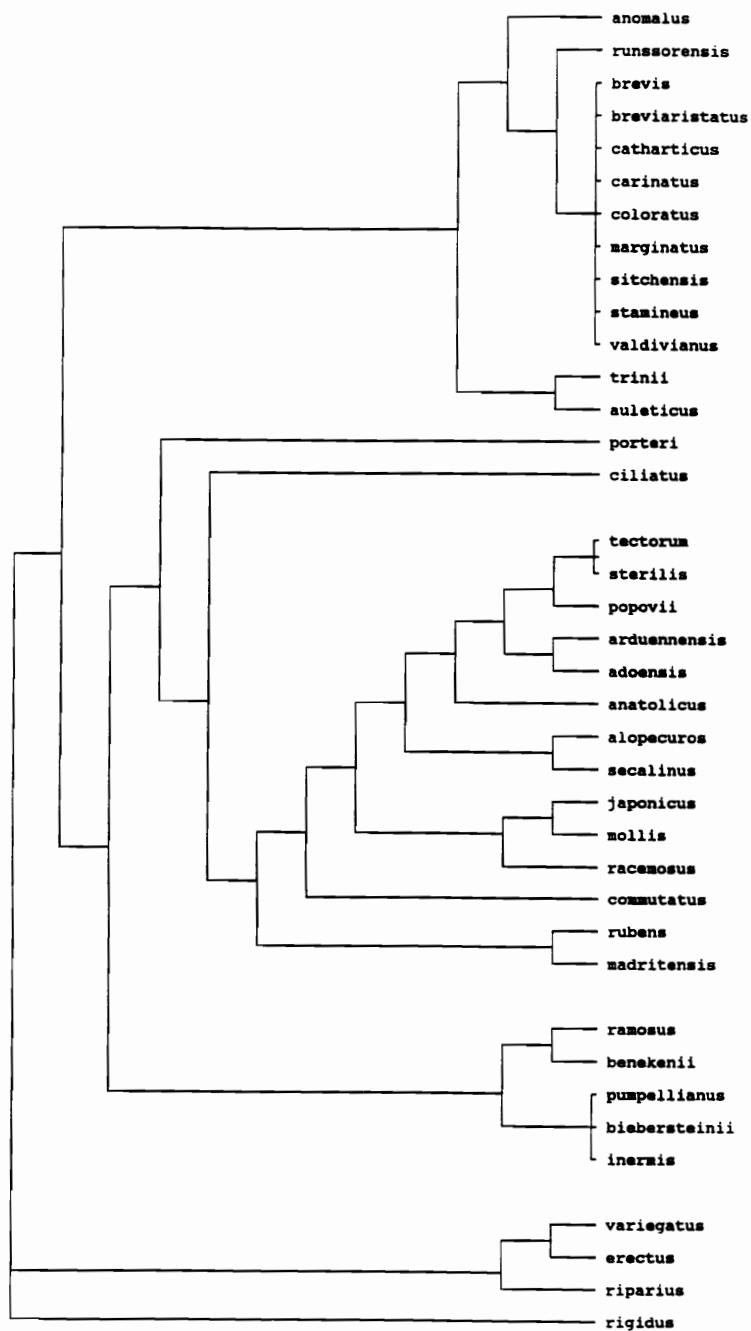


Figure 8. One of the most parsimonious trees of *Bromus* chloroplast DNA restriction sites generated by PHYLIP. The tree is 82 steps long.

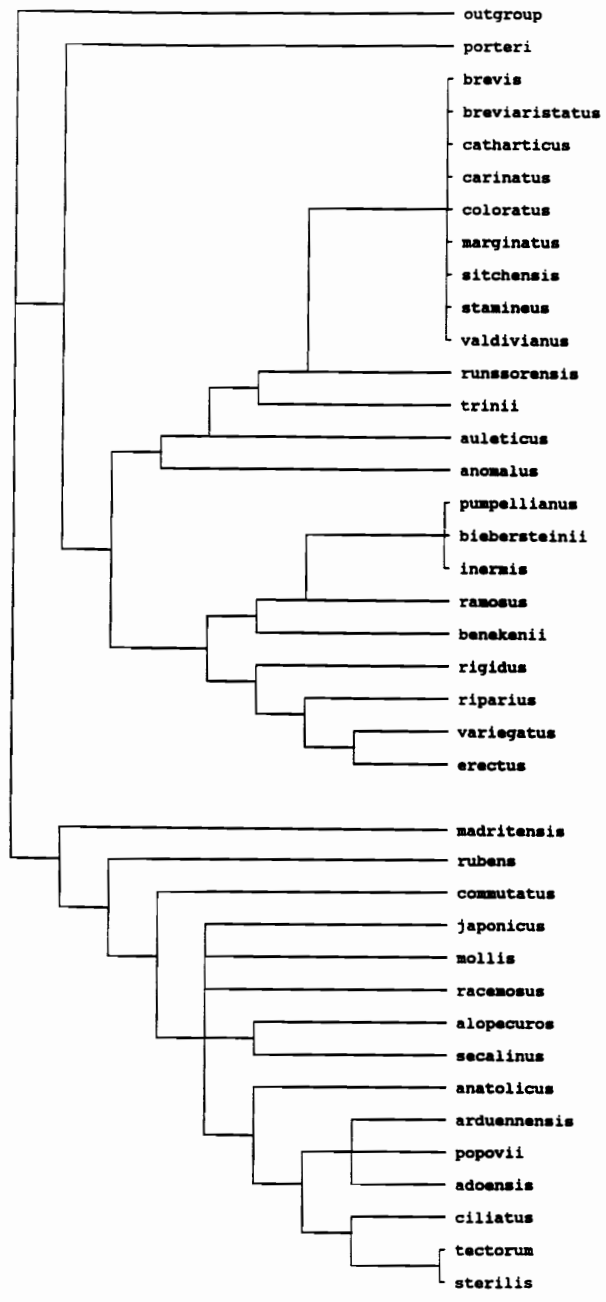


Figure 9. One of 11 most parsimonious trees of *Bromus* chloroplast DNA restriction sites obtained from HENNIG-86. The tree is 82 steps long with a consistency index of 0.46.

## *Chapter 3: Variation of ribosomal DNA in the genus Bromus (Poaceae)*

### *Summary*

Ribosomal DNA (rDNA) restriction endonuclease patterns were surveyed in 56 species of *Bromus*. *BamHI*, *BstEII*, *EcoRI* and *KpnI* restriction fragments hybridizing to cloned rDNA probes were compared. The four restriction enzymes detected a wide variety of patterns in the rDNA. The number of fragments reflected either the number of sites within the ribosomal genes for each enzyme or the presence of multiple repeat unit length variants. *BamHI* produced extensive length heterogeneity within and among species. The *EcoRI* digests were not useful phylogenetically since the enzyme generally cleaves only once per repeat unit. *EcoRI* and *KpnI* restriction patterns were useful in identifying the number of repeat unit length variants per species. The *BstEII* and *KpnI* patterns were useful in determining relationships at the subgenus level. The presence of a unique 2.1 kb *BstEII* fragment of the coding region characteristic in subgenera *Festucaria*, *Ceratochloa*, *Neobromus* and *Stenobromus* suggests close genetic relationship between the subgenera. A similar

fragment of 1.1 kb was present in subgenus *Bromus* suggesting genetic isolation from the other subgenera of *Bromus*.

Key words: *Bromus*, ribosomal DNA, restriction analysis, phylogeny

## ***Introduction***

*Bromus* L. (brome-grasses) is a cosmopolitan genus of about 130 diploid and polyploid species. The phylogenetic relationships among the different subdivisions of the genus remain inconclusive. Smith (1972) used evidence from morphology and seed protein serology to argue for a high degree of homogeneity within *Bromus* and the division of the genus into six sections. Stebbins (1981), emphasizing the distinctness of the subdivisions in *Bromus*, recognized seven distantly related subgenera: *Festucaria*, *Ceratochloa*, *Neobromus*, *Bromus*, *Stenobromus*, *Nevskiella*, and *Boissiera*. He considered the genomic relationships between the subgenera to be weak or non-existent. Tsvelev (1976) further emphasized the divergence among the subdivisions of *Bromus* by placing them in separate genera. Armstrong (1987) argued that the intrageneric subdivisions are too distinct to be considered sections, especially when compared with genera in other grass tribes, and that the treatment of the subdivisions as genera or subgenera is more consistent with other groups in the family.

The phylogenetic relationships between the subgenera have been deduced mainly from morphological and limited cytogenetical evidence. Problems can arise when phylogenies are based entirely on characters that are highly plastic or on data that cannot be interpreted unambiguously. New sources of evidence are needed to provide more insight into the genomic relationships in the genus.

The tandemly repeated, multicopy ribosomal genes have been widely used in evolutionary studies in plants (Appels and Honeycutt 1986). The rDNA is a desirable system in tracing phylogenies because of the highly conserved 18S-25S coding region, the more rapidly evolving intergenic spacer region (IGS), the high copy number of the rDNA genes, and the process of genic homogenization called concerted evolution (Doyle et al. 1984). The variation patterns observed in both length and restriction site polymorphism in the ribosomal genes have already been used to assess biosystematic relatedness in plant groups such as the *Lisianthus skinneri* complex (Sytsma and Schaal 1985), *Glycine* (Doyle and Beachy 1985), *Helianthus* (Choumane and Heizmann 1988), maize and its ancestors (Zimmer et al. 1988), sorghum, sugarcane and maize (Springer et al. 1989), *Hordeum* (Molnar et al. 1989), and rice (Cordesse et al. 1990; Sano and Sano 1990).

This study examines the variation produced from a restriction enzyme analysis of the rDNA gene family in 56 species of *Bromus*. The data are used to assess phylogenetic relationships in the genus. For the purposes of this discussion, the treatment proposed by Stebbins (1981) will be followed since it provides a comprehensive treatise of the genomic relationships between the subgenera of this large group of species.

### ***Genomic relationships between the subgenera of Bromus***

Five subgenera of *Bromus* are represented in this study. Subgenera *Stenobromus* and *Bromus* contain 10 and 40 annual or biennial species, respectively, and are indigenous to Eurasia (Armstrong 1987). These diploid and polyploid species are considered evolutionarily the most advanced in the genus (Armstrong 1987). Subgenus *Neobromus* consists of one species, *B. trinii*, native to South America. Subgenus *Ceratochloa* includes 17 recognized species located in North and South America. *Festucaria* is the largest subgenus, comprising approximately 60 species distributed in Eurasia and North to South America (Armstrong 1981, 1987).



The genomic relationships between the subgenera have been deduced primarily from chromosome pairing of a few interspecific hybrids (Stebbins 1981). All subgenera have the basic chromosome number  $x = 7$ . *Festucaria* is considered monophyletic (Stebbins 1981; Armstrong 1987). This subgenus includes a series of diploid to decaploid species based on one to five sets of the *L* genome. The smaller chromosomes found in polyploid *Festucaria* are similar in size to those found in subgenera *Neobromus* and *Ceratochloa*.

Subgenus *Ceratochloa* contains hexaploid and octoploid species, while diploid and tetraploid species are thought to be extinct. The hexaploid species are endemic to South America and are grouped under the *B. catharticus* complex. Their genome is designated as  $A_1B_1B_2$ . The octoploid species belong to the *B. carinatus* complex and are North American in distribution. This species complex contains 42 small and 14 large chromosomes and was given the genomic formula  $A_1B_1B_2L$  (Stebbins and Tobgy 1944; Stebbins 1956). Cytogenetic studies have shown that the 14 large chromosomes were derived from diploid North American members of subgenus *Festucaria* (Stebbins and Tobgy 1944; Stebbins 1947).

*Neobromus* is presumed to be distantly related to *Ceratochloa* since only one of its three genomes is reported to have a weak affinity to those of *Ceratochloa*. Cytogenetic studies also led Stebbins (1981) to conclude that the relationship between subgenera *Festucaria*, *Ceratochloa* and *Neobromus* is obscure and it is unlikely that they had a recent common origin.

Subgenera *Bromus* and *Stenobromus* are thought to be derived from primitive members of *Festucaria* (Stebbins 1981). However, based on morphology, Stebbins (1981) has stated that each subgenus originated from a different primitive species of *Festucaria*. The genetic affinity between subgenera *Bromus* and *Stenobromus* and the other subgenera is unknown.

In summary the relationships between the different subgenera of *Bromus* may either be described as distant, obscure or unknown.

## ***Materials and methods***

### ***Plant Material***

Fifty six species of *Bromus* were examined from subgenera *Festucaria*, *Ceratochloa*, *Neobromus*, *Bromus*, and *Stenobromus*. Seed material was not available for subgenera *Nevskiella* and *Boissiera*. Tables 7a and 7b list the species used, chromosome and accession numbers, and source and origin of the seeds. The plants used in this study were grown from seeds under greenhouse conditions. In all cases total DNA was isolated from single plants. In some instances, total DNA was extracted from more than one plant per species to ascertain the presence of intraspecific variation.

### ***DNA isolation and restriction endonuclease digestion***

Total DNA was extracted from 1-5 g of frozen leaf material following the procedure of Saghai-Marouf et al. (1984), with the following modifications. RNA was removed from the preparations by treatment with DNase-free pancreatic RNase and RNase T1. This was followed by one or two phenol-chloroform extractions and ethanol precipitation of the DNA. Total DNA (2-5  $\mu$ g) was digested with the restriction enzymes *BamHI*, *BstEII*, *EcoRI*, and *KpnI* following the conditions recommended by the supplier (Bethesda Research Laboratory). The genomic DNA digests were subjected to horizontal agarose gel electrophoresis in TAE buffer (Maniatis et al. 1982). Gels were stained in ethidium bromide and photographed under UV illumination. The DNA was then nicked, inducing single strand breakage, under UV for 5 minutes and transferred from the gels to Zetaprobe (BRL) using the alkaline transfer medium of Reed and Mann (1985).

### *Molecular hybridization*

The DNA molecules used as hybridization probes included two fragments, pRY12 (4.5 kb) and pRY18 (3.8 kb), inserted at the *Bam*HI site of pUC13. These two clones comprise the entire rDNA repeat from rice (cultivar Taichung 65, Japonica type ) and were generously contributed by Y. Sano of the National Institute of Genetics, Mishima, Japan. The location of the two probes in relation to the rDNA unit is illustrated in Fig. 10. The DNA probes were labelled by nick translation with <sup>32</sup>P labelled  $\alpha$  d-CTP (Rigby et al. 1977). Hybridizations with labelled probes were conducted at 65° C in 3X SSC, 20mM NaH<sub>2</sub>PO<sub>4</sub>, pH 7, 7% SDS, 10X Denhardtts, salmon sperm DNA at 100  $\mu$ g/ml. Filters were then washed three times at 65° C for 15 min in 3X SSC/0.1% SDS followed by 3 washes in 2X SSC/0.1% SDS and exposed to Kodak XAR5 X-ray film overlaid with an intensifying screen (Dupont Cronex Lightning Plus). In some cases the exposures were performed without an intensifying screen at room temperature. This was useful in identifying bands that were very close together and provided a means of estimating fragment sizes more accurately.

### *Analysis of data*

Fragment sizes were estimated using a nonlinear regression analysis computer program. Standard markers included phage lambda DNA digested with *Hind*III, and the one kilobase ladder marker (BRL). In addition, *Bam*HI digests of the plasmid pUC13 (2.6 kb) containing pRY12 and pRY18 were used as markers. The *Bst*EII rDNA restriction fragments did not total to the variant size produced by the other enzymes. Size inconsistency was also reported by Springer et al. (1989), who proposed that an unusual secondary structure of the DNA may have affected the migration of one or more fragments or that very small fragments produced in their study were not able to be detected. Similar reasons may apply in our study.

## Results

Digestion of genomic DNA from the 56 species of *Bromus* with *BamHI*, *EcoRI*, *KpnI* and *BstEII* and sequential probing of the Southern blots with pRY12 and pRY18 yielded one to seven DNA fragments per species (Table 8a-e). The number of fragments reflected either the number of sites within the ribosomal genes for each enzyme or the presence of multiple repeat unit length variants.

### Variation in *EcoRI* and *KpnI* fragments

In the *EcoRI* DNA digests, large fragments hybridized to both probes with the same intensity (Fig. 11). The molecular sizes of these fragments indicated that each band in the *EcoRI* pattern represented a complete repeat unit. This suggested that *EcoRI* cleaves once per repeat unit. Only *B. scoparius* of subgenus *Bromus* and *B. sericeus* of *Stenobromus* had an additional *EcoRI* site.

*KpnI* recognized a single cleavage site within the repeat unit in 38 species (Table 8a-e, Fig. 12). In those species, the two probes bound to high molecular weight DNA over 23 kb. The most striking feature of the *KpnI* digestions was the lack of the restriction site in a majority of the species in subgenus *Bromus* and one species, *B. laevipes*, of subgenus *Festucaria*. Only two species of subgenus *Bromus*, *B. alopecuros* and *B. popovii*, had more than one *KpnI* site. This contrasts sharply with the species of subgenus *Stenobromus* where all species except *B. rigidus* had more than one site for *KpnI*. One *KpnI* site was found in species of *Festucaria* except for *B. ciliatus*, *B. anomalus*, *B. benekenii* and *B. kalmii* where more than one site was found. Intraspecific variation in the *KpnI* site was observed in three individuals from one accession of *B. stamineus* of subgenus *Ceratochloa*.

### *Variation in BamHI fragment patterns*

Digestion with *BamHI* produced two to six DNA fragments ranging in size from ca. 4 to 8 kb that hybridized to the probes with varied intensities (Table 8a-e, Fig. 13). Smaller fragments of 0.7-1.9 kb were detected in *B. rubens*, *B. rigidus*, *B. erectus*, and *B. biebersteinii* (Table 8a-e). These fragments resulted from the presence of extra *BamHI* sites within the IGS region. Intraspecific and interspecific variation in *BamHI* fragment patterns were evident. In all accessions, a constant 3.8 kb segment representing part of the conserved coding region was present (Fig. 13). The other fragments were variable in size.

A question was raised whether the DNA fragments that hybridized with the pRY12 represent only the IGS or the IGS and the coding region. To resolve that question, the pRY12 clone was digested with *SmaI* and *HinfI*. Those enzymes have sites inside the IGS region (Sano and Sano 1990) and enabled the removal of the coding region from the pRY12 clone. The hybridization pattern obtained from using the non-coding part of the probe was identical to those obtained with complete pRY12 clone. This experiment, thus, showed that the length variants revealed by the pRY12 represent the IGS region only.

A prominent feature of the *BamHI* digests was the detection of fragments larger than 8 kb that hybridized to pRY12 and pRY18 (Fig. 13). These fragments corresponded in size to the sum of the invariant 3.8kb fragment and one of the variable fragments in each DNA sample, thus representing the complete rDNA monomeric units. These large fragments may be due to lack of a *BamHI* site caused by either nucleotide sequence alteration or to methylation that prevented cleavage. Hydrolysis of the same DNA samples with an excess amount of enzyme did not result in any further cleavage of these fragments.

Although spacer length (sl) variants had a wide range of sizes within the genus, a certain amount of clustering of sizes was observed. The most common spacer length variant is ca. 5.4 kb occurring

in over 50% of the accessions (Table 8a-e). Two other fragments of approximately 6.5 kb and 4.5 kb appeared in intermediate and low frequencies, respectively.

In *Ceratochloa*, members of the *B. carinatus* complex shared a large number of *sl* variants (Table 8a-e). *Bromus marginatus* and *B. carinatus* had identical *Bam**HI* fragment patterns. However, the major band, identified by having the darkest intensity, was 6.0 kb in *B. marginatus* and 7.3 kb in *B. carinatus*. *Bromus breviaristatus* differed from the above species by the presence of a 5.9 variant and the absence of a 7.3 variant. *Bromus sitchensis* had four fragments sizes in common with *B. carinatus* and *B. marginatus*. In the *B. catharticus* complex, *B. catharticus* and *B. valdivianus* had fragment patterns and sizes that resembled those found in species of the *B. carinatus* complex. On the other hand, *B. brevis*, *B. stamineus* and *B. coloratus* differed considerably from the *B. carinatus* complex and the rest of the *B. catharticus* complex by having only one *sl* variant (Table 8a-e). Variant size was 6.0 in the former two and 5.4 in the latter species.

Subgenus *Neobromus*, which is monotypic, including *B. trinii*, showed a *Bam**HI* pattern with two length variants of 4.4 and 4.9 kb. The *BstEII* pattern of *B. trinii* was identical to that of *B. stamineus*, subgenus *Ceratochloa*.

### ***Variation in BstEII fragment patterns***

The fragment patterns produced with *BstEII* were complex. The number of fragments produced varied from 1 to 7 (Table 8a-e). However, a number of fragments of varying sizes that hybridized to pRY12 also hybridized either moderately or strongly to pRY 18 (Fig. 14). Two invariant fragments, 1.1 kb and 2.1 kb, were detected with pRY18. The 1.1 kb fragment was characteristic of subgenus *Bromus* while the 2.1 kb fragment was present in all species of subgenera *Neobromus*, *Ceratochloa* and *Stenobromus* (Fig. 14). The 2.1 fragment was also detected in species of *Festucaria* except *B. porteri*, *B. anomalus*, *B. benekenii*, and *B. laevipes*. In *Neobromus*, *Ceratochloa* and the species of *Festucaria* that contained the non variant 2.1 kb fragment, two or three other fragments

were present that clustered around 2.5 kb and 3.5 kb. Larger fragments of 4-7 kb were present in some species of *Festucaria*. With the exception of *B. rigidus* of *Stenobromus*, one or more fragments of about 7.3 kb were present (Table 8a-e).

All species of subgenus *Bromus*, except *B. scoparius* and *B. anatolicus*, had more than one site for *BstEII* (Table 8a-e). Generally, a very wide range of fragment sizes from 1.1 to 6.4 was present in the subgenus *Bromus*. This indicated that there was a greater number of *BstEII* restriction sites in this subgenus than the other subgenera. The 1.1 kb invariant fragment characteristic of *Bromus* was not present in *B. scoparius*, *B. severtzovii*, *B. anatolicus*, *B. mollis* and *B. popovii*. Intraspecific variation was observed in *B. lanceolatus* and *B. brachystachys*.

## ***Discussion***

Ribosomal DNA repeat length heterogeneity is a common feature in both plants and animals (Appels and Honeycutt 1986; Rogers and Bendich 1987). This length variation is largely attributable to variability in the intergenic spacer as a result of the presence of different numbers of the subrepeated sequences (reviewed by Rogers and Bendich 1987). The results of this study demonstrate that rDNA repeats in the 56 species of *Bromus* vary considerably with respect to length and sequence arrangements. The genus is well represented in this study in terms of total number of species, number of species per subgenus, and geographic distribution. Therefore, the sample is a good representation of the type and level of variation found in the rDNA genes within the genus. A wide variety of patterns in the rDNA was observed with digestion by the four restriction enzymes.

### *Restriction site and length variation in rDNA*

The restriction enzymes *BstEII* and *KpnI* were particularly useful in determining relationships at the subgenus level. The restriction digests produced with *EcoRI* were not useful phylogenetically since the enzyme generally cleaves only once per repeat unit. However, the *EcoRI* as well as the *KpnI* restriction patterns were useful in identifying the number of repeat unit length variants per species.

The ladder-like restriction pattern observed in the *BamHI* digests of *B. barcensis*, certain species of *Ceratochloa* and the *BamHI* and *KpnI* digests of *B. diandrus* provided a means of approximating the subrepeat size in *Bromus*. The length variants of these species differed by 300 bp or multiples thereof. This could imply that subrepeats of approximately 300 bp occur in *Bromus*. This subrepeat size is similar to that found in rice (Sano and Sano 1988). Smaller subrepeat sizes were reported from other grass genera ranging in size from 115 bp for barley and oats (Saghai-Marooof et al. 1984), 130 bp for wheat (Appels and Dvorak 1982) and 200 bp for maize (McMullen et al. 1986). The ca. 300 bp size increment was not observed in all the species, indicating that the variation in the spacer length may be altered by mechanisms such as insertions and/or deletions. The subrepeats of the large spacer are known to change quickly in an evolutionary sense by these mechanisms (McIntyre et al. 1988).

The incomplete cuts of some of the DNAs observed in this study (Fig. 13) are probably due to methylation. This was particularly evident for the *BamHI* sites. Methylation of *BamHI* restriction sites has been reported for a number of grasses including wheat and barley (Gerlach and Bedbrook 1979; Appels et al. 1980), maize and its ancestors (Zimmer et al. 1988), barley, (Molnar et al. 1989), and rice (Cordesse et al. 1990).

The different intensities of rDNA bands within a species (Fig. 13) suggest the presence of major and minor repeat length variants. This variation at the individual level could be attributed to either differences in copy number or different degrees of availability of the *BamHI* sites in these length



classes. Variation in copy number of different variants at the individual level has been documented in flax (Cullis 1979; Cullis and Charlton 1981); and *Vicia faba* (Rogers et al. 1986).

The presence of the non-variant 3.8 kb *BamHI* fragment in all the species of *Bromus* demonstrates the ubiquitous nature of these sites. Initial *EcoRI*/*BamHI* double digestions of *B. inermis*, *B. marginatus* and *B. mollis* indicate that these conserved sites correspond in position to map type A produced for *Hordeum* (Molnar and Fedak 1989). Map type A repeats are also present in wheat and rye (Gerlach and Bedbrook 1979; Appels et al. 1980), maize, teosinte and tripsacum (Zimmer et al. 1988), sorghum and sugarcane (Springer et al. 1989), and rice (Sano and Sano 1990). This study further supports the idea proposed by Molnar and Fedak (1989) that the conserved *EcoRI* and *BamHI* sites within the coding region of the rDNA genes are widely distributed in the Poaceae. However, different *EcoRI* sites were identified in *B. scoparius* of subgenus *Bromus*. and *B. sericeus* of *Stenobromus*. The position of these sites was different in the two species. This was determined by the observation that the smaller 3.0 kb fragment of *B. scoparius* hybridized to both pRY12 and pRY18 while a similar 2.6 kb fragment of *B. sericeus* was detected only by pRY18. These results suggest that the extra *EcoRI* site is located within the IGS for *B. sericeus* and within the coding region covered by pRY18 for *B. scoparius*. This makes *B. scoparius* unique amongst the grasses studied so far in having an extra *EcoRI* site within the coding region of its rDNA genes.

### ***Evolutionary implications of rDNA variation***

The restriction patterns obtained from the enzymes *BstEII* and *KpnI* were useful in determining genetic relationships at the subgeneric level. Subgenera *Ceratochloa*, *Neobromus* and most species of *Festucaria* share very similar *BstEII* rDNA patterns (Table 8a-e). Except for four species of *Festucaria*, the three subgenera share two unique sites in the coding region that resulted in a 2.1 kb invariant fragment. Two other fragments, ca. 2.5 and 3.5 kb, were also characteristic of these subgenera. The *BstEII* restriction sites showed that *B. trinii* of subgenus *Neobromus* shared almost identical size fragments with members of *Ceratochloa*, illustrating the genetic affinity between these

two subgenera. The origin of subgenus *Neobromus* is not clear since its diploid and tetraploid ancestors are extinct. Stebbins (1981) maintained that *Neobromus* is more similar to genera related to *Bromus* such as *Pseudodanthonia* and *Metcalfia* (tribe *Aveneae*). These genera have not been examined in this study. Nevertheless, the similarity in rDNA sequences between *B. trinii* and *B. stamineus* suggests close affinity between *Neobromus* and *Ceratochloa*.

The similarities in rDNA patterns between *Festucaria* and *Ceratochloa* are supported by data from restriction site variation in the maternally inherited chloroplast genomes (Pillay and Hilu 1990). In the chloroplast DNA study, species of *Ceratochloa* were identical in cpDNA sequences and were closely related to polyploid *Festucaria*. Evidence from cpDNA also shows that *B. trinii* of *Neobromus* has a close affinity with *Ceratochloa* (Pillay and Hilu unpublished data). Therefore, results from rDNA and cpDNA studies suggest a greater affinity between the genomes of subgenera *Festucaria*, *Ceratochloa* and *Neobromus* and do not support Stebbins (1981) hypothesis that the relationships between these subgenera are obscure. These results emphasize the inherent perils of deducing genomic relatedness from chromosome behavior of hybrids especially at the polyploid level (Jackson 1984; Kaul and Murthy 1985). It is conceivable that while chromosomes may become differentiated from each other, resulting in decreased or lack of pairing, conserved genes located on them may not undergo such rapid differentiation. This is especially true if one considers a very conserved gene family as the rDNA.

The differences observed in both the *BstEII* and *KpnI* restriction patterns (Table 8a-e) suggest that the genetic affinity between subgenera *Stenobromus* and *Bromus* is weak or non-existent. Most species of *Stenobromus* produced two fragments with *BstEII*: a nonvariant 2.1 kb fragment and a variable fragment of about 7.3 kb. The nonvariant fragment in subgenus *Bromus* was 1.1 kb. Two to six other fragments with a size range of 2.5 to 7.4 were present in *Bromus*. *KpnI* has more than one restriction site in *Stenobromus* except for *B. rigidus* where only one site was present. The *KpnI* sites were absent in most species of subgenus *Bromus*. The distant relationship between *Stenobromus* and *Bromus* suggested by the rDNA data is congruent with morphological and cytological evidence (Stebbins 1981). The presence of the 2.1 kb *BstEII* fragment in the coding

sequence in most species of *Festucaria* and in all *Stenobromus*, and its absence in subgenus *Bromus* suggests that *Festucaria* is more closely related to *Stenobromus* than to *Bromus*. The question remains whether the absence of the *BstEII* sites that defined the 2.1 kb fragment is a primitive or derived state. DNA restriction patterns imply that *Stenobromus* is more likely to share common ancestry with *Festucaria* than does subgenus *Bromus*. The study thus supports Smith's (1972) statement that section *Bromus* is effectively isolated from the other sections by genetic barriers. *Stenobromus* and *Bromus* are believed to have originated from different primitive species of *Festucaria* (Stebbins 1981). Although the 1.1 kb fragment was not present in species of *Festucaria*, variation in the coding region was observed (Table 8a-e). Consequently Stebbins's hypothesis on the origin of *Stenobromus* and *Bromus* cannot be overruled, but additional evidence is needed to decide the issue. However, compared with the other subgenera (except for *B. laevipes* of *Festucaria*), most species of subgenus *Bromus* were highly polymorphic for *BstEII*. Springer et al. (1989) have indicated that a recently or narrowly derived species normally shows a similar level of spacer length variability but less restriction site polymorphism. If this statement is true, then subgenus *Bromus* could be regarded as a primitive component of the genus *Bromus*. This would be contrary to Stebbins' (1981) hypothesis that *Neobromus*, *Ceratochloa* and *Festucaria* differentiated in the Pliocene while subgenera *Bromus* and *Stenobromus* did so in the Pleistocene and Recent.

The diploid species of *Festucaria* were more variable in rDNA patterns than the polyploids (Table 8a-e). The variability found in the rDNA genes of the diploid species of *Festucaria* (*B. porteri*, *B. ciliatus*, *B. anomalus*, *B. laevipes* and *B. kalmii*) parallels that observed in the cpDNA of *B. porteri*, *B. ciliatus* and *B. anomalus* (Pillay and Hilu 1990). This raises the question of possible co-evolutionary changes between the nuclear and chloroplast genomes of these species. Such changes have been reported for tobacco (Kung et al. 1982) and maize (Timothy et al. 1979).

In *Festucaria*, the unique *BstEII* 2.1 kb fragment was absent in three of the five diploid species. These diploid species are considered to be closely related. Four of the five species of subgenus *Bromus* that did not contain the unique 1.1 kb *BstEII* fragment (*B. scoparius*, *B. anatolicus*, *B.*

*alopecuros*, *B. popovii*) were also diploids. This probably implies that some of the diploids have lost the restriction site since restriction site losses are considered more likely than gains (Giannasi and Crawford 1986). Further detailed studies of subgenus *Bromus* are required to define other species relationships.

In subgenus *Ceratochloa*, the *B. carinatus* and *B. catharticus* complexes were almost identical in the *BstEII* patterns but were separable with the *BamHI* pattern except for *B. catharticus* and *B. valdivianus*, which grouped with the *carinatus* complex. *Bromus catharticus* and *B. valdivianus* have also been shown to have extremely short satellite arms, which are common in members of the *B. carinatus* group (Schulz-Schaeffer 1960). The similarity of the rDNA fragment patterns of the *B. carinatus* species complex suggests that they are closely related. Stebbins (1981) has indicated that delimitation of species in this group is extremely difficult on the basis of external morphology or the degree of reproductive isolation. This work thus supports Stebbins' (1981) suggestion that the octoploid species of the *B. carinatus* complex should be united into a single polymorphic species. The similar fragment sizes observed in species of the *B. carinatus* complex and in *B. catharticus* and *B. valdivianus* of the *catharticus* complex suggests that the latter species might be involved in the parentage of the *B. carinatus* complex. These data are therefore consistent with the evolutionary model of the origin of the *B. carinatus* complex (Stebbins 1981).

It is not clear why some members of *Ceratochloa*, especially members of the *B. catharticus* complex (*B. brevis*, *B. stamineus* and *B. coloratus*) had only one spacer length variant while all the other members consistently had five variants. Members of *Ceratochloa* are allopolyploids containing either three (*B. catharticus* complex) or four genomes (*B. carinatus* complex). This may possibly explain the presence of a large number of variants for some species of *Ceratochloa*. In this case species of *Ceratochloa* with single variants (*B. brevis*, *B. stamineus*, *B. coloratus*) have either undergone extensive interlocus homogenization of their rDNA genes or are displaying the phenomenon of nucleolar dominance (Dvorak 1990). Variation in the length of the spacer is believed to arise through unequal crossing over between the short tandem repeats of different rDNA genes (Szostak and Wu 1980; Smith 1985; Rogers et al. 1986). The lack of variation in the rDNA spacer of species

in rice has been explained by a possible breakdown in the mechanisms which produce such variation (Cordesse et al. 1990). Such an explanation may also be true in the case of *B. brevis*, *B. stamineus* and *B. coloratus*.

In conclusion, considerable amount of variation was observed, particularly with *BamHI*, indicating a potential use of these DNA markers in the systematics and evolution of *Bromus* at the population and interspecific levels if larger samples are used. Variation at the coding region, resolved by *BstEII*, was useful in interpreting evolutionary relationships among the subgenera.

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## *References*

- Appels R, Dvorak J (1982) The wheat ribosomal DNA spacer region: its structure and variation in populations and among species. *Theor Appl Genet* 63:337-348
- Appels R, Honeycutt RL (1986) rDNA evolution over a billion years. In: Dutta SK (ed) *DNA systematics II. Plants*. CRC Press, Boca Raton, Florida, pp 81-135
- Appels R, Gerlach WL, Dennis ES, Swift H, Peacock WJ (1980) Molecular and chromosomal organization of DNA sequences coding for the ribosomal RNAs in cereals. *Chromosoma* 78:293-311

- Armstrong KC (1981) The evolution of *Bromus inermis* and related species of *Bromus* sect. *Pnigma*. *Bot Jahrb Syst* 102:427-443
- Armstrong KC (1987) Chromosome evolution in *Bromus*. In: Tsuchiya T, Gupta TK (eds) *Chromosome engineering in plant genetics and breeding*. Elsevier Science Publ., Amsterdam (in press)
- Choumane W, Heizmann P (1988) Structure and variability of nuclear ribosomal genes in the genus *Helianthus*. *Theor Appl Genet* 76:481-489
- Cordes F, Second G, Delseny M (1990) Ribosomal gene spacer length variability in cultivated and wild rice species. *Theor Appl Genet* 79:81-88
- Cullis CA (1979) Quantitative variation of rRNA genes in flax genotrophs. *Heredity* 42:237-246
- Cullis CA, Charlton L (1981) The induction of ribosomal DNA changes in flax. *Pl Sci Lett* 20:213-217
- Doyle JJ, Beachy RN, Lewis WH (1984) Evolution of rDNA in *Claytonia* polyploid complexes. In: Grant WF (ed) *Plant systematics*. Academic Press, New York, pp 321-341
- Doyle JJ, Beachy RN (1985) Ribosomal gene variation in soybean (*Glycine*) and its relatives. *Theor Appl Genet* 70:369-376
- Dvorak J (1990) Evolution of multigene families: the ribosomal RNA loci of wheat and related species. In: Brown ADH, Clegg MT, Kahler AL, Weir BS (eds) *Plant population genetics, breeding, and genetic resources*. Sinauer, Sunderland, Massachusetts, pp 83-97
- Gerlach WL, Bedbrook J (1979) Cloning and characterization of ribosomal RNA genes from wheat and barley. *Nucleic Acid Res* 71:8698-1885
- Gianassi DE, Crawford DJ (1986) Biochemical systematics. In: Hecht MK, Wallace B, Prance GT (eds) *Evolutionary biology*, Vol 20, Plenum Press, New York, pp 25-248
- Jackson RC (1984) Chromosome pairing in species and hybrids. In: WF Grant (ed) *Plant biosystematics*. Academic Press, Toronto, pp 67-86
- Kaul MLH, Murthy TGK (1985) Mutant genes affecting higher plant meiosis. *Theor Appl Genet* 70:449-466
- Kung SD, Zhu YS, Shen GF (1982) *Nicotiana* chloroplast genome III. 64:80-85 Chloroplast DNA evolution. *Theor Appl Genet* 61:73-79
- Maniatis T, Fritsch EF, Sambrook J (1982) *Molecular Cloning: A Laboratory Manual*. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York
- McIntyre CL, Clarke BC, Appels R (1988) DNA sequence analysis of the ribosomal spacer regions in the *Triticeae*. *Pl Syst Evol* 160:91-104
- McMullen MD, Hunter DB, Phillips RL, Rubenstein I (1986) The structure of the maize ribosomal DNA spacer region. *Nucleic Acid Res* 14:4953-4968
- Molnar SJ, Fedak G (1989) Polymorphism in ribosomal DNA repeat units of 12 *Hordeum* species. *Genome* 32:1124-1127

- Molnar SJ, Gupta PK, Fedak G, Wheatcroft R (1989) Ribosomal DNA repeat unit polymorphism in 25 *Hordeum* species. *Theor Appl Genet* 78:387-392
- Pillay M, Hilu KW (1990) Chloroplast DNA variation in diploid and polyploid species of *Bromus* (Poaceae) subgenera *Festucaria* and *Ceratochloa*. *Theor Appl Genet* 80:326-332
- Reed KC, Mann DA (1985) Rapid alkaline transfer of DNA from agarose gels to nylon membranes. *Nucleic Acid Res* 13:6207-7221
- Rigby PWJ, Dieckmann M, Rhodes C, Berg P (1977) Labelling deoxyribonucleic acid to high specific activity in vitro by nick translation with DNA polymerase I. *J Mol Biol* 113:237-251
- Rogers SO, Bendich AJ (1987) Ribosomal RNA genes in plants: variability in copy number and in the intergenic spacer. *Pl Mol Biol* 9:509-520
- Rogers SO, Honda S, Bendich AJ (1986) Variation in the ribosomal RNA genes among individuals of *Vicia faba*. *Pl Mol Biol* 6:339-345
- Saghai-Marouf MA, Soliman KM, Jorgensen RA, Allard RW (1984) Ribosomal DNA spacer-length polymorphisms in barley: Mendelian inheritance, chromosomal location, and population dynamics. *Proc Natl Acad Sci USA* 81:8014-8018
- Sano Y, Sano R (1990) Variation of the intergenic spacer region of ribosomal DNA in cultivated and wild rice species. *Genome* 33:209-218
- Sano Y, Sano R (1988) Ribosomal DNA spacer length polymorphisms in rice. In: Abstract S, 16th Int Congr Genet. *Genome* 30 (Suppl. I): Abstract 35-31-6
- Schulz-Schaeffer J (1960) Cytological investigations in the genus *Bromus* III. The cytotaxonomic significance of the satellite chromosomes. *J Heredity* 51:269-277
- Smith G (1985) Evolution of repeated DNA sequences by unequal crossover. *Science* 191:528-535
- Smith P (1972) Serology and species relationships in annual bromes (*Bromus* L. sect. *Bromus*). *Ann Bot* 36:1-30
- Springer PS, Zimmer EA, Bennetzen JL (1989) Genomic organization of the ribosomal DNA of sorghum and its close relatives. *Theor Appl Genet* 77:844-850
- Stebbins GL (1947) The origin of the complex of *Bromus carinatus* and its phylogeographic implications. *Contr Gray Herb* 165:42-55
- Stebbins GL (1956) Cytogenetics and evolution in the grass family. *Am J Bot* 43:890-905
- Stebbins GL (1981) Chromosomes and evolution in the genus *Bromus* (Gramineae). *Bot Jahrb Syst* 102:359-379
- Stebbins GL, Tobgy HH (1944) The cytogenetics of hybrids in *Bromus* 1. Hybrids within the section "*Ceratochloa*". *Am J Bot* 31:1-11
- Sytsma KJ, Schaal BA (1985) Phylogenetics of the *Lisianthus skinneri* (Gentianaceae) species complex in Panama utilizing DNA restriction fragment analysis. *Evolution* 39:594-608
- Szostak JW, Wu R (1980) Unequal crossing-over in the ribosomal DNA of *Saccharomyces cerevisiae*. *Nature* 284:426-430

- Timothy DH, Levings III CS, Pring DR, Conde MF, Kermicle JL (1979) Organelle DNA variation and systematic relationships in the genus *Zea*: teosinte. *Proc Natl Acad Sci USA* 76:4220-4224
- Tsvelev NN (1976) Grasses of the Soviet Union Part 1. Nauka Publ., Leningrad [Translated from Russian] pp 298-339
- Zimmer EA, Jupe ER, Walbot V (1988) Ribosomal gene structure, variation, and its inheritance in maize and its ancestors. *Genetics* 120:1125-1136



**Table 7a. List of Bromus species, their chromosome and accession numbers, sources and origin of seed material used in this study**

Species	Chr No.	Accn No.	Source of material	origin
Subgenus <i>Festucaria</i>				
1 <i>B. anomalus</i> Rupr.	14*	232200	P.I. Station, Pullman	USA
2 <i>B. ciliatus</i> L.	14*	387908	P.I. Station, Pullman	Belgium
3 <i>B. kalmii</i> Gray	14	71-130	K. Armstrong, Canada	Canada
4 <i>B. laevipes</i> Shear	14	81-16	K. Armstrong, Canada	USA
5 <i>B. porteri</i> (Coulst.) Nash	14*	74-34	K. Armstrong, Canada	Canada
6 <i>B. barcensis</i>	28	233931	P.I. Station, Pullman	Canada
7 <i>B. benekenii</i> (Lange) Trimen	28	440181	P.I. Station, Pullman	Italy
8 <i>B. ramosus</i> Huds.	28	253299	P.I. Station, Pullman	Yugoslavia
9 <i>B. tomentellus</i> Boiss.	28	384825	P.I. Station, Pullman	Iran
10 <i>B. tomentosus</i> Trin.	28	384832	P.I. Station, Pullman	Iran
11 <i>B. auleticus</i> Trin. ex Nees	42*	162779	P.I. Station, Pullman	Argentina
12 <i>B. macranthus</i> E. Desv.	42	264400	P.I. Station, Pullman	Argentina
13 <i>B. runssorensis</i> K. Schum.	42	273869	P.I. Station, Pullman	Ethiopia
14 <i>B. variegatus</i> M. Bieb.	42	325233	P.I. Station, Pullman	Soviet Union
15 <i>B. erectus</i> Huds.	56	383616	P.I. Station, Pullman	Turkey
16 <i>B. inermis</i> Leyss.	56*		Sharp Bros. Seed Co.	USA
17 <i>B. pumpellianus</i> Scribn.	56	70-140	K. Armstrong, Canada	Canada
18 <i>B. biebersteinii</i> Roem. et Schult.	70	372614	P.I. Station, Pullman	Canada
19 <i>B. riparius</i> Rehm.	70	440214	P.I. Station, Pullman	Soviet Union
Subgenus <i>Ceratochloa</i>				
20 <i>B. brevis</i>	42	384465	P.I. Station, Pullman	Argentina
21 <i>B. catharticus</i> Vahl	42*		G. L. Stebbins, Ca	USA
22 <i>B. coloratus</i> Steud.	42	202696	P.I. Station, Pullman	Chile
23 <i>B. stamineus</i> Desv.	42	238251	P.I. Station, Pullman	Yugoslavia
24 <i>B. valdivianus</i> Phil.	42	368864	P.I. Station, Pullman	Australia
25 <i>B. carinatus</i> H. & A.	56*		Sharp Bros. Seed Co.	USA
26 <i>B. breviaristatus</i> Buckl.	56	392355	P.I. Station, Pullman	Soviet Union
27 <i>B. marginatus</i> Nees	56		Jacklin Seed Co., Idaho	USA
28 <i>B. sitchensis</i> Trin.	56	202534	P.I. Station, Pullman	Belgium

\* Chromosomes numbers confirmed by squashing technique.

Table 7b. List of *Bromus* species, their chromosome and accession numbers, sources and origin of seed material used in this study.

Species	Chr. No.	Accn. No.	Source of material	origin
Subgenus <i>Stenobromus</i>				
29 <i>B. fasciculatus</i> Presl.	14*	253735	P.I. Station, Pullman	Iraq
30 <i>B. tectorum</i> L.	14*	483443	P.I. Station, Pullman	USA
31 <i>B. sericeus</i> Drob.	14	317422	P.I. Station, Pullman	Afghanistan
32 <i>B. sterilis</i> L.	14*	442456	P.I. Station, Pullman	Belgium
33 <i>B. madritensis</i> L.	28*	442459	P.I. Station, Pullman	Belgium
34 <i>B. rubens</i> L.	28*	487414	P.I. Station, Pullman	USA
35 <i>B. diandrus</i> Roth	42		G. L. Stebbins, Ca	USA
36 <i>B. rigidus</i> Roth	42*	442462	P.I. Station, Pullman	Belgium
Subgenus <i>Bromus</i>				
37 <i>B. alopecuroides</i> Poir.	14*	202674	P.I. Station, Pullman	Jordan
38 <i>B. anatolicus</i> Boiss. et Heldr.	14*	204861	P.I. Station, Pullman	Turkey
39 <i>B. arduennensis</i> (Lej.) Crep.	14*	442454	P.I. Station, Pullman	Israel
40 <i>B. brachystachys</i> Horn.	14	220582	P.I. Station, Pullman	Afghanistan
41 <i>B. brizaeformis</i> Fisch. et Meyer	14	238243	P.I. Station, Pullman	Sweden
42 <i>B. danthoniae</i> Trin.	14	381133	P.I. Station, Pullman	Iran
43 <i>B. intermedius</i> Guss.	14	220579	P.I. Station, Pullman	Afghanistan
44 <i>B. japonicus</i> Thunb.	14*	442461	P.I. Station, Pullman	Belgium
45 <i>B. popovii</i> Drob.	14	283198	P.I. Station, Pullman	Soviet Union
46 <i>B. scoparius</i> L.	14	254882	P.I. Station, Pullman	Iraq
47 <i>B. adoensis</i> Hochst.	28	202531	P.I. Station, Pullman	Belgium
48 <i>B. commutatus</i> Schrad.	28*	205287	P.I. Station, Pullman	Turkey
49 <i>B. lanceolatus</i> Roth	28*	317420	P.I. Station, Pullman	Afghanistan
50 <i>B. mollis</i> L.	28*	469232	P.I. Station, Pullman	USA
51 <i>B. pseudodanthoniae</i> Drob.	28	211004	P.I. Station, Pullman	Afghanistan
52 <i>B. secalinus</i> L.	28*	442463	P.I. Station, Pullman	Belgium
53 <i>B. racemosus</i> L.	28	362114	P.I. Station, Pullman	Yugoslavia
54 <i>B. oxyodon</i> Schrenk	28	300567	P.I. Station, Pullman	Soviet Union
55 <i>B. severtzovii</i> Regel	28	314144	P.I. Station, Pullman	Soviet Union
56 <i>B. hordeaceus</i> L.	28	250005	P.I. Station, Pullman	Iran
Subgenus <i>Neobromus</i>				
57 <i>B. trinii</i> Desv.	42	202154	P.I. Station, Pullman	Argentina

\* Chromosomes numbers confirmed by squashing technique.

Table 8a. Ribosomal restriction fragments from digestions of total DNA of *Bromus* species.

Fragment sizes (kb)	BamHI								BstEII						
	0-1	1-2	2-3	3-4	4-5	5-6	6-7	7-8	2-3	3-4	4-5	5-6	6-7	7-8	
Subgenus <i>Festucaria</i>															
<i>B. porteri</i>				3.8		5.4			2.5		4.9				
<i>B. ciliatus</i>				3.8	4.8	5.0			2.1				6.7		
<i>B. kalmii</i>				3.8		5.1			2.1				6.7		
						5.6							6.9		
<i>B. laevipes</i>				3.8			6.3			3.2	4.8	5.4			
							6.8			3.2			5.8	6.3	
<i>B. anomalus</i>				3.8		5.6				3.2			6.5		
						5.9									
<i>B. auleticus</i>				3.8		5.4			2.1	3.3					
									2.3						
<i>B. macranthos</i>				3.8	4.8				2.1	3.2					
									2.2						
<i>B. ramosus</i>				3.8		5.4			2.1			5.1			
						5.7			2.5						
<i>B. benekenii</i>				3.8			6.3		2.5		4.7				
							6.7								
<i>B. tomentellus</i>	0.8			3.8		5.4	6.3		2.1				6.1		
						5.7	6.8		2.5						
									2.7						
<i>B. tomentosus</i>				3.8			6.3		2.1	3.6					
							6.8		2.6						
<i>B. barcensis</i>	0.8			3.8	4.1	5.4	6.3	7.3	2.1	3.3					
						5.8	6.8		2.3						
									2.5						
<i>B. runssorensis</i>				3.8		5.6	6.6		2.1	3.3					
						5.8			2.4	3.4					
<i>B. variegatus</i>	0.8			3.8	4.7	5.0	6.2		2.1	3.6					
					4.8	5.3	6.7		2.6						
							6.9								
<i>B. erectus</i>	0.8			3.8	4.7	5.1			2.1	3.1					
					4.8				2.6						
<i>B. inermis</i>				3.8			6.0		2.1	3.3					
							6.3		2.5						
							6.6		2.6						
							6.8								
<i>B. pumpellianus</i>				3.8		5.4			2.1	3.4					
						5.6			2.4						
									2.7						
<i>B. biebersteinii</i>	0.8			3.8	4.8	5.3	6.0		2.1	3.6					
					4.9	5.4	6.3		2.6						
<i>B. riparius</i>	0.8			3.8		5.2	6.0		2.1						
						5.4	6.3		2.6	3.6					

Table 8b. Ribosomal restriction fragments from digestions of total DNA of Bromus species.

Fragment sizes (kb)	BamHI					BstEII					
	3-4	4-5	5-6	6-7	7-8	2-3	3-4	4-5	5-6	6-7	7-8
Subgenus <i>Ceratchloa</i>											
<i>B. marginatus</i>	3.8		5.7	6.0	7.3	2.1	3.4				
				6.3		2.3					
				6.6							
<i>B. carinatus</i>	3.8		5.7	6.0	7.3	2.1	3.4				
				6.3		2.3					
				6.6							
<i>B. breviaristatus</i>	3.8		5.7	6.0		2.1	3.4				
				5.9	6.3	2.3					
				6.8							
<i>B. sitchensis</i>	3.8		5.7	6.0	7.3	2.1	3.4				
				6.8	7.8	2.3					
<i>B. catharticus</i>	3.8		5.4	6.0		2.1	3.4				
				5.6		2.3					
				5.7							
<i>B. valdivianus</i>	3.8		5.4	6.0		2.1	3.4				
				5.6	6.8		3.5				
				5.7							
<i>B. brevis</i>	3.8			6.0		2.1	3.4				
							3.5				
<i>B. stamineus</i>	3.8		5.4			2.1	3.4				
						2.3	3.6				
<i>B. coloratus</i>	3.8			6.0		2.1	3.4				
						2.3					
Subgenus <i>Neobromus</i>											
<i>B. trinii</i>	3.8	4.4				2.1	3.2				
		4.9				2.3	3.3				
Subgenus <i>Stenobromus</i>											
<i>B. tectorum</i>	3.8		5.4			2.1				7.3	
			5.6								
<i>B. sterilis</i>	3.8		5.2			2.1			6.9		
<i>B. rubens</i>	3.5	4.4	5.4			2.1			6.7	7.3	
	3.8	4.8	5.6								
<i>B. fasciculatus</i>	3.8	4.8	5.1	6.8		2.1				7.3	
			5.6			2.7					
<i>B. madritensis</i>	3.8	4.8	5.1	6.3		2.1				7.1	
<i>B. sericeus</i>	3.8		5.6			2.1				7.3	
			5.9								
<i>B. rigidus</i>	3.8	4.4	5.1			2.1	4.6				
		4.8	5.6			2.7					
<i>B. diandrus</i>	3.8		5.2	6.1		2.1					
			5.3	6.6							
			5.6	6.8							

Table 8c. Ribosomal restriction fragments from digestions of total DNA of *Bromus* species.

fragment sizes (kb)	BamHI					BstEII						
	3-4	4-5	5-6	6-7	7-8	1-2	2-3	3-4	4-5	5-6	6-7	7-8
Subgenus <i>Bromus</i>												
<i>B. adoensis</i>	3.8			6.6				nd				
<i>B. scoparius</i>	3.8		5.6			1.1	2.4		4.1	5.4	6.4	
		4.9	5.9									
<i>B. hordaceus</i>	3.8			6.6		1.1	2.8		4.9	5.9		
<i>B. lanceolatus</i>	3.8		5.8	6.3		1.1	2.9	3.2		5.4	6.6	
<i>B. severtzovii</i>	3.8			6.0			2.5	3.3			6.1	
	3.6			6.4								
<i>B. anatolicus</i>	3.8			6.0								
				6.6								
<i>B. brachystachys</i>	3.8			6.3		1.1	2.9		4.9		6.1	
				6.6								
<i>B. brizaeformis</i>	3.8		5.7	6.3		1.1		3.1		5.0	6.1	
<i>B. secalinus</i>	3.8		5.6	6.3		1.1	2.8			5.0		
<i>B. japonicus</i>	3.8		5.8	6.0		1.1	2.5	3.6				
<i>B. alopecuroides</i>	3.8		5.6	6.0			2.8			5.6		
<i>B. mollis</i>	3.8		5.6			1.1	2.7		4.1	5.2		
<i>B. commutatus</i>	3.8			6.0		1.1	2.7			5.1	6.1	
				6.4			2.8				6.6	
				6.8								
<i>B. intermedius</i>	3.8			6.0	7.9	1.1	2.4	3.3		5.1	6.1	
				6.6						5.5	6.6	
<i>B. danthoniae</i>	3.8			6.4	7.5	1.1	2.9	3.1	4.9	5.1		
					7.8					5.9		
<i>B. pseudodanthoniae</i>	3.8			6.4	7.5	1.1	2.9	3.1	4.9	5.1		
										5.9		
<i>B. popovii</i>	3.8		5.6					3.5	4.9			
			5.7									
<i>B. racemosus</i>	3.8			6.0		1.1	2.7	3.1		5.1		
				6.3						5.8		
Subgenus <i>Festucaria</i>												
<i>B. porteri</i>					8.6					10.9		
<i>B. ciliatus</i>	3.0		5.0							10.8		
<i>B. kalmii</i>	3.6	4.8								10.1		
	3.8											
<i>B. laevipes</i>			23*							10.5		
<i>B. anomalus</i>	3.3		5.5				7.5					
<i>B. auleticus</i>				7.4							11.1	
<i>B. macranthos</i>					8.0			9.5				
<i>B. ramosus</i>				7.7		9.3					11.1	
<i>B. benekenii</i>		4.2	5.6								11.8	12.2
<i>B. tomentellus</i>						9.9	10.6					12.2
<i>B. tomentosus</i>							10.1		10.5			14.1
							10.6				11.4	

Table 8d. Ribosomal restriction fragments from digestions of total DNA of *Bromus* species.

fragment sizes (kb)	KpnI					EcoR						
	7-8	8-9	9-10	10-11	9-10	10-11	11-12	12-13				
<i>B. barcensis</i>		8.9	9.2		9.7	10.5						
<i>B. runssoroensis</i>			9.5	10.1		10.9						
			9.9				11.1					
							11.8					
<i>B. variegatus</i>			9.5	10.1			11.1					
							11.8					
<i>B. erectus</i>			9.5	10.1		10.9	11.1					
<i>B. inermis</i>			9.6	10.1		10.9		12.2				
<i>B. pumpellianus</i>			9.4			10.9	11.1					
			9.6									
<i>B. biebersteinii</i>			9.6			10.9						
<i>B. riparius</i>			9.6			10.5						
Subgenus <i>Ceratchloa</i>												
<i>B. marginatus</i>			9.4	10.1		10.5	11.4					
			9.6				11.9					
<i>B. carinatus</i>			9.6	10.4		10.7	11.9					
			9.9			10.9						
<i>B. breviaristatus</i>			9.4			10.5	11.1					
			9.9									
<i>B. sitchensis</i>		8.1	9.5			10.5	11.9	12.8				
<i>B. catharticus</i>		8.1				10.5						
<i>B. valdivianus</i>	7.7	8.3				10.5	11.9					
<i>B. brevis</i>	7.9					10.5						
<i>B. stamineus</i>	7.5	8.1			9.8	10.5						
<i>B. coloratus</i>		8.1				10.5						
Subgenus <i>Neobromus</i>												
<i>B. trinii</i>			9.2									
Species												
Fragment sizes (kb)	2-3	3-4	4-5	5-6	9-10	2-3	3-4	6-7	8-9	9-10	10-11	11-12
Subgenus <i>Stenobromus</i>												
<i>B. tectorum</i>	2.7		4.1							9.4		
	2.8											
<i>B. sterilis</i>	2.8		4.1							9.0		
<i>B. rubens</i>	2.8	3.0	4.1							9.4		
<i>B. fasciculatus</i>	2.7		4.1							9.4		
	2.8											
<i>B. madritensis</i>	2.7		4.1					6.8	8.7	9.8		
	2.8											
<i>B. sericeus</i>	2.7		4.1			2.6		6.8		9.4		
	2.8											
<i>B. rigidus</i>					9.4					9.4		
<i>B. diandrus</i>		3.8	4.1	5.1					8.8		10.7	
			4.5	5.3								11.9
			4.8									

Table 8e. Ribosomal restriction fragments from digestions of total DNA of *Bromus* species.

Species	KpnI					EcoR				
	3-4	5-6	9-10	10-11	11-12	3-4	5-6	8-9	9-10	10-11
Subgenus <i>Bromus</i>										
<i>B. adoensis</i>	23*									10.1
<i>B. scoparius</i>	23*					3.0	5.8		9.4	
<i>B. hordaceus</i>	23*									10.1
<i>B. lanceolatus</i>	23*									10.1 10.2
<i>B. severtzovii</i>	23*								9.2	
<i>B. anatolicus</i>	23*								9.2	
									9.9	
<i>B. brachystachys</i>	23*								9.5	
									9.9	
<i>B. brizaeformis</i>	23*								9.2	
									9.4	
<i>B. secalinus</i>					11.1				9.1	
									9.4	
<i>B. japonicus</i>					11.1				9.3	
<i>B. alopecuroides</i>		5.1							9.2	
		5.6							9.3	
<i>B. mollis</i>			9.1						9.1	
<i>B. commutatus</i>				10.6					9.3	
									9.6	
<i>B. intermedius</i>	23*								9.4	10.3
									9.6	
<i>B. danthoniae</i>	23*								9.6	10.1
<i>B. pseudodanthoniae</i>	23*								9.6	10.1
<i>B. popovii</i>	3.8	5.1							9.2	
<i>B. racemosus</i>	23*								9.2	

\* indicates fragment size greater than 23 kb

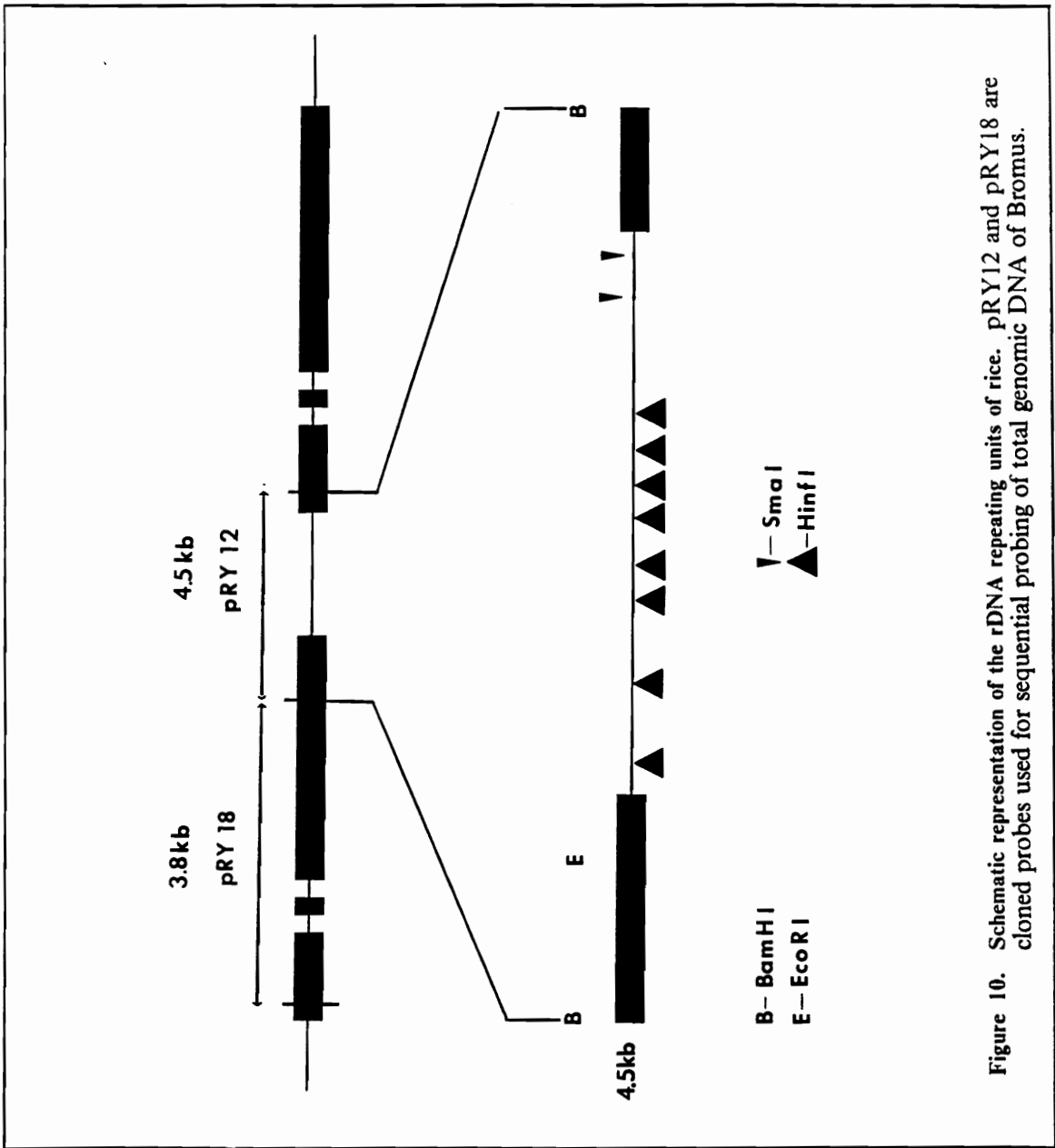
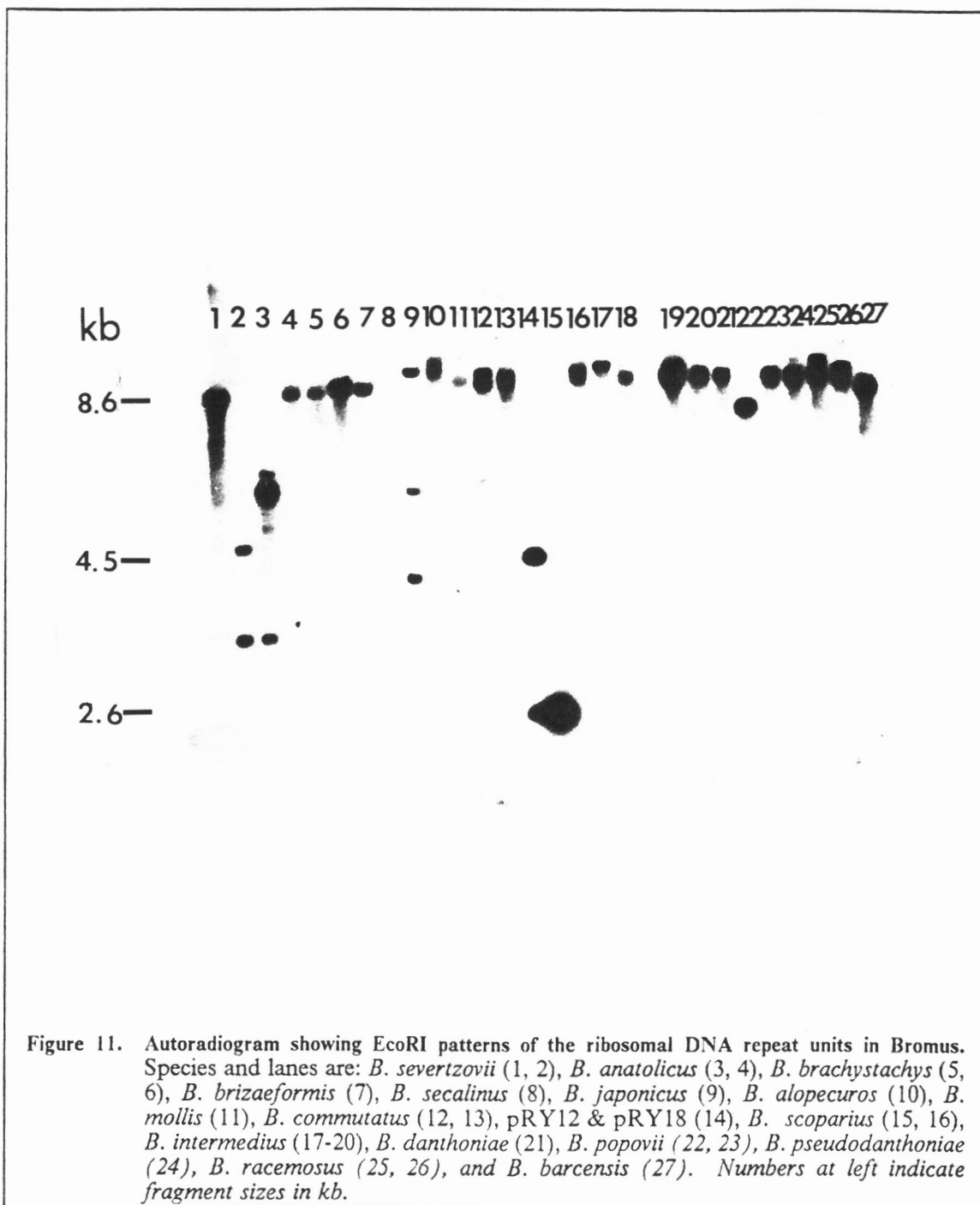


Figure 10. Schematic representation of the rDNA repeating units of rice. pRY12 and pRY18 are cloned probes used for sequential probing of total genomic DNA of *Bromus*.





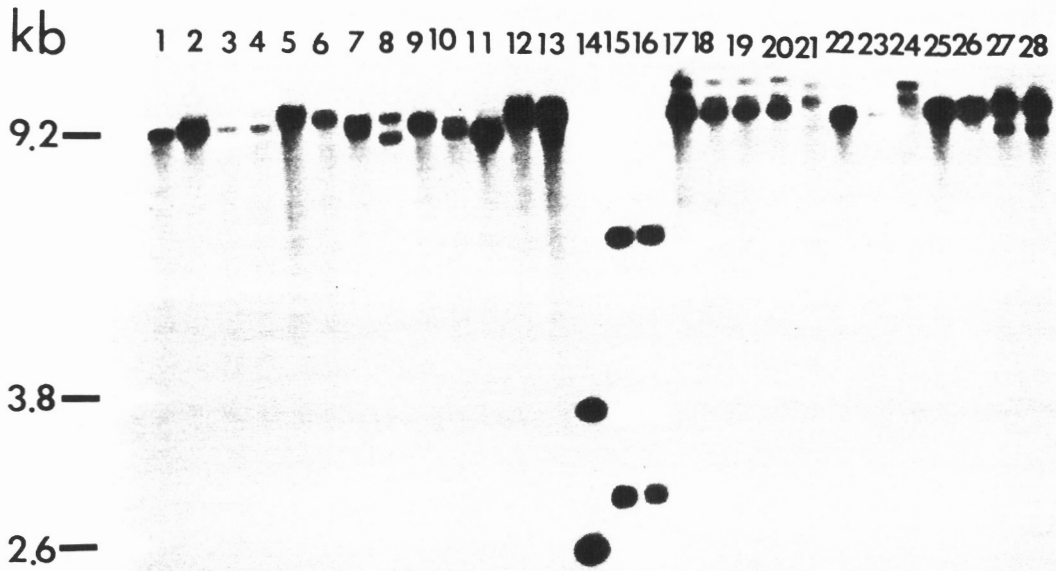


Figure 12. Autoradiogram showing KpnI patterns of the ribosomal DNA repeat units in *Bromus*. Species and lanes are: *B. porteri* (1), *B. ciliatus* (2), *B. anomalus* (3), *B. auleticus* (4, 5), *B. ramosus* (6- 8), *B. benekenii* (9), *B. tomentellus* (10), *B. variegatus* (11), *B. runssorensis* (12), *B. erectus* (13), pRY12 (14), pRY18 (15), *B. inermis* (16,17), *B. pumpellianus* (18,19), *B. biebersteinii* (20), *B. riparius* (21,22), *B. macranthos* (23), *B. marginatus* (24, 25), *B. carinatus* (26), *B. breviaristatus* (27), and *B. catharticus* (28). Numbers at left indicate fragment sizes in kb.

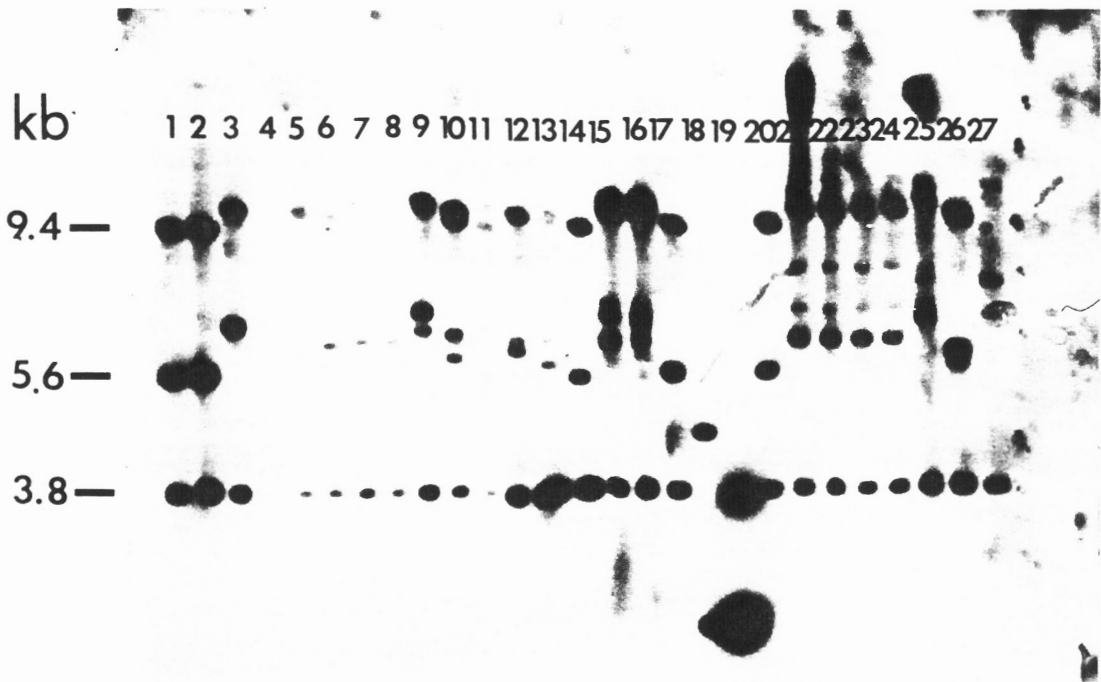


Figure 13. Autoradiogram showing polymorphism in BamHI fragments hybridizing to pRY12 and pRY18. Species and lanes are: *B. scoparius* (1,2,16,19), *B. oxyodon* (3), *B. lanceolatus* (4), *B. severtzovii* (5), *B. anatolicus* (6,7), *B. brachystachys* (8), *B. brizaeformis* (9), *B. secalinus* (10), *B. japonicus* (11), *B. alopecuros* (12), *B. mollis* (13), *B. commutatus* (14,15), pRY12 (17), pRY18 (18), *B. intermedius* (20-23), *B. danthoniae* (24), *B. popovii* (25), and *B. pseudodanthoniae* (26). Numbers at left indicate fragment sizes in kb.



Figure 14. *BstEII* digests of genomic DNAs showing size variation of the ribosomal DNA repeat units in *Bromus*. Species and lanes are: *B. tectorum* (1,2), *B. sterilis* (3,4), *B. rubens* (5), *B. fasciculatus* (6,7), *B. madritensis* (8,9), *B. sericeus* (10,11), *B. rigidus* (12,13), pRY12 and pRY18 (14), *B. rigidus* (15-17), *B. diandrus* (18,19), *B. adoensis* (20), *B. scoparius* (21,22), *B. oxyodon* (23-25), *B. hordaceus* (26,27), and *B. lanceolatus* (28) Numbers at left indicate fragment sizes in kb.

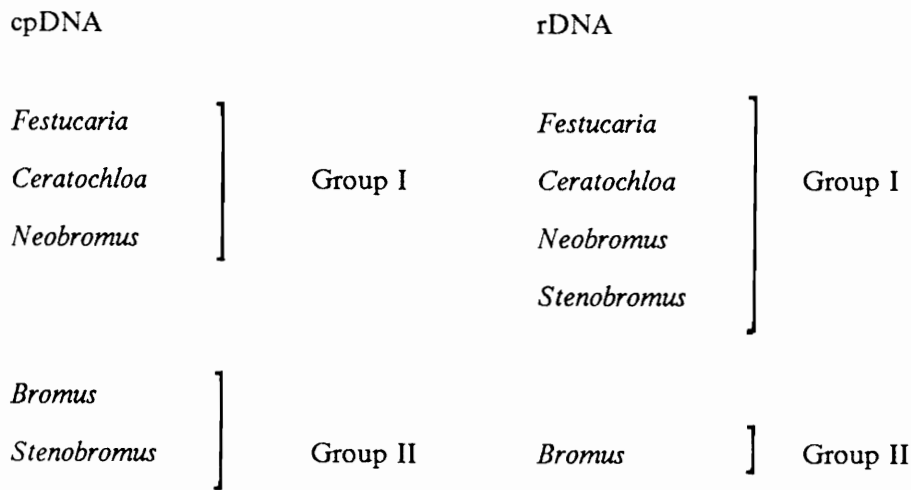
## *Conclusion*

Plant systematics is a multi-disciplinary science and uses data from a broad field of science including morphology, anatomy, biochemistry, physiology, ecology, geography, cytology and molecular biology. In recent years, there has been a surge of research activities utilizing DNA, both nuclear and organellar, to exploit phylogenetic relationships of plants. One of the objectives of plant systematics is to provide an understanding of evolutionary processes and relationships.

In this research cpDNA and rDNA were analyzed in a phylogenetic context in *Bromus*. The DNA results provided new insights into the systematics and evolution of the genus and were, generally, not in agreement with previously proposed relationships. The three major groupings of the genus, based on morphological, cytogenetical and serological data, are shown below.

Sections	Subgenera	Genera
<i>Pnigma</i>	<i>Festucaria</i>	<i>Bromopsis</i>
<i>Ceratochloa</i>	<i>Ceratochloa</i>	<i>Ceratochloa</i>
<i>Neobromus</i>	<i>Neobromus</i>	<i>Neobromus</i>
<i>Bromus</i>	<i>Bromus</i>	<i>Bromus</i>
<i>Genea</i>	<i>Stenobromus</i>	<i>Anisantha</i>

The major intrasubgeneric divisions of *Bromus* based on cpDNA and rDNA are as follows:



Both cpDNA and rDNA indicated that *Bromus* could be divided into two major intrasubgeneric groups (Groups I and II). However, the groups, defined by the two types of DNA studied, differed in their composition of taxa. Both cpDNA and rDNA suggested that subgenera *Festucaria*, *Ceratochloa* and *Neobromus* are closely related. Further, rDNA results showed that subgenus *Stenobromus* is closely allied with *Festucaria*, *Ceratochloa* and *Neobromus*. The rDNA results indicated that subgenus *Bromus* was genetically isolated from the other four subgenera. In contrast, the cpDNA results suggested that subgenera *Stenobromus* and *Bromus* clustered together and were relatively distant from the other three subgenera. The DNA results are not in agreement with Stebbins' (1981) statement that the chromosomes of *Festucaria*, *Ceratochloa* and *Neobromus* are so dissimilar, that a recent common origin is unlikely or difficult to imagine. The very slow rate of cpDNA evolution is providing an earlier evolutionary history of *Bromus* when compared with markers from the more rapidly evolving nuclear DNA. This is a probable explanation for the discrepancy between Stebbins' results and the estimated phylogeny obtained from cpDNA.

The differences in compositions of groups I and II determined by cpDNA and rDNA need further attention. These groups differ primarily by the shifting position of subgenus *Stenobromus*, which falls in group I under rDNA but is in group II under cpDNA. Another anomaly, between the

results obtained from cpDNA and rDNA, concerns the relative evolutionary status of subgenus *Bromus*. The cpDNA trees suggest that subgenus *Bromus* is an evolutionary advanced taxon, while rDNA results suggest that the subgenus is the most primitive of all subgenera. These anomalous results are probably due to some of the assumptions made in this study about presence/absence of restriction sites and the very slow rate of cpDNA evolution. It has to be emphasized that the criteria of primitiveness/advancement are based on assumptions of the loss/gain of restriction sites. It is assumed that it is easier to lose a restriction site than to gain one. The cpDNA phylogenetic trees are based on shared derived restriction sites. The rDNA data were analyzed on the basis of the presence/absence of the unique 2.1 kb fragment which separates subgenus *Bromus* from the other subgenera. If one considers that the mutation rate of chloroplast genes is about half the rate observed in nuclear genes, it may be suggested that the cpDNA is providing a more accurate account of the evolutionary history of *Bromus*. Further, the evolutionary time scale, for the origin of subgenus *Bromus*, provided by Stebbins (1981) coupled with the fact that the annual habit is usually considered an advanced state seems to support the cpDNA data concerning the evolutionary status of subgenus *Bromus*. Perhaps, a more detailed study of the rDNA of *Bromus*, with a battery of restriction enzymes, would provide a clearer answer of the status of subgenus *Bromus*.

The results of this study emphasize the multifarious nature of plant systematics since they suggest the need for research in other disciplines before the total picture of phylogeny and systematics of *Bromus* could be drawn. Conclusions about the systematics of *Bromus* could be supported by data from other disciplines such as morphology, immunology, chemotaxonomy and perhaps further cytological data obtained from techniques such as chromosome banding.

Although morphological data have been used to differentiate intrageneric groups in *Bromus*, a detailed study of morphological characters has not been documented. Many of the keys and species descriptions seem to be reproductions from previous literature. Therefore, I feel that a detailed morphological study of *Bromus*, probably in a cladistic sense, would be useful. However, caution has to be exercised when dealing with certain morphological characters since some of them display a

high degree of phenotypic plasticity. The primary morphological characters used to distinguish intrageneric groups in *Bromus* are the size and shape of spikelets, lemmas and awns, and the differences in nervature of glumes and lemmas. Soderstrom and Beaman (1966) have stated that the number of nerves on glumes and lemmas is not always constant. Similarly, Bor (1968) has pointed out some of the problems associated with identification of *Bromus* species from spikelet characters. For example, spikelet characters are very variable in the same species when they are collected from desert habitats or from banks of irrigation channels. The only morphological character that is known to be genetically controlled is the twisted awns of the lemmas in *B. trinii* (Smith 1970). Since the genetic basis of the other frequently used morphological characters are unknown, a morphological study of the genus will probably be useful if the data are looked at in combination with data from other characters.

Another area of research that could be useful in the systematics of *Bromus* is immunology. In this respect, immunological and electrophoretic studies of prolamins, a class of storage proteins unique to grasses, are probably a good example. Serological studies, involving seed storage proteins, have already been conducted in *Bromus* (Smith 1969, 1972). However, these studies involved crude extracts containing albumin and globulin proteins. Antisera from such protein extracts were not useful in distinguishing specific subgenera probably because of the very conservative nature of these proteins. Immunological studies of this type would be more useful if highly specific antisera could be used.

I have provided examples of two areas of research which could, perhaps, provide more information on the systematics of *Bromus*. The genus *Bromus* presents many fine opportunities for research in many other disciplines.

Finally, Hennig (1966 p 29) has stated that "the phylogenetic system is a task whose goal is as unattainable as that of any other science". He adds that what we tentatively call the phylogenetic system can never be anything final. Such a provisional system can be justified on the grounds that



currently known facts and methods makes it probable that it represents phylogenetic relationships more accurately than any other system. I fully concur with this viewpoint.

# *Vita*

## **MICHAEL PILLAY**

### **PERSONAL DATA**

Date of birth: June 27, 1950  
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Marital status: Married, three children  
Nationality: Indian South African  
Home address: S 185 Shawnee Apt, Blacksburg, Virginia 24060

### **EDUCATION**

#### ***Ph. D. Biology, 1991.***

Virginia Polytechnic Institute and State University (VPI & SU), Blacksburg, Virginia 24061

Dissertation Title : DNA Systematic Studies in the genus *Bromus* (Poaceae)

#### ***M.S. Agronomy, December 1986***

Louisiana State University and Agricultural and Mechanical College, Baton Rouge, Louisiana

Thesis Title: Cytogenetic and Field Evaluation Studies in *Lotononis bainesii* Baker and *L. angolensis* Welw.

***B.Sc. (Hons) Botany, 1983***

University of Durban-Westville, South Africa.

Research Projects:

1. A comparative study of *Cyperus textilis* and *C. sexangularis*.
2. Karyotype studies in the genus *Kniphofia*.

***B. A. Geography and Economics, 1980***

University of South Africa.

***B. Ed. Education, 1977***

University of South Africa.

***U.H.D.E., 1974***

University Higher Diploma in Education (Teacher certification)

University of Durban-Westville, South Africa

***B. Sc. Botany & Zoology, 1972***

University of Durban-Westville, South Africa

**PROFESSIONAL EXPERIENCE**

***Academic Advisor.***

University Academic and Advising Center, VPI & SU. (August 1989-May 1990).

***Teaching Assistant.***

Dept. of Biology, VPI & SU. General/Principles of Biology lab. 1987 -1989; Plant Systematics (Spring, 1989); Aquatic Vascular Plants (Fall, 1989)

***Teacher.***

Department of Internal Affairs (Education), South Africa.

General Science and Senior Biology, January 1974 - August 1984

Promoted to Head of Department (Biology), January 1985.

**HONORS/AWARDS**

Graduate Teaching Assistantship, Department of Biology, VPI & SU. 1987-1990

Sigma Xi small research grant, \$300/, plus matching funds from Department of Biology, VPI & SU. 1987

Graduate Research Development Project grant, \$250/, plus matching funds from Department of Biology, VPI & SU. 1988

Scholarship from South African Education Program under aegis of Institute of International Education (August 1984-June 1986)

**PUBLICATIONS**

Pillay M, Hilu KW (1990) Chloroplast DNA variation in diploid and polyploid species of *Bromus* (Poaceae) subgenera *Festucaria* and *Ceratochloa*. *Theor Appl Genet* 80:326-332

Pillay M, Thro AM (1988) Chromosome morphology in the pasture legume *Lotononis bainesii* and the related species *L. angolensis*. *Cytologia* 53:617-625

Thro AM, Weir AT, Torres K, Flores HE, Pillay M (1987) Production of plantlets from tissue cultures of *Lotononis*, a South African veld legume. *J Grassl Soc South Afr* 4:151-153

## **PRESENTATIONS**

Pillay M, Hilu KW (1988) Systematic and evolutionary studies in *Bromus* (Poaceae, grass family) using a molecular approach. Virginia Academy of Science Meeting, Charlottesville, Virginia, May 1988

## **AFFILIATIONS**

Member Virginia Academy of Science

A handwritten signature in black ink, reading "Michael Hilu". The signature is written in a cursive style with a large, looping 'M' and 'H'.