ZHENBANG CHEN
MOLECULAR AND CYTOGENETIC CHARACTERIZATION OF APOSPOROUS-APOMIXIS IN *PENNISETUM SQUAMULATUM*
(Under the Direction of Dr. PEGGY OZIAS-AKINS)

Apomixis is an asexual reproduction system through which plants propagate themselves through seeds without fertilization. Progenies are genetically identical to their maternal parent, morphologically uniform, and stable between generations. Employment of apomixis could revolutionize plant breeding programs and hybrid production. Unfortunately, apomixis has not been reported in any of our major crops. The transfer of apomixis from wild relatives into crops was hindered by linkage drag of undesired traits for the last 30 years. Molecular studies indicated that aposporous embryo sac development, one type of apomixis, was associated with a complex chromosome unit, which was defined as an Apospory-Specific Genomic Region (ASGR) in *Pennisetum squamulatum*, a relative of pearl millet (*P. glaucum*). Serious suppression of recombination was observed in the ASGR. Molecular and cytogenetic studies were carried out to investigate the extent of recombination suppression over the genome of *P. squamulatum*, to identify the ASGR-carrier chromosome, and to follow the transfer of this chromosome through multiple backcross (BC) generations.

A linkage map was established for the polyploid genome of *P. squamulatum* with amplified fragment length polymorphism (AFLP) markers. The linkage map comprised 182 single-dose markers, 44 linkage groups, and covered 2600 cM. The estimated genome size of *P. squamulatum* was 4632 cM with an expected genome coverage of 73.7%. Four AFLP markers associated with the trait for aposporous embryo sac development were clustered between close flanking markers on a single linkage group. The total map distribution of AFLP markers indicated that recombination suppression was limited to the ASGR.

One chromosome from *P. squamulatum* that carried the ASGR was identified with fluourescence *in situ* hybridization (FISH) using apomixis-linked markers. The ASGR was located on the distal end of a metacentric chromosome. There was no other chromosome in *P. squamulatum* that showed hybridization signal indicating the hemizygosity of the ASGR in *P. squamulatum*. Using genomic *in situ* hybridization (GISH), three chromosomes that hybridized with *P. squamulatum* genomic DNA were detected in a BC_3 generation with a recurrent background of tetraploid pearl millet. The *P. squamulatum* chromosomes in BC_5, BC_6, and BC_7 ranged from one to three. The expression of aposporous embryo sac development was associated with the ASGR-carrier chromosome and no change in reproductive phenotype was detected with the presence of other *P. squamulatum* chromosomes. The *P. squamulatum* chromosomes in backcrosses behaved as univalents and no associations between *P. squamulatum* chromosomes themselves or with *P. glaucum* chromosomes were observed.

KEY WORDS: Apomixis, Genetic map, *Pennisetum*, AFLP, FISH, GISH

MOLECULAR AND CYTOGENETIC CHARACTERIZATION OF APOSPOROUS-APOMIXIS IN *PENNISETUM SQUAMULATUM*

by

ZHENBANG CHEN

B.S., Shanxi Agricultural University, China, 1982

M.S., Henan Agricultural University, China, 1985

A Dissertation Submitted to the Graduate Faculty of The University of Georgia in Partial Fulfillment of the Requirements for the Degree

DOCTOR OF PHILOSOPHY

ATHENS, GEORGIA

2002

MOLECULAR AND CYTOGENETIC CHARACTERIZATION OF APOSPOROUS-APOMIXIS IN *PENNISETUM SQUAMULATUM*

by

ZHENBANG CHEN

Approved:

Major professor: Peggy Ozias-Akins

Committee: Wayne W. Hanna
Kelly R. Dawe
H. Roger Boerma
William M. Randle

Electronic Version Approved:

Gordhan L. Patel
Dean of the Graduate School
The University of Georgia
May, 2002

DEDICATION

To my parents, parents in-law, my wife, my son and daughter

for their understanding, encouragement, support and love.....

ACKNOWLEDGMENTS

I have been so lucky since I become involved in this program because I have had so many nice and helpful people working around me and standing firmly behind me. It would not have been possible for me to get to this point without any one of them. Although I can not mention them all, here, I would like to mention some of them and convey my appreciation through them to others not mentioned here. I am so grateful to Dr. Ozias-Akins; it was she who encouraged and supported me so that this work could be carried out. It was her enormous patience that kept this work going. It was her rich knowledge that made this work meaningful, and it was her great patience that kept her reading and improving my writing. I want to thank Dr. W. Hanna for his constant encouragement and strong fortitude, which helped me to keep going with this work again and again. He has been generous with his time, experimental materials, and any thing I needed for my study. Both of their attitudes toward scientific research set a great model for my professional career.

I would like to say thanks to Dr. Kelly R. Dawe for his generous help with my AFLP work, and to Dr. H. Roger Boerma and Dr. William M. Randle for their generous help with my study when I took my classes in Athens. I appreciate the time they took to answer my questions whenever and wherever I needed help. I thank Dr. Wayne Parrott for his help with my cytogenetic work.

I would also like to acknowledge Dr. Roche, Dr. Goel, and Dr. Akiyama for their help during my research; they were a joy to work with. Many thanks to Anne Bell and Evelyn Perry for their assistance with lab supplies.

TABLE OF CONTENTS

Page

CHAPTER 1

INTRODUCTION

Apomixis is an asexual reproduction system through which plants propagate themselves through seeds (Nogler 1984). It has been reported in more than 35 families and over 300 different species (Bashaw and Hanna 1990). Depending on the origin of embryo sacs, apomixis can be further divided into three types: adventitious embryony, diplospory, and apospory (Nogler 1984). *Pennisetum squamulatum* Fresen, a relative of the cultivated crop pearl millet (*P. glaucum* (L.) R. Br.), produces seeds through aposporous apomixis. It was believed to be an excellent donor of apomixis because of its obligate mode of reproduction and 4-nucleate embryo sac that facilitated the identification of apospory (Dujardin and Hanna 1984). In apomictic reproduction, unlike sexual reproduction, unreduced eggs are generated through mitosis, and they can develop into embryos without fertilization (Nogler 1984; Wen et al. 1998). In the life cycle of an obligate apomict, there is no ploidy level change from diploid to haploid to diploid. No genetic information is added to the embryos from the sperm. Progenies are genetically identical to their maternal parent, morphologically uniform, and stable between generations. Employment of apomixis could revolutionize plant breeding programs and hybrid production (Bashaw and Hanna 1990). Unfortunately, apomixis has not been reported in any of our major crops. The transfer of apomixis from wild relatives has been attempted over the last 30 years but has been hindered with the linkage drag of undesired traits, [from *Tripsacum* into maize (Petrov et al. 1979; Kindiger and Sokolov 1997; Savidan et

al. 1995), from *Pennisetum squamulatum* into pearl millet (Dujardin and Hanna 1983; Hanna et al. 1993)]. Apomixis along with more than one of the *P. squamulatum* chromosomes has been successfully transferred to the pearl millet gene pool (Ozias-Akins et al. 1993).

Genetic transformation with a well characterized gene for apomixis could promise a bright future for utilization of apomixis in crops; however, molecular studies have indicated that aposporous embryo sac development is associated with a complex chromosome unit, which was defined as an Apospory-Specific Genomic Region (ASGR). Serious suppression of recombination was observed in the ASGR (Ozias-Akins et al. 1998), which has almost made the strategy of map-based cloning impractical.

Six years ago when I first started to work on apomixis in Dr. Ozias-Akins' Lab, I thought that I could easily succeed with a beautiful genetic map for apomixis because I was so confident about my ability and was provided with an excellent foundation of a large mapping population and many molecular markers for our favorite trait. It did not take very long to prove that my favorite trait was not as simple a trait as what I and many others thought. After 18 months of hard work, I got no recombination from 4764 data scores. So I did not even get a chance to try Mapmaker, though I often thought that statistics was one of my favorite subjects (it proved wrong again in my qualification). All markers were unquestionably mapped on the same locus without even a single calculation. The result was embarrassing to me very much at the moment. I started to wonder and think seriously about my working model. I tried to get an explanation for my "one locus map" and a possible genetic model for my favorite trait.

Lack of recombination between two or even a few molecular markers could be reasonably explained as close linkage, especially when a large number of plants have been pooled for detecting the closely linked markers. In this case, 16 each of apomictic and sexual plants were pooled. The chance of excluding a molecular marker of 1 cM away is 15% [1-(1-0.1)^{16}]. A genetic distance of 1 cM still should be in the power of detection with a mapping population of 397 individuals, and it would be improbable for 12 molecular markers to all reside within a 1 cM window. Among the 12 markers involved, 11 of them were developed from RAPD markers. For a 10-mer oligo, the frequency of matching sequence along the DNA molecule for a given genome is about 1/Mb (1/4^{10}). The probability for 11 RAPD markers to fall at the same locus would be very small. So the chromosome region harboring apomixis and the molecular markers should not be a small region. Chromosome structural aberrations such as deletion, translocation, or inversion could result in the fixation of a super allele, but they are often associated with high sterility. The investigation of pollen viability in *P. squamulatum*, the donor for our favorite trait, did not support the assumption of these types of chromosomal aberration. However, introgression of an alien chromosomal fragment could result in recombination suppression, without much loss of fertility, if the insertion did not seriously disturb the genome.

If there is a chromosome responsible for apomixis and this chromosome is unique and has no homologous chromosomes in the genome of the donor, the result of recombination suppression could be explained as well. Like the Y chromosome in humans to determine sex, there may be a chromosome in *P. squamulatum* to determine

the mode of reproduction. If this is true, the characterization of the ASGR should be carried out on a chromosomal level.

With the encouragement of Dr. Ozias-Akins, characterization of apomixis in *P. squamulatum* was carried out employing both molecular and cytogenetic methods: (1) With AFLP markers, a genetic linkage map of *P. squamulatum* was established. Four more AFLP markers completely associated with apomixis were identified. They were located near the end of linkage group 1, which suggests that the region responsible for apomixis is not an entire chromosome, but a chromosome segment. (2) With fluorescence *in situ* hybridization, both the apomixis-related chromosome and the putative apomixis-related region were identified. The results with both experimental methods were congruent.

References:

Bashaw EC, Hanna WW (1990) Apomictic reproduction. In Reproductive Versatility in the Grasses. G.P. Chapman, ed (Cambridge University Press). pp. 100-130

Dujardin M, Hanna WW (1983) Apomictic and sexual pearl millet x *Pennisetum squamulatum* hybrids. J Hered 74:277-279

Dujardin M, Hanna WW (1984) Microsporogenesis , reproductive behavior, and fertility in five *Pennisetum* species. Theor Appl Genet 67:197-201

Hanna WW, Dujardin M, Ozias-Akins P, Lubbers E, Arthur L (1993) Reproduction, cytology, and fertility of pearl millet X *Pennisetum squamulatum* BC_4 . J Heredity 84:213-216

Kindiger B, Sokolov V (1997) Progress in the development of apomictic maize. Trends in Agronomy 1:75-94

Nogler GA (1984) Gametophytic apomixis. Pp 475-518 in *Embryology of Angiosperms*, edited by B. M. Johri. Springer-Verlag, Berlin

Ozias-Akins P, Lubbers EL, Hanna WW, McNay JW (1993) Transmission of the apomictic mode of reproduction in *Pennisetum*: Co-inheritance of the trait and molecular markers. Theor Appl Genet 85:632-642

Ozias-Akins P, Roche D, Hanna WW (1998) Tight clustering and hemizygosity of apomixis-linked molecular markers in *Pennistetum squamulatum* implies genetic control of aporpory by a divergent locus that may have no allelic form in sexual genotypes. Proc Natl Acad Sci USA 95:5127-5132

Petrov DF, Belousova NI, Fokina ES (1979) Inheritance of apomixis and its elements in corn-*Tripsacum* hybrids. Genetica 15:1827-1836

Savidan Y, Grimanell D, Leblanc O (1995) Widecross transfer of apomixis to maize: to dream an impossible dream? In Harnessing Apomixis. An international conference to address a topic of far-reaching significance. A New Frontier in Plant Sci, September 25-27. College Station, Taxas. p.20

Savidan Y (2000) Apomixis: Genetics and Breeding. Plant Breed Review 18:14-86

Wen XS, Ye XL, Li YQ, Chen ZL, Xu SX (1998) Embryological studies on apomixis in *Pennisetum squamulatum*. Acta Botanica Sinica 40:598-605

CHAPTER 2

LITERATURE REVIEW

2.1 Overview

Apomixis is a natural asexual reproductive process in angiosperm plants where seeds form without meiosis and fertilization of the egg (Nogler 1984a). Unexpected outcomes of reproduction in female plants were reported as early as the mid-nineteenth century. As related by Gustafson (1946), J. Smith first observed apomixis in 1841 in *Alchornea ilicifolia*, a dioecious species of *Euphorbiaceae*. Strasburger (in Gustafsson 1946) later showed that the high seed set in *Alchornea ilicifolia* resulted from adventitious embryo formation instead of embryo development from unfertilized egg cells. Other dioecious species such as *Chara crinita* and *Antennaria alpine*, with no male plants described, were subsequently reported to reproduce asexually through seeds (Gustafsson 1946). Apospory, one form of gametophytic apomixis, first was observed in the higher plant *Hieracium* subgenus *Pilosella* (Gustafsson 1946). Unreduced megaspore mother cells (diplospory) were found in European blackberry (*Rubus*) by Lidforsis (in Asker and Jerling 1992).

The term, apomixis, was first adopted by Winkler (in Nogler 1984a) to replace the term apogamy. According to the types of life cycles, both plants and animals can be characterized as amictic, referring to organisms without sexual differentiation and fertilization; amphimictic, referring to organisms with sexual differentiation and fertilization; and apomictic, referring to organisms with sexual differentiation but not

fertilization for embryo production. Tackholm subdivided apomictic species into two groups of agamospermous and vegetatively reproducing species (Gustafsson 1946). Nogler (1984a) defined apomixis as asexual reproduction through seed, which is now the most widely accepted definition (Savidan 2000). Apomixis can be further divided into three types: diplospory, apospory and adventitious embryony.

In diplospory, the megaspore mother cell divides mitotically (*Antennaria* Type) or through a modified, restitution-type meiosis (*Taraxacum* type) and gives rise to the egg sac initial (Nogler 1984a). This initial cell divides mitotically three times to develop into an unreduced, mature eight-nucleate embryo sac (Asker and Jerling 1992). Eight-nucleate embryo sacs contain one egg cell, two synergids, a central cell with two nuclei, and three antipodals. The configuration of this egg sac is similar to that found in sexual plants. However, all nuclei of the antipodals, central cell and egg apparatus in apomictic embryo sacs are genetically identical with their mother plant. The egg cell develops into an embryo without fertilization (Nogler 1984a).

Apospory refers to the type of embryo sac formation whose origin is a nucellar cell. A few cells near the center of nucellar tissue and around the megaspore mother cell can enlarge to form aposporous initials. Such initials can divide mitotically and develop simultaneously with the megaspore mother cell to form unreduced embryo sacs adjacent to a reduced embryo sac. Sexual and aposporous development can exist on the same plant and even in the same ovule. Aposporous initials usually develop faster than megaspore mother cells because the former do not have to go through meiosis. If the development of aposporous initials outpaces megasporogenesis or megagametogenesis, the sexual development can terminate. However, it is not clear whether the formation of

an aposporous embryo sac directly or indirectly causes degeneration of sexual embryogenesis. When aposporous embryo sacs are initiated late relative to sexual megagametogenesis, both sexual and aposporous embryo sacs may form in the same ovule. All nuclei of the aposporous embryo sac are genetically identical to the mother plant.

Apomixis has been observed in over 300 species from at least 35 different plant families (Hanna and Bashaw 1987). It is restricted to the reproductive processes of adventitious embrony, diplospory and apospory. Most apomicts are from three plant families, *Asteraceae, Rosaceae* and *Poaceae.* In *Poaceae,* over 95% percent of apomicts show four-nucleate apospory in tribes of *Paniceae* and *Andropogoneae* (Brown and Emery 1958; Nogler 1984a; Bashaw and Hanna 1990). In *Pennisetum*, a member of the tribe, *Paniceae*, 14 species have been reported to reproduce asexually (Schmelzer 1997). All apomictic species are polyploids with a basic chromosome number of $x = 9$, except for *P. massaicium* Stapf with $x = 8$. Apomicts occur as both euploid and aneuploid genotypes in *Pennisetum*. In *Cenchrus ciliaris* (Bashaw 1962), *P. flaccidum* Griseb (Mehra and Remanandan 1973), *P. massaicum* (Jauhar 1981), and *P. orientale* L.C.M. Rich. (Jauhar 1981), whenever diploid plants were found, they were sexual. The reproductive phenotypes of *P. frutescens* Leeke, *P. latifolium* Spreng., *P. macrourum* Trin., *P. setaceum* (Forsk.) Chiov., *P. squamulatum* Fresen. and *P. villosum* R.Br.Ex Fresen. were apomictic, except for a sexual tetraploid sample of *P. flaccidum* (Mehra and Remanandan 1973).

The significance of the application of apomixis in plant breeding was discussed by Bashaw and Hanna (1990). In apomictic plants, the embryos are generated from

unreduced egg cells in the maternal ovule tissues. They do not result from the fusion of male and female gametes except for BIII hybrids (hybrids that result from fertilization of an unreduced egg). The seeds from apomictic plants contain embryos that have a genetic makeup identical to that of the mother plants, no matter how heterozygous their genetic background (Koltunow 1993). Heterozygosity, therefore, can be fixed generation after generation in plants with apomixis. Apomictic F1 hybrid plants with desirable traits could retain heterosis and these traits generation after generation without segregation and selection, which is the most tedious and time-consuming work in any conventional breeding program (Hanna and Bashaw 1987). Introduction of apomixis could revolutionize plant breeding programs by simply eliminating the process of progeny stabilization. Any progeny with an ideal phenotype and with apomixis could be treated as a true-breeding line to be tested and evaluated for potential release. The potential use of apomixis in plant breeding, especially in hybrid production, is increasingly being recognized as more information on apomixis becomes available.

Although apomixis is widespread in nature, it has not been reported in any of our economically important crops. To transfer apomixis deliberately, it is critical to understand the genetics of apomixis and the regulatory mechanisms underlying apomixis (Ramulu et al. 1999).

2.2 Embryological Study of Apomixis in *Pennisetum*

As mentioned above, gametophytic apomixis is an asexual reproduction system where the embryo sac develops through mitosis (Nogler 1984a). This process describes the outcome of both diplospory and apospory which are distinguished by the type of cell from which the embryo sac originates. In *Pennisetum*, a 4-nucleate embryo sac is formed

through apospory (Bashaw and Hanna 1990). Initially, the development of the megaspore mother cell (MMC) in an aposporous plant is the same as that in a sexual plant (Bashaw and Hanna 1990). The difference between totally sexual and apomictic plants can be observed only after the aposporous embryo sac initials start to develop. In *P. squamulatum*, a single archesporial cell enlarges and develops into a MMC which later divides meiotically to form a triad (Wen et al. 1998). The undivided MMCs or triads soon degenerate while one to several nucellar cells in proximity to the degenerating cell(s) begin to enlarge. One of the enlarged nucellar cells near the micropyle becomes vacuolated and develops into an uninucleate aposporous embryo sac. This uninucleate embryo sac then undergoes two mitotic divisions to form a four-nucleate embryo sac. One cell located near the micropylar end forms the egg, one cell located beside the egg cell forms a synergid, and the other two nuclei reside in a central cell located toward the chalazal end of the embryo sac. Sometimes more than one nucellar cell will develop at about the same speed and form multiple aposporous embryo sacs.

There are no antipodal cells formed in the *Panicum*-type embryo sac facilitating the identification of apospory in this species compared with those that produce embryo sacs that closely resemble 8-nucleate sexual embryo sacs. The time of initiation of the apomictic process is very important (Nogler 1984a). Aposporous initials directly enter mitosis to generate embryo sacs while MMCs first generate megaspores through meiosis prior to embryo sac development. Aposporous development, therefore, should require less time than sexual development, and the process of sexual development usually is outpaced and arrested in obligate apomixis (Nogler 1984a). In such cases, sexual development will terminate and only aposporous embryo sacs will be formed. Both

aposporous and sexual embryo sacs may be observed in the same ovule if the initiation of apospory is not early enough to take over the sexual embryo sac development, which would result in facultative apomixis. Diplosporous embryo sacs and aposporous embryo sacs may rarely coexist in the same individuals (Savidan 2000).

Unreduced egg cells in *P. squamulatum* can develop into a functional embryo without fertilization. Depending on the initiation time point of embryogenesis, embryo developments were classified as pre-genesis embryo: embryos formed without fertilization, 1 to 2 days before anthesis; and late-genesis embryo: derived from unfertilized egg cells, 3 to 4 days after anthesis (Wen et al. 1998). In the early-genesis embryo type, the synergid initiates degeneration and the egg cell starts the first division with the division plate parallel to the long axis of the embryo sac 1 or 2 d before anthesis. Two nuclei of the central cell merge together and form a uninucleate central cell. After the egg cell has divided several times and developed into a globular embryo, the central cell nucleus starts to divide. For the late-genesis embryo type, 1 to 2 d after the anthesis, the central cell nuclei already started division to form free-nuclear endosperm in the embryo sac while no obvious changes could be observed in both the egg cell and synergid. In some cases, the synergid divided into two cells before they degenerated. Vacuoles could be observed in all egg cells before they differentiated into embryos. As soon as embryogenesis was initiated, subsequent developmental processes were comparable to those found in sexual plants. In aposporous apomixis, the central cell nucleus divided to form free-nuclear endosperm after fertilization. Cell walls were formed after free-nuclear division.

In *Pennisetum ciliare* (L.) Link, the egg cells in aposporous embyo sacs started to divide before the pollen tubes reached the female gametophyte (Vielle et al. 1995). The degeneration of the egg apparatus was very quick and cell walls formed over the egg cell plasma membrane toward the chalazal end 4 h after pollination.

In a backcross of *P. glaucum* x *P. squamulatum* to pearl millet, endosperm development was observed to arrest 4-6 d after pollination and was concluded to be one reason for low seed set. The success of endosperm development was reported to correlate with a 2 maternal : 1 paternal genome ratio (Morgan et al. 1998). In the normal sexual process, a 2*n* central cell usually develops into endosperm after fertilization with a 1*n* sperm cell, during which a 2 maternal : 1 paternal genome ratio is established. In *Pennisetum*, the aposporous central cell can be either 2*n* (1 polar nucleus) or 4*n* (2 polar nuclei) (Bashaw and Hanna 1990). After fertilization with a 1*n* sperm, the ratio of maternal : paternal genomes is either 2 : 1 or 4 : 1. When apomixis is introduced into the genetic background of a sexual species, the higher maternal : paternal ratio does not appear to be viable (Morgan et al. 1998). In *P. squamulatum*, multiple embryo sacs tend to abort during embryogenesis and cause about 30% abortion of embryos during the anthesis stage (Wen et al. 1998). This phenomenon of genome-ratio effects is not observed in diplosporous *Tripsacum* species (Grimanelli et al. 1997). Fertilized central cells with genome ratios of 4 : 1, 4 : 2, 8 : 1 or 8 : 2 are as viable as in normal sexual plants with a 2 : 1 ratio.

2.3 Regulation of Apomixis

An early hypothesis for the induction of apomixis was the stimulation of egg cells by necrohormone from dying cells or tissues in the vicinity of the egg cell. The

necrohormone was thought to be responsible for inducing diplospory and the development of unreduced egg cells into embryos without fertilization (Gustafsson 1946; Asker and Jerling 1992). This theory was soon rejected because apomictic development often occurs earlier than the degeneration of cells in an ovule.

Apomixis is often associated with polyploidy and heterozygosity in many species, which easily lead to the hypothesis that apomixis was the result of polyploidy and heterozygosity of genetic background (discussed in Gustafsson 1946). Almost all apomicts are polyploid in nature (Nogler 1984a). The polyploidy level can be as high as dodecaploid in some apomicts, and different levels of polyploidy can often be found in the same species. In the genus *Pennisetum* only polyploids have been confirmed to reproduce through apomixis while their diploid relatives produce their progenies sexually (Schmelzer 1997). Tetraploid is the most common cytotype of apomictic plants. Triploids are rare in apomicts and are mainly found in the *Taraxacum* and *Ixeris* types of diplospory (Nogler1984). *P. setaceum* is the only triploid species in *Pennisetum* found to reproduce through apomixis (Simpson and Bashaw 1969). The observation of Muntzing (1940) that reducing the level of polyploidy of *Poa pratensis* by haploid parthenogenesis made possible recovery of sexual offspring from apomictic plants, strongly supported the polyploidy theory championed by Strasburger that polyploidy was one of the causes of apomixis besides hybridity. However, the polyploidy theory as a cause of apomixis can be argued against by the observation that genes controlling apomixis may not be transmitted through haploid gametes thus enforcing polyploidy (Nogler 1984a).

Also, in only rare cases has artificial doubling of chromosome number led to a change in reproductive phenotype. Increasing the ploidy of diploid plants of *Paspalum*

notatum by colchicine treatment induced facultative apomictic reproduction in three individual plants. It was proposed that gene(s) controlling apomixis existed at the diploid level but could not be expressed until the allele dosage had increased beyond a threshold (Quarin et al. 2001).

More recent research on the transfer of apomixis from *Pennisetum* and *Tripsacum* into pearl millet (Dujardin and Hanna 1983a, 1984a, 1984b, 1986) and maize (Leblanc et al. 1995, 1996), respectively, even showed that apomixis does not necessarily require polyploidy. *P. squamulatum* is aposporous with a hexaploid genome (Dujardin and Hanna 1983a). Crosses between pearl millet (*P. glaucum*) ($2n = 4x = 28$) and *P. squamulatum* were made to transfer apomixis into pearl millet (Dujardin and Hanna 1985, 1989). Highly apomictic backcross $(BC)_3$ plants with 27 or 29 chromosomes were selected. Apomixis was maintained here when the ploidy level was reduced from hexaploid in *P. squamulatum* to a few chromosomes in a tetraploid *P. glaucum* background. Only a few chromosomes were transferred, and they should not function as a whole genome set in the sexual tetraploid genetic background of *P. glaucum* as they did in their donor. A polyhaploid plant was discovered in an F_1 hybrid population of *P. glaucum* x *P. squamulatum* (Dujardin and Hanna 1986). This plant produced 95% of aposporous embryo sacs, had 21 chromosomes, and likely originated by pathenogenesis of a reduced gamete from an apomictic F_1 plant. Cytogenetic investigation showed that there were 4 bivalents and 13 univalents at meiosis, which led to the conclusion that this plant did not contain a complete genome from either parental species. A tetraploid diplosporous *Tripsacum* species ($2n = 4x = 72$) was used as the male parent to transfer apomixis into maize (Leblanc et al. 1995, 1996). Apomictic plants of 28 chromosomes,

10 from maize and 18 from *Tripsacum* representing the haploid component from each species, were selected. In *Hieracium aurantiacum* a diploid apomictic plant was identified that produced aposporous embryo sacs, and endosperm formation was autonomous (Bicknell 1997). In *Brachiaria decumbens*, both diploid and tetraploid cytotypes were facultative apomicts and no diploid sexual types were found yet in this species (Naumova et al. 1999). The expression of aposporous embryo sacs in the diploid was much lower (10% to 15%) than that in tetraploids (80% to 95%). Similar results of low diplospory expression (0%-3%) in diploids were observed with a cross between diploid (2n=18) sexual *Erigeron strigosus* and triploid (2n = 27) apomictic *Erigeron annuus* (Noyes 2000). Therefore, it seems that polyploidy is not invariably associated with the occurrence of apomixis, rather apomixis contributes to the stability of polyploidy and heterozygosity (Asker and Jerling 1992).

Apomixis is genetically controlled (Nogler 1984a; Asker and Jerling 1992). However, attempts to determine the genetic regulation of apomixis through crossing have met with great difficulties. Besides the rare occurrence of sexual plants in some apomictic populations, other factors which have limited genetic study include facultative apomixis, polyploidy, difficulty in identifying apomictic progeny in segregating populations (Bashaw and Hanna 1990; Hussey and Bashaw 1995), and possible selection against megaspores carrying genes for apomixis (Roche et al. 2001a). Low viability of pollen in some apomictic plants (Gustafsson 1946) also could lead to erroneous estimation of segregation ratios because of unbalanced meiotic division, which could result in a biased pollen population. As mentioned above, apomixis in nature always has been found to be connected with heterozygosity and polyploidy (Nogler 1984a), and

some apomictic plants, especially with a high level of polyploidy, showed a tendency to revert to sexuality by haploid parthenogenesis (Muntzing 1958). Therefore heterozygosity and polyploidy dominated the early concepts of the factors that caused apomixis (Bashaw and Hanna 1990). Much effort has been made in crossing sexual and related apomictic forms and extensive analyses have been published (Asker and Jerling 1992), though perhaps 95% of the data available on this subject are inconclusive (Savidan 1990). However, the majority agrees that one or a few dominant gene(s) regulate asexual reproduction in species with aposporous apomixis.

Different genetic models have been postulated with different species and crops (Nogler 1984a; Richards 1986; Bashaw and Hanna 1990; Koltunow 1993; Sherwood et al. 1994). Most of the recent genetic analyses of crosses between sexual and apomictic plants have indicated dominant inheritance of apomixis versus sexuality (Table 2.1). Powers (1945) proposed a model for at least three recessive alleles in *Parthenium argentatum*. This model was based on the assumption that at least three components of apomixis were separately inherited (failure of reduction, failure of fertilization, and parthenogenetic egg cell development). This model was never tested experimentally. Hanna et al. (1973) postulated that in *Panicum maximum* sexuality was dominant to apomixis and that mode of reproduction was probably controlled by at least two loci. Contradictory conclusions were reached in an independent study with *Panicum maximum* by Savidan and Pernes (1982) who postulated that apomixis was inherited as one dominant supergene based on their extensive crossing and backcrossing experiment. Crosses between sexual and apomictic dandelions (*Taraxacum*) showed multigenic control of diplosporous apomixis in *Taraxacum* (Van Dijk et al. 1999). In *Erigeron*

annuus, parthenogenesis was reported to segregate independently from diplosporous embryo sac formation and each trait, parthenogenesis and diplosporous apomixis, was regulated by single locus (Noyes and Rieseberg 2000).

One dominant gene underlying apomictic development over sexuality has been the most popular recent hypothesis among apomixis researchers (reviewed by Grossniklaus 2001). Monogenic inheritance of aposporous apomixis was observed in two *Hieracium* species (Bicknell et al. 2000). Genetic analysis of a *Paspalum simplex* mapping population indicated that apomixis was regulated by a large chromosome fragment, which was inherited as a single dominant genetic unit (Pupilli et al. 2001; Caceres et al. 2001). The study of Taliaferro and Bashaw (1966) on genetic regulation of aposporous apomixis in buffelgrass proposed an epistatic model where locus B for sexual reproduction was epistatic over A for aposporous apomixis and they showed disomic inheritance. Sherwood et al. (1994) proposed a single-locus model for buffelgrass where apomixis showed a random chromatid, tetrasomic inheritance.

The expression of apomixis also may be modified through a dosage effect of "aposporous factors" (Nogler 1984a). Some plants with fewer aposporous factors tended to be facultative or the degree of apomictic expression was susceptible to environmental influence. While in diploid cytotypes of *Taraxacum*, the dosage effect of diplosporous factors was described not to be able to be achieved as in polyploids and sexual reproduction dominated the development (Mogie 1992). Similar dosage effects on apomixis were found in *Bothriochloa-Dichanthium* (Harlan et al. 1964; d'Cruz and Reddy 1971), *Beta lomatogona* (Cleiji et al. 1976), *Poa pratensis* (Akerberg and

Binggefors 1953; Almgard 1966), *Eragrostis curvula* (Voigt and Burson 1981) and in *Hieracium* (Koltunow 2000).

The exposure of a recessive lethal factor which was associated with a dominant apomixis gene (A) was postulated with the study on *Ranunculus auricomus* (Nogler 1984b). This model assumed that a recessive lethal factor (A^-) always associated with the dominant apomictic gene A and in haploid gametes, expression of the recessive A^- locus would make the haploid gametes with A A^- inviable. Under the condition of polyploidy, reduced microspores would at least be diploid and the dominant allele at the A^- locus would allow the gametes to function. Therefore, a diploid apomict would not exist under natural conditions. This hypothesis was supported by the data from an F_1 mapping population of *P. glaucum* and *P. squamulatum* (Ozias-Akins et al. 1998). Alternative to gametic lethality, selection against diploid zygotes also can occur as shown in *Hieracium* (Bicknell et al. 2000). In both *P. squamulatum* and *Paspalum simplex*, the segregation ratio of apospory to sexual reproduction fit that of tetrasomic inheritance (Pupilli et al. 1997; Ozias-Akins et al. 1998). Genetic analysis of diplosporous apomixis in tetraploid *Tripsacum* showed a similar result (Grimanelli et al. 1998a, 1998b).

In summary, apomixis is believed to be controlled by one or a few genetic factors. Most experiments support the one-locus model for aposporous apomixis. The discrepancy between published results may be due to different genetic backgrounds among species.

2.4 Transfer of apomixis to crop plants

Apomixis usually is found in wild relatives of economically important crops. *Elymus rectisetus* (Nees in Lehm.) Love and Connor is a relative of wheat (*Triticum aestivum* L.),

Tripsacum dactyloides (L.) is a relative of maize, and many species with apomictic reproduction in the genus *Pennisetum* are relatives of cultivated pearl millet (*P. glaucum*) (Bashaw and Hanna 1987). The transfer of apomixis from wild relatives into cultivated crops has attracted much effort. Apomixis from *Tripsacum* was transferred into a maize genetic background (Petrov et al. 1979; Leblanc et al.1996; Kindiger and Sokolov1997). An apomictic hybrid, G-278, with 20 maize chromosomes and 18 *Tripsacum* chromosomes was obtained from an interspecific cross between maize and *Tripsacum*. With maize as a pollen donor, G-278 was tested for its reproductive mode. Among its offspring, 84% was of the maternal type (Petrov et al. 1979). In a different group, one plant with 28 chromosomes and 12 other plants with 38 chromosomes were obtained. Genomic *in situ* hybridization showed that the 28-chromosome plant had 10 chromosomes (*n*) from maize and 18 chromosomes (*n*) from *Tripsacum*. The other 12 plants contained 20 chromosomes (2*n*) from maize and 18 chromosomes (*n*) from *Tripsacum.* The number of *Tripsacum* chromosomes could not be further reduced because high male sterility occurred in plants with less than 28 chromosomes (Savidan et al. 1995).

Dujardin and Hanna have carried out extensive breeding efforts with apomictic *Pennisetum* species and hybrids since the late 1970s (Dujardin and Hanna 1983a, 1983b, 1984a, 1984b, 1985, 1986, 1988, 1989; Hanna and Dujardin 1982, 1986, 1987; Hanna et al.1993; Bashaw and Hanna 1990). To develop an apomictic pearl millet, Dujardin and Hanna used a double cross of *P. glaucum* x *P. purpureum* and *P. glaucum* x *P. squamulatum* as pollen donor and tetraploid millet ($2n = 4x = 28$) as the recurrent female parent. An obligate apomictic backcross three (BC_3) plant (K169-46) with a genome of

29 chromosomes was selected from 1053 progeny (Dujardin and Hanna 1989). Nine BC_4 plants were used for extensive investigations on chromosome behavior during meiosis and mitosis, male fertility, and reproductive mode (Hanna et al. 1993). More than one of the *P. squamulatum* chromosomes has been successfully transferred to the pearl millet gene pool (Ozias-Akins et al. 1993). Because of the morphological similarity of chromosomes among different species of *Pennisetum,* the number of *P. squamulatum* chromosomes in the genomes of BC_3 and BC_4 could not be definitively determined in these studies even though such information is very important for further investigation of the genetic regulation of apomixis.

BC_1 plants with 54- and 60-chromosomes were generated by crossing wheat as male with a hybrid as female. This hybrid was obtained from a reduced wheat egg cell fertilized by an unreduced *Elymus rectisetus* sperm from a cross of wheat (*Triticum aestivum* L., $2n = 6x = 42$, AABBDD) and apomictic wheatgrass [*E. rectisetus* (Nees in Lehm.) A. Love and Connor, $2n = 6x = 42$, SSYYWW] accessions (Liu et al. 1994). The pollen stainability was 10% and 5% in 54- and 60-chromosome BC_1 plants, respectively. The authors believed that $2n$-gametes from the *E. rectisetus* accession facilitated hybrid and BC_1 formation.

Four crosses were made between three Australian apomictic *Elymus* taxa as female and three cereal grains: wheat, rye, and barley as males (Torabinejad and Mueller 1993). One F_1 hybrid plant was obtained from a cross of *E. scabrus* var 'plurinervis' x *Triticum aestivum,* 4 F_1 hybrid plants from *E. scabrus* var 'scabrus' x *Secale cereale,* 13 F_1 hybrid plants from *E. scabrus* var 'plurinervis' x *Hordeum vulgare,* and 2 F_1 hybrid plants, including one BIII, type from *E. rectisetus* x *H. vulgare*. All hybrids were sterile.

Mean chromosome pairings were investigated and univalents were consistently observed during meiotic metaphase-I. The development of embryo sacs in the BIII hybrid was facultatively apomeiotic. Spike morphology in the *E. scabrus* x *T. aestivum* and *E. scabrus* x *H. vulgare* hybrids was intermediate to their parents and *E. scabrus* x *S. cereale* and *E. rectisetus* x *H. vulgare* looked like their maternal parents.

Outside of Poaceae, apomixis genes were introduced from *Manihot neusana* Nassar to a cultivated cassava, *M. esculenta* Crantz through interspecific hybridization. The aposporous reproduction was confirmed to be transferred in the offspring with morphological uniformity, embryonic observation and molecular marker analysis (Nassar et al. 1998; 2000).

2.5 Molecular markers and plant genetics

2.5.1 Marker types

There are basically two kinds of molecular markers, protein/isozymes and DNA sequences. Here we consider only the DNA markers because (i) they now are the most commonly used markers for plant genetics; (ii) DNA molecular markers are abundant and independent from environmental influences and epistatic interactions; and (iii) they demonstrate Mendelian inheritance. DNA markers mainly include Restriction Fragment Length Polymorphisms (RFLP) (Botstein et al. 1980), Randomly Amplified Polymorphic DNA (RAPD) (Williams et al. 1990; Welsh and McClelland 1990), which can be developed into sequence tagged site (STS) markers, microsatellites or Simple Sequence Repeats (SSR) (Litt and Luty 1989) and Amplified Fragment Length Polymorphism (AFLP) (Vos et al. 1995). DNA polymorphisms have been exploited extensively from RFLP to AFLP and the latter literally could generate an unlimited number of molecular

markers for genetic studies and plant improvement (Young et al. 1999). RFLP, RAPD, SSR and AFLP have all been used to determine inter- and intra-specific genetic diversity, construct molecular maps of crops, and tag important agronomic traits using specially designed mapping populations. (Williams et al. 1990; Winter and Kahl 1995; Jones et al. 1997b; Van DerNest et al. 2000; Tan and Zhang 2001; Kolliker et al. 2001).

None of the molecular marker types is perfect and the choice of an appropriate marker should depend on the objectives of the study, technical considerations, availability of appropriate laboratory facilities and the cost of development and application. The main features of these four marker types are summarized in Table 2.2. However, many of the molecular linkage maps have been made with two or more different types of markers for saturating the maps (Nachit et al. 2001; Fossati et al. 2001; Mengon et al. 2000; Shah et al. 2000).

AFLP combines the reproducibility of STS and the sampling of multiple fragments of RAPD (Lin and Kuo 1995; Vos et al. 1995). It does not require the development of target-specific primers based on primer sequence information as for SSR. However the consistency of AFLP could be as high as SSR because of the adaptor design. Primer pairs for SSRs have to be developed for each locus, considerable resources must be invested, and one SSR reaction samples only one locus. RAPDs can sample from more than one site simultaneously, but because of the low annealing temperature, amplification patterns may vary from laboratory to laboratory and from experiment to experiment (Johns et al. 1997). AFLP mainly includes three steps: (1) Digestion of genomic DNA with two different restriction enzymes; one is a 6-base-pair cutter and the other is a 4-base cutter. (2) Ligation of a short piece of known sequence

DNA (adaptor) to the ends of digested DNA fragments. The adaptor sequences for the two different digested ends are different. Because the genomic DNA is completely digested with the restriction enzymes, every fragment should ligate to an adaptor at each end. (3) PCR amplification of the ligation products with two primers, each of which matches the corresponding sequences of adaptors plus an extension of 1 - 3 anonymous bases. The primers are 18-20 nucleotides long and the PCR is then carried out under very high stringency conditions. The number of nucleotides extending from the 3' ends of adaptor sequences depends on the size of genome under investigation. Only the fragments matching completely the sequences of primers will be amplified and the PCR products are part of genomic DNA because of the extension of the nucleotides. One sixteenth of the possible fragments would be amplified if the primers had a single nucleotide extension for each end and 1/4096 are amplified with a 3-nucleotide extension. Because the digestion sites are distributed randomly over the genome, the final PCR products are also a random sample from the entire genome. To this point of view, AFLP is similar to RAPD. Since the primers used in AFLP are 18 to 20 nucleotides, which are 10 nucleotides longer than the primers used in RAPD reactions, PCR of AFLPs can be carried out under very high stringency like the PCR for SSRs. The result is thus similar to SSRs in reproducibility. AFLP requires more steps than other PCR-based marker types, although digestion and ligation can be carried out simultaneously. For complex templates, amplification requires two steps, preselective amplification for enriching only one fraction of the ligated fragments and reducing the background, and a selective amplification with labeled primers (see Fig. 2.1).

Reproducibility of RAPD, AFLP and SSR markers has been tested with the same set of DNA and primers in several laboratories simultaneously (Jones et al. 1997a). RAPDs showed the most inconsistency among different laboratories. Both AFLPs and SSRs were amplified in all laboratories with the SSR alleles slightly differing in their sizes. Shah et al. (2000) compared RFLP, RAPD, and SSR markers for their ability to detect polymorphism between two wheat cultivars, Cheyenne and Wichita. Six out of 10 SSRs tested showed polymorphism between varieties and 50% of the polymorphic SSRs showed polymorphism for chromosome 3A, which was reported to carry genes conditioning yield components, plant height, and anthesis date. Three out of 77 STSs showed polymorphism between varieties and only one of them showed polymorphism for chromosome 3A. Seventy-seven percent of 52 RFLPs tested showed polymorphism between two varieties and 42% of them were polymorphic for chromosome 3A. Twenty percent of 40 RAPDs showed polymorphism for both varieties and none of them were polymorphic for chromosome 3A. The ability to detect the polymorphism between the two tested wheat varieties was RFLP ~ SSR > STS > RAPD. McGregor et al. (2000) investigated the polymorphism among tetraploid potato (*Solanum tuberosum* L.) cultivars with AFLP, SSR, and RAPD. Polymorphisms could be detected for varieties with each type of marker while the mean number of profiles generated per primer (or primer pair) per cultivar, referred to as Genotype Index (GI), was different. With potato, AFLP had the highest GI (GI = 1), followed by multi-locus SSR (GI = 0.77), RAPD (GI = 0.53), and single-locus SSR (GI = 0.36).

Even with the same type of marker, markers developed with techniques that sample different components of the genome might have different characteristics (Brown

et al. 1996). For example, out of 323 SSR-markers, 194 developed from genomic library screening showed greater polymorphism (83.8%) than 129 (54.0%) derived from rice-expressed sequence tags (ESTs) in public DNA databases when tested between parental pairs of six inter-subspecific crosses and one inter-specific cross of rice (Cho et al. 2000). Both groups proved to be similar with regard to the number of alleles per locus, polymorphism information content (PIC), and allele size ranges.

AFLP has been increasingly employed since it was introduced (Vuylsteke et al. 1999; Bert et al. 1999; O'Hanlon et al. 2000; Cho et al. 2000; Dong et al. 2001; Busso et al. 2000; Groh et al. 2001; Marsan et al. 2001). The popularity of this technique is also largely because of the unlimited abundance of amplified bands (Russell et al. 1997; Young et al. 1999). By changing the number of extension nucleotides, the combination of different nucleotides, and restriction enzymes, one can generate different sets of AFLPs and produce an unlimited number of markers. A total of 650 *Eco*RI/*Mse*I-AFLP markers and 189 *Pst*I/*Mse*I-AFLP markers were generated with 41 primer combinations in soybean (Young et al 1999). In maize (Vuylsteke et al. 1999), AFLP markers generated using *Eco*RI/*Mse*I and methylation-sensitive *Pst*I/*Mse*I deviated significantly from a random distribution. The clustered regions were co-localized well with the putative centromeric regions of the chromosomes. For soybean (Young et al. 1999), distortion of *Eco*RI/*Mse*I-AFLP markers was also significant but *Pst*I/*Mse*I-AFLP markers were randomly distributed over the genome. The deviation of *Eco*RI/*Mse*I-AFLP markers was caused by lack of recombination in certain regions of the genome.

AFLPs can sample more fragments than RAPDs for each reaction. The profile of each run from AFLP and RAPD is significantly different. RAPD uses 10-mer oligos to

sample from the genome while AFLP uses the 6-mer and 4-mer restriction sites simultaneously. The frequency of RAPD sites over a genome sequence is $1/4^{10}$. It is $17/4^6$ ($1/4^6 + 1/4^4$) for AFLP sites. The sample size of AFLP is theoretically over 4000 times larger than that of RAPD. So AFLP should be much more efficient. AFLPs and RAPDs were used in a study on inheritance of apomixis in *Poa pratensis* with the strategy of bulked segregant analysis (Barcaccia et al. 1998). Thirteen polymorphic AFLP loci were detected with 36 primer combinations while only 3 polymorphic loci were found with 48 RAPD primers. Among those polymorphic loci, 8 AFLPs were linked to parthenogenesis and only 1 RAPD locus cosegregated with the trait. However, the polymorphism of AFLP markers depends upon the resident polymorphism within a species. The abundance could be very low in certain cases (Bai et al. 1999).

2.5.2 Application of molecular markers

2.5.2.1 Mapping

There have been at least 212 molecular marker-based maps established for over 78 economically important species from 66 different genera including monocots, dicots and gymnosperms (reviewed by Riera-Lizarazu et al. 2001). Molecular genetic linkage maps are established for rice (*Oryza sativa* L.) [with RFLP/AFLP/SSLP (Simple Sequence Length Polymorphism)] (McCouch 2001), maize (*Zea mays* L.) (with RFLP and AFLP) (Vuylsteke et al. 1999; Coe et al. 2001), tomato (*Lycopersicon esculentum*) (Tanksley et al. 1992; Chunwongse et al. 1997), with ryegrass (*Lolium perenne*) (with AFLP) (Bert et al. 1999), tef [*Eragrostis tef* (Zucc.) Trotter] (with AFLP) (Bai et al. 1999), sorghum (with RFLP and AFLP) (Peng et al. 1999; Boivin et al. 1999), sugarcane (*Saccharum* spp.) (with RFLP, SSR and AFLP) (Cordeiro et al. 2000; Hoarau et al. 2001), hexaploid oat

(with AFLP) (Groh et al. 2001), eggplant (*Solanum melongena* L.) (with RAPD and AFLP) (Nunome et al. 2001), pepper (with RFLP, SSR and AFLP) (Huang et al. 2001; Kang et al. 2001), soybean [*Glycine max* (L.) Merr.] (with SSR) (Diwan and Cregan 1997; Yamanaka et al. 2001). More than 30 maps are published for maize alone, 8 for tomato and 7 for wheat.

More and more studies are employing AFLPs to either saturate the existing genetic map or construct new and high-density maps. Two high-density AFLP linkage maps of maize were constructed based on: (1) a B73 x Mo17 recombinant inbred population and (2) a D32 x D145 immortalized F-2 population. AFLP markers were generated with the enzyme combinations of *Eco*RI/*Mse*I and *Pst*I/*Mse*I. A total of 1539 and 1355 AFLP markers have been mapped in the two populations, respectively (Vuylsteke et al. 1999). A durum wheat (*Triticum turgidum* L., var. durum) linkage map was constructed with 306 markers including 138 RFLPs, 26 SSRs, 134 AFLPs, five SSPs (seed storage proteins), and three known genes (Nachit et al. 2001). The map is 3598 cM long, with an average distance between markers of 11.8 cM, and 12.1% of the markers deviated significantly from the expected Mendelian ratio of 1 : 1. The molecular markers were evenly distributed between the A and B genomes.

Mapping in polyploids has lagged behind diploids because of the complex behavior of markers (Wu et al. 1992). The inheritance of loci in polyploids is very complex and methods for analyzing segregation data from polyploid populations are still under development. Single-dose restriction fragment (SDRF) markers, which exist on only one of the homologous chromosomes, segregate in a testcross at a ratio of 1:1. They can be treated the same as markers from a diploid population. Single-dose PCR-based

markers also can be selected according to the segregation ratio of 1:1. By selecting single-dose markers, only a portion of the genome is sampled, and this portion may be very small for autopolyploids because the majority of loci in the genome could be multiplex. Although this is not a perfect method because it can only sample a part of the genome and includes largely dominant markers, it provided a simple way to map a complicated genome such as *Saccharum spontaneum* (Da Silva et al. 1993; D'Hont et al. 1994; Mudge et al. 1996; Grivet et al. 1996). An AFLP genetic map for sugarcane based on a selfed population of cultivar R570 was established with a total of 887 single-dose AFLP markers generated with 37 primer combinations (Hoarau et al. 2001). The map included 120 cosegregation groups (CGs) with markers linked in coupling and with a cumulative length of 5,849 cM covering an estimated one-third of the total genome length. The isolated minor CGs indicated that the accessibility of some chromosome regions is difficult. Analysis of repulsion-phase linkages suggested a high preferential pairing for 13 CG pairs. Out of the 120 CGs, 34 could be assigned to one of the 10 homo(eo)logy groups, which already were defined with a previous RFLP map (Hoarau et al. 2001).

Genetic mapping of two F_1 autotetraploid alfalfa populations (*Medicago sativa* L.) was carried out with 82 single-dose restriction fragments (Brouwer and Osborn 1999). Deviation of markers from Mendelian inheritance (4-9%) was much less in the tetraploid populations than previously reported for diploid populations (18-54%). For a basic chromosome number of 8 in alfalfa, 4 homologous cosegregation groups were detected for 7 of the 8 expected linkage groups. An integrated map of the homologous cosegregation groups included 88 loci on seven linkage groups covering 443 cM. The

application of SSRs in mapping of tetraploid alfalfa was investigated by Diwan et al. (2000). Ten SSRs were used to compare the performance of these markers with diploid and tetraploid F_2 populations. Two to four alleles were detected in the tetraploid population for each SSR and were inherited tetrasomically. Mapping with the single-dose alleles (SDA), linkage groups were comparable between tetraploid and diploid populations. An AFLP linkage map was constructed for autotetraploid potato with two segregating populations (Bradshaw et al. 1998). The map from population 12701ab1 contained 17 groups of 4 or more markers and 13 minor groups of 2 or 3 markers. The total length was 990.9 cM with the longest linkage group of 96.6 cM. With the mapping population of Stirling, 9 groups were made up of 4 or more markers and 17 minor groups comprised 2 or 3 markers. The total map length was 484.6 cM.

A linkage map was constructed with 306 AFLP markers for triploid diplosporous *Erigeron annuus* (Noyes and Rieseberg 2000). Among 47 linkage groups, two linkage groups, LG-P and LG-D were associated with the two components of diplosporous apomixis, parthenogenesis and diplospory. Four markers were significantly associated with parthenogenesis and 11 were completely associated with diplospory.

2.5.2.2 Fingerprinting

Markers have been widely used to determine the genetic diversity or relationships among different varieties and species for germplasm evaluation or phylogenetic studies (Kumar 1999). Abdalla (2001) studied the genetic diversity and relationships of diploid and tetraploid cottons using AFLP. Variation among apple cultivars could be revealed with both RAPD and AFLP markers (Goulao and Oliveira 2001). A high degree of genetic diversity within each of the cultivated populations in tetraploid alfalfa could be detected

with RAPD and SSR molecular markers. They were believed to be a powerful tool for approaching genetic variation and genetic relationships (Mengoni et al. 2000). Dendrograms were constructed with SSRs to study the phylogenetic relationships among *Avena* species (Li et al. 2000a). The dendrograms based on SSR data alone or the combination of SSR and RAPD data matched very well with the pedigree information for soybean (Doldi et al. 1997).

AFLP markers were employed to assess the genetic diversity among 21 established natural and nine synthetic varietes and lines of *Brassica juncea* from different origins (Srivastava et al. 2001). Genetic diversity within and among landraces of pearl millet (*Pennisetum glaucum*) under farmers' management in West Africa was studied for the influence of farmer management with 163 AFLP loci (Busso et al. 2000). The isolation of ideotypes (preferred genotype) with unique genetic backgrounds occurred due to different farmers' preferences, and therefore the genetic diversity due to human's practice should be considered for collection strategies for genebanks and regional genetic evaluations.

2.5.2.3 Marker Assisted Selection

Mapping is not just for the sake of making a map for each plant. The ultimate goal is to apply it to guide effective exploration of the genetic resources. Map-based cloning has been proved to be one means to isolate genes (Tanksley et al. 1995; Kumar 1999). Marker-assisted selection often refers to the application of molecular markers in the selection of traits which are difficult to access directly by phenotype screening. Accumulating genes through conventional breeding was difficult, especially for minor modifier genes, but it becomes practical with the assistance of molecular markers (Chen

et al. 2001a). The characteristics of food chemistry have primarily been selected though complicated chemical analysis with a large amount of material and could only be carried out at late stages of plant development (Fennema 1996). With molecular marker assistance, such traits can be more easily selected on a much larger population. Two SSRs were identified to tag the waxy gene in *Oryza sativa* L. ssp. indica, japonica and wild rice (*O. rufipogon*) (Tan and Zhang 2001). The high correlation between repeat number at each locus and amylose content level could be used for quality improvement in rice breeding programs. The selection could even start at any stage of development and could proceed without any concern for environmental influence on amylose content.

A set of molecular markers for glutenin (Glu-A1 and Glu-D1) genes was applied to assist the selection in backcross progenies to improve the glutenin quality in Spanish wheat (de Bustos et al. 2001). The selection was also assisted by using allele-specific PCR (AS-PCR) markers to recover the genetic background of the recurrent parent. In maize, SSRs could be used to identify progeny with the genetic background of specific parental lines (Smith and Senior 2000). AFLP markers were used to map and characterize quantitative trait loci (QTLs) for grain yield and two grain-related traits in maize (Marsan et al. 2001). The increase of the genome coverage by saturation of the existing genetic map with more molecular markers revealed new and closer QTLs and resulted in selection with higher precision.

2.5.3 Molecular Markers and Apomixis

The ability to interchange at will obligate asexual and sexual reproduction in crop breeding material would allow plant breeders to quickly develop superior varieties and to fix heterosis in a single parent (Hanna and Bashaw 1987; Jefferson 1993; Van Dijk and

Van Damme 2000). Transfer of apomixis through conventional methods from wild relatives to cultivated crops has been very complicated and time consuming because of wide-hybridization and the expected simultaneous introduction of other undesirable characteristics. High sterility is one very serious and common consequence of chromosome transfer in maize-*Tripsacum* crosses and millet-*Pennisetum* crosses (Leblanc et al.1996; Hanna et al. 1993). Gene cloning and genetic transformation could introduce only the gene(s) of apomixis, and meanwhile avoid other drawbacks of conventional hybridization.

Since the expression product of an apomixis gene(s) has not yet been identified, a crucial step in cloning the gene(s) is to locate the position on the corresponding chromosome(s). Map-based cloning has successfully been adopted in rice (Spiegelman et al. 2000), and tomato (Ronen et al. 2000) as well as other plant species. The difficulty of accessibility to apomixis hindered the mapping for this trait, besides the polyploid nature of apomictic species; however, substantial efforts toward mapping of apomixis have been made in different species (Leblanc et al. 1995; Gustine et al. 1997; Barcaccia et al. 1998; Pessino et al. 1997, 1998, 1999; Grimanelli et al. 1998b; Ozias-Akins et al. 1998; Noyes and Rieseberg 2000; Pupilli et al. 2001). Markers identified for different species are summarized in Table 2.3. Over 50 markers, including RAPDs, RFLPs and AFLPs, have been identified as linked with apomixis.

To locate apomixis gene(s), genetic mapping has been conducted with different approaches. RFLP and RAPD markers have been the most common type used for genetic analysis of apomixis (Ozias-Akins et al. 1993, 1998; Pessino et al. 1998). AFLP markers have more recently been adopted in apomixis studies (Noyes and Rieseberg

2000). The comparative maps revealed that the region responsible for aposporous apomixis in *Brachiaria* was syntenic with the short arm of chromosome 5 in maize (Pessino et al. 1997) and rice chromosome 2 (Pessino et al. 1998). In *Paspalum simplex*, the chromosome region conditioning apomixis is syntenic with the telomeric region of the long arm of rice chromosome 12 (Pupilli et al. 2001). The apospory-specific genomic region was conserved between *Pennisetum squamulatum* and *C. ciliaris* (Roche et al. 1999) or even among different apomictic species in the genus *Pennisetum* (Lubbers et al. 1994). For diplosporous apomixis in *Tripsacum*, the markers associated with diplosorous embryo sac development showed synteny with the long arm of chromosome 6 in maize. In other work on *Tripsacum*, a region on the short arm of maize chromosome 5 was proposed to be associated with apomixis, and this region contains genes related the initiation of meiosis (Blakey et al. 2001). One of the components for diplosporous apomixis in *Erigeron annuus*, parthenogenesis, was reported to be inherited independently from diplospory (Noyes and Rieseberg 2000). In several mapping studies, markers associated with apomictic embryo sac development have been clustered on the genetic map (Grimanelli et al. 1998b; Ozias-Akins et al. 1998; Noyes and Rieseberg 2000; Pupilli et al. 2001) and inherited as a single genetic unit to regulate the development of apomixis (Ozias-Akins et al. 1998; Pupilli et al. 2001).

2.5.4 Recombination suppression

Genetic mapping studies of apomixis in both monocots and dicots have been hindered by low genetic recombination associated with the locus for apomictic embryo sac formation (Ozias-Akins et al. 1998; Noyes and Rieseberg 2000). There are several ways in which recombination suppression can occur. The chromosome region near a centromere usually

shows very low recombination. High-density mapping of tomato and potato demonstrated phenomenal recombination suppression in the regions corresponding to centromeres, centromeric heterochromatin and some telomeres while random distribution of molecular markers was observed over all the other regions of the genome in both species (Tanksley et al. 1992). The suppression in those regions caused more than 10-times less recombination frequencies than other regions of the chromosomes. Similar reduction of recombination frequency in tomato centromeric regions was observed in a separate study (Frary et al. 1996). In this case, 8 and 10 molecular markers were clustered in the putative centromeric region of chromosomes 7 and 9, respectively. No recombination could be detected with a F_2 population of 1620 plants. Genetic lethality also can result in apparent recombination suppression. If any recombination near the apomixis locus produces inviable microspores, the mapping result will show suppression of recombination. Heterozygous inversions and deletions can also limit recombination inside and around the relative regions by causing regional asynapsis because the homologous chromosomes could not pair in the inverted or deleted regions (Singh and Singh 1989). However, pairing between homologous segments in the inverted regions can be accomplished by forming an inversion loop if the inversion region is large enough (Schulz-Schaefer 1980). Crossovers inside the loop often cause deletions and result in inviable gametes. Only the gametes without recombination in this region would produce the offspring, thus recombination would remain undetected.

2.6 *In situ* hybridization

In situ hybridization (ISH) was first developed by two different groups simultaneously for detecting the location of RNA on a cytological preparation slide (Gall and Pardue

1969; John et al. 1969). This method subsequently was adopted mainly for detecting the location of DNA sequences or genes on chromosome preparations (reviewed by Jiang and Gill 1994b).

2.6.1 Basic technique of ISH

The principal for ISH is the pairing of nucleotides through hydrogen-bonds. As we all know that DNA is a molecule made of 4 different nucleotides A, G, C, and T; A pairs with T through two H-bonds and G with C through three H-bonds. Two complementary single-stranded DNA sequences pair with each other, through a process called annealing, to form double-stranded DNA which is a more stable form with lower energy. When double-stranded DNA absorbs heat or other forms of energy, the H-bonds are broken and double-stranded DNA separates to form single-stranded DNA, a process called denaturation. More stable annealing occurs among longer DNA sequences with higher sequence identity thus more energy would be needed to separate or denature the DNA sequence (Fig. 2.2A). From this basic property of DNA, many methods of manipulating DNA molecules have been developed, such as sequencing, RFLP, RAPD, PCR and ISH.

ISH enables us to establish the location of a DNA fragment or gene (probe) with its physical linkage on chromosomes, RNA expression pattern in cells or tissues during development, or dispersion in different genomes (Schwarzacher and Heslop-Harrison 2000). By hybridization of labeled DNA or RNA fragments, one can detect the location of genes on chromosomes or RNA expression in cells or tissues on cytological preparations. *In situ* hybridization thus connected cytogenetics with molecular genetics.

The original ISH technique used radioisotopes to label probes (Gall and Pardue 1969; John et al. 1969). The isotopic probe gives a very strong signal and is very

sensitive. Hybridization signal must be detected by photographic emulsion over the surface of the preparation. This technique has been used for detecting single copy DNA sequences and for physical mapping of genes (Ferguson-Smith 1991). The disadvantages of isotopic label were low resolution, safety issues, and lengthy exposure times (weeks or months). More immediate results could be obtained with colorimetric methods when the signal was detected by colored precipitates formed from enzyme substrates. This technique avoided the safety issues and long exposure time encountered with isotopic ISH, but the resolution was low (Schwarzacher and Heslop-Harrison 2000). With improvement of resolution and the advantages of high contrast, speed, and safety as well as the detection of multiple-targets simultaneously, nonisotopic ISH has become a widely used method. Since nonisotopic ISH was first developed in 1982 (Langer-Safer et al. 1982), it has been greatly improved for higher resolution and efficiency (Pinkel et al. 1986; Viegas-Péquignot et al. 1991).

Probes can be labeled with biotin-16-dUTP or digoxigenin-11-dUTP. Hybridization signals then are detected by enzymatic reporter molecules, commonly horseradish peroxidase or alkaline phosphatase conjugated avidin/streptavidin (Langer-Safer et al. 1982), or by streptavidin or antibody conjugated fluorochromes (Pinkel et al. 1986). Probes also can be directly labeled with fluorophores, a range of which emit most colors under appropriate excitation wavelengths and no additional detection steps are needed after hybridization. After the probes are labeled, they can be applied on the chromosome preparations for hybridization. In the diagram, the nucleotide U is labeled with a hapten (shown as red triangle) (Fig. 2.2B). The signal is commonly low with directly labeled probes. For low-copy DNA sequences, the signal must be amplified or

enhanced with anti-fluorophore antibodies. Because the probe can be labeled with different haptens (mostly biotin or digoxigenin), nonisotopic ISH can be used for detecting multiple-targets simultaneously in order to find the physical order of different genes on chromosomes (Leitch et al. 1991; Mukai et al. 1993; Jiang and Gill 1994b; Cheng et al. 2001). Based on the type of probes used, ISH can be classified as GISH (Dornam et al. 1985; Pinkel et al. 1986), using total genomic DNA as a probe or FISH, using selected DNA fragments as a probe. Recently, DNA strands or fibers fixed on slides were used to improve the resolution and determine the physical distances between two different DNA fragments (Heng et al. 1992; Wiegant et al. 1991; Parra and Windle 1993; Jackson et al. 1998; Cheng 2001). Fiber FISH can also be used to detect overlapping clones, chromosomal rearrangements or the size of DNA loci (Jackson et al. 1998).

The process of ISH must be gauged to maintain the integrity of both labeled probe and chromosomes. Both must be denatured to start the reaction (Fig. 2.2C). Denaturation usually is carried out at high temperature (80^{o}C), but the denature time cannot be very long or the chromosome preparation will be damaged, especially when the chromosome preparation is reused for several ISH experiments.

Hybridization typically is carried out at 37-42^{o}C for 8- 24 hours. The reaction must be kept in a moist container to avoid drying. Since hybridization is also an annealing process, it is very important to set the proper temperature. Higher stringency (higher temperature) will reduce mismatches but also will reduce hybridization, while lower stringency will increase mismatches and hence the background (Fig. 2.2D). Because the reaction is dynamic, a trial and error process may be required to identify the

optimum hybridization conditions. Altering stringency during washing also can assist with the removal of the background and mismatched probe. Detection of fluorochromes (shown as green stars in the diagram) requires the use of a fluorescence microscope and a recording mechanism such as sensitive high speed film or a cooled CCD camera (Fig. 2.2E).

ISH has been integrated with other cytogenetic techniques, particularly chromosome banding which enables the identification of targeted fragments on special chromosomes (rye, Hutchinson and Seal 1983; human, Lawrece et al. 1990; wheat, Jiang and Gill 1993). A modified N-banding-ISH/GISH sequential procedure gave best results. Similarly, a modified C-banding - ISH/GISH procedure also gave satisfactory results (Jiang and Gill 1993).

2.6.2 Application of ISH in plants

2.6.2.1 Location of high-copy and repetitive DNA sequences

High-copy number DNA such as dispersed- or tandem-distributed repetitive DNA sequences are abundant over the genome and often easy to detect on chromosomes with ISH. Many types of repeat DNA fragments from rye and wheat have been analyzed with ISH (Bedbrook et al. 1980; Appels et al. 1981; Jiang and Gill 1993). A repetitive DNA sequence was located on a specific rye chromosome by isotopic ISH incorporated with a C-banding technique (Hutchinson and Seal 1983). Fluorescence ISH was introduced for plant species to detect the location of a repeat DNA sequence on wheat chromosomes (Rayburn and Gill 1985). From distribution patterns of the repetitive DNA sequences on chromosomes, almost all chromosomes from wheat could be identified. All chromosomes from the B-genome, 3 from D-genome, and 1 from A-genome could be

identified by their hybridization patterns using a 120-bp repeat sequence as probe. With multicolor ISH, Jiang and Gill (1994a) identified the location of a new rRNA gene on a specific wheat chromosome.

Two kinds of high-copy-number repeat rDNAs (45S and 5S rDNA) have been frequently used as probes for FISH to provide physical markers for specific chromosomes (Mukai et al. 1993; Fukui et al. 1998; Tagashira and Kondo 2001). Referring to the hybridization pattern of those physical markers, chromosomes, which were difficult to identify morphologically, could be distinguished from each other. Genome analysis with rDNAs has been done for wheat (Ma et al. 1994), *Nicotiana kawakamii* Y. Ohashi (Nakamura et al. 2001), *Brassica* (Fukui et al. 1998), *Amaranthus caudatus* L. (Kolano et al. 2001), and maize (Sadder and Weber 2001).

2.6.2.2 Location of low-copy DNA sequences

It is far more difficult to detect low-copy DNA sequences than high-copy or repeated DNA sequences. The hybridization signal of low-copy DNA on chromosomes is usually very weak and needs a highly sensitive detection system. However, larger fragments of low-copy DNA have been successfully localized on chromosomes. A 17-kb T-DNA sequence was located on a chromosome of *Crepis capillaris* (Ambros et al. 1986) and a 13.5-kb legumin gene on a chromosome of pea (*Pisum sativum*) (Simpson et al. 1988). FISH was used to characterize the chromosomal location of the transgene insertion in rice plants (Dong et al. 2001). A low-copy 0.7 kb DNA sequence in rye (*Secale cereale* L.) was located on a rye chromosome (Gustafson et al. 1990). But for small (<10kb) DNA probes, the detection frequency of hybridization signals was very low even after the signal enhancement (Jiang and Gill 1994b). With a cooled charged

coupled device (CCD) camera integrated with the computer, the sensitivity of fluorescence signal detection increased 30 times (Wiegant et al. 1991). Koch et al. (1989) tried to increase the intensity of signals by PCR on chromosome preparations. This method has worked well for repetitive DNA (Kubalakova et al. 1997). Tyramide-FISH (Tyr-FISH) was developed to amplify the hybridization signals by the deposition of fluorochrome-labeled tyramide by an enzymatic reaction (Raap et al. 1995). It was introduced into the cytogenetic study of plants by Khrustaleva and Kik (2001) for FISH signal-amplification. With Tyr-FISH it was possible to detect target T-DNA sequences as small as 710 bp on plant metaphase chromosomes.

2.6.2.3 Genomic *in situ* Hybridization

Genomic *in situ* hybridization (GISH) has been shown to be an effective method for differentiating closely related genomes (reviewed by Jiang and Gill 1994b; Bisht and Mukai 2001; Chen et al. 2001b). With GISH, high stringency hybridization conditions and a high ratio of block- to probe-DNA should be used to reduce the false positive signals (Ran et al. 2001). Cytogenetic investigation with GISH on the allotetraploid *Eleusine coracana* genome using genomic DNA from various diploid species of the genus as probes revealed that *E. indica* and *E. floccifolia* were the most probable genome donors (Bisht and Mukai 2001). Two putative parental genomes of *Poa jemtlandica*, from *P. alpina* and *P. flexuosa*, were clearly distinguished with GISH (Brysting et al. 2000). With GISH, pairing between homeologous chromosomes from different genomes of *Triticum aestivum-Thinopyrum intermedium* hybrids could be identified and hence the genetic relationship between different genomes was established (Chen et al. 2001b). In the hexaploid hybrid ($ABDJJ_{(s)}S$) of wheat and *Th. intermedium*, chromosomes from the

same species tended to pair with each other. The frequency of pairing among *Th. intermedium* chromosomes was reported to be higher than that among wheat chromosomes, which was described as a closer relationship among the three genomes, J, $J_{(s)}$, and S, of *Th. intermedium* than among chromosomes from A-, B- and D-genomes, of *T. aestivum*. Furthermore, a high frequency of pairing of S-genome chromosomes with J- and J(s)-genome chromosomes was observed during meiosis, indicating that chromosomes from the S-genome were more closely related to chromosomes from J- and J(s)-genomes than were the J and J(s) chromosomes to each other. The chromosome preparations from polyploid species *Elytrigia pycnantha* ($2n = 6x = 42$) and *Thinopyrum junceiforme* ($2n = 4x = 28$) and their hybrid population ($2n = 5x = 35$) were probed with genomic DNA from *Thinopyrum elongatum* (Host) D.R. Dewey (E genome, $2n = 14$), *Th. bessarabicum* (Savul. & Rayss) A. Love (J genome, $2n = 14$), *Pseudoroegneria stipifolia* (Czern. ex Nevski) Love (S genome, $2n = 14$), and *Agropyron cristatum* (L.) Gaertner (P genome, $2n = 14$) to study the genome origin of these polyploids (Refoufiet al. 2001). According the hybridization pattern, a genome constitution (SSPPEES)-P-S-E-S-E-S was proposed for *E. pycnantha*, where –P-S referred to the hybridization of S-genome DNA to the centromere of one chromosome from the P-genome and E-S-E-S referred to the hybridization of S-genome DNA to centromeres of two chromosomes from the E-genome. Based on these results, a genome formula of EEEE for *Th. junceiforme* and (SPEEE)-E-S-E-S for the hybrid were proposed.

GISH has proved to be a most effective and accurate method for monitoring the introgression of alien chromatin into a recipient species (reviewed by Jiang et al. 1994c). Integration of alien chromatin into the wheat genome was investigated with GISH. An

average frequency of 1.9% chromosomal translocations between wheat and *Dasypyrum. villosum* was detected with GISH in callus cells (Li et al. 2000b). GISH was used to study the genome constitution of haploid durum wheat (Dogramac-Altuntepe and Jauhar 2001). Seven A-genome chromosomes and six B-genome chromosomes could be identified unambiguously in a 5D(5B) substitution line using *Triticum urartu* genomic DNA for the A-genome probe and *Aegilops speltoides* DNA for the B-genome probe. A translocation between 4A and 7B was also identified with 24% of the long arm of the translocation chromosome coming from 7B.

Seven *Triticum aestivum* (2*n* = 6*x* = 42, AABBDD) /*Thinopyrum bessarabicum* (2*n* = 2*x* = 14, (EEb)-E-b) disomic addition lines (2*n* = 44 = 21 II ABD + 1 II E-b) were investigated using GISH with *Th. bessarabicum* genomic DNA as probe (Zhang et al. 2002). The experiment concluded that six out of the seven addition lines were introduced with one pair of *Th. bessarabicum* chromosomes each while the other additional line actually was a translocation line between the two parents. The translocation chromosome was made up with four-fifths of a *Th. bessarabicum* chromosome and one-fifth of a wheat chromosome segment.

2.6.2.4 Pachytene chromosome ISH

Pachytene chromosomes usually are 10-20 times longer than metaphase chromosomes (Zhong et al. 1996; Cheng et al. 2001). The increased length gives higher resolution between different probes with fluorescence *in situ* hybridization. The resolution between FISH signals ranges from 1-3 Mb with human mitotic metaphase chromosomes and it can be improved with pachytene chromosomes since the pachytene chromosomes are less condensed and the distance between a given pair of sites is larger than that with mitotic

chromosomes (Zhong et al. 1996). Pachytene chromosomes often showed a well differentiated pattern of brightly fluorescing heterochromatin segments when stained with DAPI. *Medicago truncatula* ($2n = 2x = 16$) was karyotyped with pachytene chromosomes using information on the positions of three repetitive sequences (5S rDNA, 45S rDNA and the MtR1 tandem repeat) (Kulikova et al. 2001) as determined by FISH. Using FISH of bacterial artificial chromosome (BAC) clones (with two to five BACs per linkage group), the genetic linkage groups have been assigned to their corresponding chromosomes. The resolution in euchromatic parts of *M. truncatula* pachytene chromosome 5 was 60 kb, which was obtained by comparing the cytological distances between FISH signals of clones of a BAC contig with their corresponding physical distance. Rice pachytene chromosome FISH was carried out with rice BAC clones (Cheng et al. 2001). A total of 17 rice BAC clones was localized on rice chromosome 10. These BAC clones were selected based on the positions of RFLP markers on the existing genetic linkage map, and each BAC clone was tagged by its corresponding RFLP marker. The physical map of pachytene chromosome 10 could thus be integrated with the genetic map of rice chromosome 10.

2.6.2.5 Fiber-FISH

Stretched DNA fibers are more accessible to probes and detection reagents than metaphase or pachytene chromosomes, which usually are highly condensed and packed with proteins. Hybridization efficiency is thus improved with DNA fibers (Weier 2001). With extended DNA fibers prepared from interphase nuclei, the resolution of a physical distance of 0.7 kb was reached with two DNA probes from the 45S ribosomal gene on extended fibers of tomato (Fransz et al.1996). The resolution of fiber-FISH ranges from

2.78 to 3.3 kb/μm in both mammalian and plant species (Fransz et al. 1996; Jackson et al. 1998) because the stretching of DNA fibers can vary between centromeric regions and other chromosome regions. With two-color labeled probes, three closely clustered chemokine genes were located within a length of 32 kb on mouse and 29 kb on human DNA fibers (Erdel et al. 2001).

Fiber FISH has proved to be effective for revealing gene organization and rearrangements (Suto et al. 2000). A human blood group locus (RND), which had been considered either absent or grossly deleted in RND negative phenotypes, was shown to be the result of complex rearrangements, including partial deletion, duplication, and recombination, in this locus.

Fiber-FISH can provide detailed data about overlaps or gaps between clones and precisely locate the positions of sequence tagged sites or expressed sequences (Weier 2001). Physical gaps of 31 kb and 500 kb were detected on *A. thaliana* chromosome II by hybridizing the flanking BAC clones onto *A. thaliana* DNA fibers (Jackson et al. 1998). *A. thaliana* DNA fibers of up to 1.71 Mb in length could be tracked when hybridized with BAC clones. This can be very important to investigate the gaps in physical mapping by contig assembly because any gap larger than this distance would be difficult to connect.

Fiber-FISH also has been useful for detecting the integration pattern of plasmids in transgenic lines (Jackson et al. 2001). Three basic integration patterns were observed from the fiber-FISH experiments: Type I, large randomly repeated integration; Type II, large tandem integrations interspersed with unknown DNA; and Type III, small insertions, possibly interspersed with unknown DNA.

2.7 Molecular and cytogenetic characterization of aposporous apomixis will provide critical information for further study on apomixis

Apomixis has been a topic of research for more than a century. The trait exists mainly in uncultivated species and is genetically controlled by one or at most a few major genes. Transfer of apomixis from wild relatives into economically important crops would revolutionize crop improvement and hold tremendous potential for agriculture. Limited progress has been made in transferring apomixis through conventional methods for the last three decades while molecular biology and improvement through biotechnology promise a brighter future in this area.

Molecular genetic studies on apomixis support that aposporous embryo sac formation is regulated by one super genetic unit, even though the result might be inconclusive because of the potential selection of gametes in apomictic plants. The clustering of many molecular markers and the phenomenon of hemizygosity in this region imply that structural aberrations on a chromosomal level might be related to apomixis, or at least the investigation on a chromosomal level is needed for further understanding of the mechanism underlying apomixis.

References:

Abdalla AM, Reddy OUK, El-Zik KM, Pepper AE (2001) Genetic diversity and relationships of diploid and tetraploid cottons revealed using AFLP. Theor Appl Genet 102:222-229

Akerberg E, Bingefors S (1953) Progeny studies in the hybrid *Poa pratensis* X *Poa alpina.* Hereditas 39:125-136

Almgard E. (1966) Experiments with *Poa* III. Ann Agric Coll Sweden 32:3-64

Ambros PE, Matzke MA, Matzke AJM (1986) Detection of a 17 kb unique sequence (T-DNA) in plant chromosomes by *in situ* hybridization. Chromosoma 94:11-18

Appels R, Dennis ES, Smyth DR, Peacock WJ (1981) Two repeated DNA sequences from the heterochromotic region of rye (*Secale cereale*) chromosomes. Chromosoma 84:265-277

Asker SE, Jerling L (1992) Apomixis in plants. (Boca Raton: CRC Press)

Bai G, Tefera H, Ayele M, Nguyen HT (1999) A genetic linkage map of tef [*Eragrostis tef* (Zucc.) Trotter] based on amplified fragment length. Theor Appl Genet 99:599-604

Barcaccia G, Mazzucato A, Albertini E, Zethof J, Gerats A, Pezzotti M, Falcinelli M (1998) Inheritance of parthenogenesis in *Poa pratensis* L.: auxin test and AFLP linkage analyses support monogenic control. Theor Appl Genet 97:74-82

Bashaw EC (1962) Effects of irradiation on apomictic common dallisgrass. Crop Sci 2:501

Bashaw EC, Hanna WW (1990) Apomictic reproduction. In reproductive versatility in the grasses. G.P. Chapman, ed (Cambridge University Press). pp. 100-130

Bedbrook JR, Johns J, O'Dell M, Thompson RD, Flavell RB (1980) A molecular description of telomeric heterochromotin in *Secale* species. Cell 19:545-560

Bert PF, Charmet G, Sourdille P, Hayward MD, Balfourier F (1999) A high-density molecular map for ryegrass (*Lolium perenne*) using AFLP markers. Theor Appl Genet 99:445-452

Bicknell RA (1997) Isolation of a diploid, apomictic plant of *Hieracium aurantiacum*. Sexual Plant Reprod 10:168-172

Bicknell RA, Borst NK, Koltunow AM (2000) Monogenic inheritance of apomixis in two *Hoeracium* species with distinct development mechanisms. Heredity 84:228-237

Bisht MS, Mukai Y (2001) Genomic *in situ* hybridization identifies genome donor of finger millet (*Eleusine coracana*). Theor Appl Genet 102:825-832

Blakey CA, Goldman SL, Dewald CL (2001) Apomixis in *Tripsacum*: Comparative mapping of a multigene phenomenon. Genome 44:222-230

Boivin K, Deu M, Rami JF, Trouche G, Hamon P (1999) Towards a saturated sorghum map using RFLP and AFLP markers. Theor Appl Genet 98:320-328

Botstein D, White RL, Skolnick M, Davis RW (1980) Construction of a genetic linkage map in man using restriction fragment length polymorphisms. Amer J Hum Genet 32:314-331

Bradshaw JE, Meyer RC, Milbourne D, McNicol JW, Phillips MS, Waugh R (1998) Identification of AFLP and SSR markers associated with quantitative resistance to Globodera pallida (Stone) in tetraploid potato (*Solanum tuberosum* subsp. tuberosum) with a view to marker-assisted selection. Theor Appl Genet 97:202-210

Brouwer DJ, Osborn TC (1999) A molecular marker linkage map of tetraploid alfalfa (*Medicago sativa* L.). Theor Appl Genet 99:1194-1200

Brown SM, Hopkins MS, Mitchell SE, Senior ML, Wang TY, Duncan RR, Gonzalezcandelas F, Kresovich S (1996) Multiple methods for the identification of polymorphic simple sequence repeats (SSRs) in Sorghum [*Sorghum-bicolor* (L) moench]. Theor Appl Genet 93:190-198

Brown WV, Emery WHP (1958) Apomixis in Gramineae: *Panicoideae*. Amer J Bot 45:253-263

Brysting AK, HolstJensen A, Leitch I (2000) Genomic origin and organization of the hybrid *Poa jemtlandica* (Poaceae) verified by genomic *in situ* hybridization and chloroplast DNA sequences Annals Bot. 85:439-445

Busso CS, Devos KM, Ross G, Mortimore M, Adams WM, Ambrose MJ, Alldrick S, Gale MD (2000) Genetic diversity within and among landraces of pearl millet (*Pennisetum glaucum*) under farmer management in West Africa. Genetic Resources And Crop Evolution 47:561-568

Caceres ME, Matzk F, Busti A, Pupilli F, Arcioni S (2001) Apomixis and sexuality in *Paspalum simplex*: characterization of the mode of reproduction in segregating progenies by different methods. Sexual Plant Reprod 14:201-206

Chen DH, Nelson RJ, Wang GL, Mackill DJ, Ronald PC (2001a) Use of DNA markers in introgression and isolation of genes associated with durable resistance to rice blast. In Phillips RL, Vasil IK (ed) DNA-Baseed Markers in Plants (2nd ed.). Kluwer Academic Publishers, Dordrecht, pp 49-57

Chen Q, Conner RL, Laroche A, Ahmad F (2001b) Molecular cytogenetic evidence for a high level of chromosome pairing among different genomes in *Triticum aestivum - Thinopyrum intermedium* hybrids. Theor Appl Genet 102:847-852

Cheng Z, Presting GG, Buell CR, Wing RA, Jiang J (2001) High-resolution pechytene chromosome mapping of bacterial artificial chromosomes anchored by genetic markers reveals the centromere location and the distribution of genetic recombination among chromosome 10 of rice. Genetics 157:1749-1757

Cho YG, Ishii T, Temnykh S, Chen X, Lipovich L, McCouch SR, Park WD, Ayres N, Cartinhour S (2000) Diversity of microsatellites derived from genomic libraries and GenBank sequences in rice (*Oryza sativa* L.). Theor Appl Genet 100:713-722

Chunwongse J, Doganlar S, Crossman C, Jiang J, Tanksley SD (1997) High-resolution genetic map of the Lv resistance locus in tomato. Theor Appl Genet 95:220-223

Cleiji G, Bock TS, M de. Lekker B. (1976) Crosses between *Beta vulgaris L.* and *Beta lomaogona* F. At M. Euphytica 25:539-547

Coe E H, Polacco ML, David G, McMullen MD (2001) Maize molecular maps: Markers, bins, and database. In Phillips RL, Vasil IK (ed) DNA-Based Markers in Plants (2^{nd} ed.). Kluwer Academic Publishers, Dordrecht, pp 255-284

Cordeiro GM, Taylor GO, Henry, RJ (2000) Characterisation of microsatellite markers from sugarcane (Saccharum sp.), a highly polyploid species. Plant Sci 155:161-168

d'Cruz R, Reddy PS (1971) Inheritance of apomixis in *Dichanthium*. Indian J Genet Plant Breed 31:451-460

D'hont A, Lu YH, De Leon DG, Grivet L, Feldmann P, Lanaud C, Glaszmann JC (1994) A molecular approach to unraveling the genetics of sugarcane, a complex polyploid of the Andropogoneae tribe. Genome 37:222-230

Da Silva JAG., Sorrells ME, Birnquist WL, Tanksley SD (1993) RFLP linkage map and analysis of *Saccharum spontaneum.* Genome 36:782-791

de Bustos A, Rubio P, Soler C, Garcia P, Jouve N (2001) Marker assisted selection to improve HMW-glutenins in wheat. Euphytica 119:69-73

Diwan N, Bouton JH, Kochert G, Cregan PB (2000) Mapping of simple sequence repeat (SSR) DNA markers in diploid and tetraploid alfalfa. Theor Appl Genet 101:165-172

Dogramac-Altuntepe M, Jauhar PP (2001) Production of durum wheat substitution haploids from durum x maize crosses and their cytological characterization. Genome 44:137-142

Doldi M L,Vollmann J, Lelley T (1997) Genetic diversity in soybean as detected by RAPD and microsatellite analysis. Plant Breed 116:331-335

Dong JJ, Kharb P, Teng WM, Hall TC (2001) Characterization of rice transformed via an *Agrobacterium*-mediated inflorescence approach. Mol breed 7:187-194

Dornam DM, GelinasR, Myerson D (1985) Detection of specific chromosomes in somatic cell hybrid. Somatic Cell Mol Genet 11:571-577

Dujardin M, Hanna WW (1983a) Apomictic and sexual pearl millet x *Pennisetum squamulatum* hybrids. The Journal of Heredity 74:277-279

Dujardin M, Hanna WW (1983b) Meiotic and reproductive behavior of facultative apomictic BC1 offspring derived from *Pennisetum americanum-P. orientale* interspecific hybrids. Crop Sci 23:156-160

Dujardin M, Hanna WW (1984a) Microsporogenesis , reproductive behavior, and fertility in five *Pennisetum* species. Theor Appl Genet 67:197-201

Dujardin M, Hanna WW (1984b) Pseudogamous parthenogenesis and fertility of a pearl millet X *Pennisetum orientale* apomictic derivative. J Heredity 75:503-504

Dujardin M, Hanna WW (1985) Cytology and reproduction of reciprocal backcrosses between pearl millet and sexual and apomictic hybrids Crop Sci 25:59-62

Dujardin M, Hanna WW (1986) An apomictic polyhaploid obtained from a pearl millet X *Pennisetum squamulatum* apomictic interspecific hybrid. Theor Appl Genet 72:33-36

Dujardin M, Hanna WW (1988) Cytology and breeding behavior of a partially fertile triploid pearl millet. J of Heredity 79:216-218

Dujardin M, Hanna WW (1989) Developing apomictic pearl millet-characterization of BC_3 plant. J Genet Breed 43:145-151

Erdel M, Theurl M, Meyer M, Duba HC, Utermann G, Werner-Felmayer G (2001) High-resolution mapping of the human 4q21 and the mouse 5E3 SCYB chemokine cluster by fiber-fluorescence *in situ* hybridization. Immunogenetics 53:611-615

Fennema OR, (1996) Food chemistry. 3rd ed. New York: M. Dekker

Ferguson-Smith MA (1991) Invited edition: Putting the genes back into cytogenetics. Amer J Hum Genet 48:179-182

Fossati LM, Castiglione SFO, Scienza A, Sala FT (2001) The use of AFLP and SSR molecular markers to decipher homonyms and synonyms in grapevine cultivars: the case of the varietal group known as "Schiave". Theor Appl Genet 102:200-205

Fransz PF, Alonso-Blanco C, Liharska TB, Peeters AJM, Zabel P, de Jong JH (1996) High-resolution physical mapping in *Arabidopsis thaliana* and tomato by fluorescence *in situ* hybridization to extended DNA fibers. Plant J 9:421-430

Frary A, Presting GG, Tanksley SD (1996) Molecular mapping of the centromeres of tomato chromosomes 7 and 9. Mol Gen Genet 250:295-304

Fukui K, Nakayama S, Ohmido N, Yoshiaki H, Yamabe M (1998) Quantitative karyotyping of three diploid Brassica species by imaging methods and localization of 45S rDNA loci on the identified chromosomes. Theor Appl Genet 96:325-330

Gall JG, Pardue ML (1969) Formation and detection of RNA-DNA hybrid molecules in cytological preparation. Proc Natl Acad Sci USA 63:378-383

Goulao L, Oliveira CM (2001) Molecular characterisation of cultivars of apple (Malus x domestica Borkh.) using microsatellite (SSR and ISSR) markers. Euphytica 122:81-89

Grimanelli D, Hernandez M, Perotti E, Savidan Y (1997) Dosage effects in the endosperm of diplosporous *Trisacum* (Poaceae). Sexual Plant Reprod 10:279-282

Grimanelli D, Leblanc O, Espinosa E, Perotti E, González de León D, Savidan Y (1998a) Non-Mendelian transmission of apomixis in maize-*Tripsacum* hybrids caused by a transmission ratio distortion. Heredity 80: 40-47

Grimanelli D, Leblanc O, Espinosa E, Perotti E, DeLeon DG, Savidan Y (1998b) Mapping diplosporous apomixis in tetraploid *Tripsacum*: one gene or several genes? Heredity 80:33-39

Grivet L, Dhont A, Roques D, Feldmann P, Lanaud C, Glaszmann JC (1996) Genetic map in sugarcane. Genetics 142:987-1000

Groh S, Zacharias A, Kianian SF, Penner GA, Chong J, Rines HW, Phillips RL (2001) Comparative AFLP mapping in two hexaploid oat populations. Theor Appl Genet 102:876-884

Grossniklaus U, Nogler GA, Van Dijk PJ (2001) How to Avoid Sex: The Genetic Control of Gametophytic Apomixis. Plant Cell 13:1491-1498

Gustafson JP, Butter E, McIntyre CL, (1990) Physical mapping of a low-copy DNA sequence in rye (Secale cereale L). Proc Natl Acad Sci USA 87:1899-1902

Gustafsson A (1946) Apomixis in higher plants. Lunds Univ Arsskr Avd

Gustine D L, Sherwood RT, Huff DR (1997) Apospory-linked molecular markers in buffelgrass. Crop Sci 37:947-951

Hanna WW, Powell JB, Millot JC, Burton GW (1973). Cytology of obligate sexual plants in *Panicum maximum* Jacq. and their use in controlled hybrids. Crop Sci 13:695

Hanna WW, Dujardin M (1982) Apomictic interspecific hybrids between pearl millet and *Pennisetum* orientale L. C. Rich. Crop Sci 22:857-859

Hanna WW, Dujardin M (1986) Cytogenetics of *Pennisetum schweinfurthii* Pilger and its hybrids with pearl millet. Crop Sci 26:449-453

Hanna WW, Bashaw EC (1987) Apomixis: its identification and use in plant breeding. Crop Sci 27:1136-1139

Hanna WW, Dujardin M, Ozias-Akins P, Lubbers E, Arthur L (1993) Reproduction, cytology, and fertility of pearl millet X *Pennisetum squamulatum* BC_4 . J Heredity 84:213-216

Harlan JR, Brooks MH,Borgaonkar DS, deWet JMJ (1964) Nature and inheritance of apomixis in *Bothriochloa* and *Dichanthium*. Bot Gaz 125:41-46

Heng HHQ, Aquire J, Tsui LC (1992) High-resolution mapping of mammalian genes by *in situ* hybridization to free chromatin. Proc Natl Acad Sci USA 89:9509-9513

Hoarau JY, Offmann B, D'Hont A, Risterucci AM, Roques D, Glaszmann JC, Grivet L (2001) Genetic dissection of a modern sugarcane cultivar (*Saccharum* spp.). I. Genome mapping with AFLP markers. Theor Appl Genet 103:84-97

Huang SW, Zhang BX, Milbourne D, Cardle L, Yang GM, Guo JZ (2001) Development of pepper SSR markers from sequence databases. Euphytica 117:163-167

Hussey MA, Bashaw EC, (1995) Breeding Apomictic Forage and Turf Grasses. In Harnessing Apomixis. An international conference to address a topic of far-reaching significance, A New Frontier in Plant Science. September 25-27. College Station, Taxas. pp18

Hutchinson J, Seal AG (1983) A sequential *in situ* hybridization and C-banding technique. Heredity 51:507-509

Jackson SAJ, Wang ML, Goodman HM, Jiang JM (1998) Application of fiber-FISH in physical mapping of Arabidopsis thaliana. Genome 41:566-572

Jackson SA, Zhang P, Chen WP, Phillips RL, Friebe B, Muthukrishnan S, Gill BS (2001) High-resolution structural analysis of biolistic transgene integration into the genome of wheat. Theor Appl Genet 103:56-62

Jauhra PP (1981) Cytogenetics and breeding of pearl millet and related species. Alan R. Liss Inc., New York

Jefferson RA (1993) Strategic development of apomixis as a general tool for agriculture, pp 207-217 in Wilson KJ (ed) Proc Intl Workshop on Apomixis in Rice. The Rockefeller Foundation. New York and China National Center for Biotechnology Development. Beijing

Jiang JM, Gill BS (1993) Sequential chromosome-banding and in-situ hybridization analysis. Genome 36:792-795

Jiang JM, Gill BS (1994a) New 18S·26S ribosomal RNA gene loci: chromosomal landmarks for the evolution of polyploid wheat. Chromosoma 103:179-185

Jiang JM, Gill BS (1994b) Nonisotopic *in situ* hybridization and plant genome mapping: the first 10 years. Genome 37:717-725

Jiang JM, Gill BS (1994c) Different species-specific chromosome translocations in *Triticum timopheevil* and *T. turgidum* supports diphyletic origin of polyploid wheat. Chromosome Res 2:59-64

John HA, Birnstiel ML, Jone KW (1969) RNA-DNA hybrids at the cytological level. Nature (London) 223:582-587

Jones CJ, Malcevschi A, Marmiroli N, Aert R, Volckaert G, Rueda J, Linacero R, Vazquez A, Karp A, Edwards KJ, Castaglione S, Winfield MO, Sala F, Vandewiel C, Bredemeijer G, Vosman B, Matthes M, Daly A, Brettschneider R, Bettini P, Buiatti M, Maestri E (1997a) Reproducibility testing of RAPD, AFLP and SSR markers in plants by a network of Euripean laboratories. Mol Breed 3:381-390

Jones N, Ougham H, Thomas H (1997b) Markers and mapping- we are all genetics now. New Phytologist 137:165-177

Kang BC, Nahm SH, Huh JH, Yoo HS, Yu JW, Lee MH, Kim BD (2001) An interspecific (*Capsicum annuum* x *C-chinese*) F-2 linkage map in pepper using RFLP and AFLP markers. Theor Appl Genet 102:531-539

Khrustaleva LI, Kik C (2001) Localization of single-copy T-DNA insertion in transgenic shallots (*Allium cepa*) by using ultra-sensitive FISH with tyramide signal amplification. Plant J 25:699-707

Kindiger B, Sokolov V (1997) Progress in the development of apomictic maize. Trends in Agronomy 1:75-94

Koch JE, Kolvraa S, Petyersen KB, Gregersen N, Bolund L (1989) Oligonucleotyde-priming methods for the chromosome-specific lebeling of alpha satellite DNA *in situ.* Chromosoma 98:259-265

Kolano B, Pando LG, Maluszynska J (2001) Molecular cytogenetic studies in *Chenopodium quinoa* and *Amaranthus caudatus*. Acta Societatis Botanicorum Poloniae 70:85-90

Kolliker R, Jones ES, Drayton MC, Dupal MP, Forster JW (2001) Development and characterisation of simple sequence repeat (SSR) markers for white clover (*Trifolium repens* L.). Theor Appl Genet 102:416-424

Koltunow AM (1993) Apomixis: Embryo sac and embryos formed without meiosis or fertilization in ovules. Plant Cell 5:1425-1437

Koltunow AM (2000) The genetic and molecular analysis of apomixis in the model plant *Hieracium*. Acta Biologica Cracoviensia Series Botanica 42:61-72

Kubalakova M, Macas J, Dolezel J (1997) Mapping of repeated DNA sequences in plant chromosomes by PRINS and C-PINTS. Theor Appl Genet 94:758-763

Kulikova O, Gualtieri G, Geurts R, Kim DJ, Cook D, Huguet T, de Jong JH, Fransz PF, Bisseling T (2001) Integration of the FISH pachytene and genetic maps of *Medicago truncatula*. Plant J 27:49-58

Kumar LS (1999) DNA markers in plant improvement: An overview. Biotechnology Advances 17:143-182

Langer-Safer PR, Levine M, Ward DC (1982) Immunological method for mapping genes on *Drosophila* polytene chromosomes. Proc Natl Acad Sci USA 79:4381-4385

Lawrence JB, Singer RH, Mcneil JA (1990) Interphase and metaphase resolution of different distance within the human dystrophia gene. Science (Washington, D.C.) 249:928-932

Leblanc O, Grimanelli D, Islam-Faridi N, Berthaud J, Savidan Y (1996) Reproductive behavior in Maize-*Tripsacum* polyhaploid plants: implications for the transfer of apomixis into Maize. J Heredity 87:108-111

Leblanc O, Grimanelli D, Gonzales-de-Leon D (1995) Detection of the apomictic mode of reproduction in maize-*Tripsacum* hybrids using maize RFLP markers. Theor Appl Genet 90:1198-1203

Leitch IJ, Leitch AR, Heslop-Harrison JS (1991) Physical mapping of plant DNA sequences by simultaneous *in situ* hybridization of two different labeled fluorescent probes. Genome 34:329-333

Li CD, Rossnagel BG, Scoles GJ (2000a) The development of oat microsatellite markers and their use in identifying relationships among Avena species and oat cultivars. Theor Appl Genet 101:1259-1268

Li HJ, Guo BH, Li YW, Du LQ, Jia X, Chu CC (2000b) Molecular cytogenetic analysis of intergeneric chromosomal translocations between wheat (*Triticum aestivum* L.) and *Dasypyrum villosum* arising from tissue culture. Genome 43:756-762

Lin L, Kuo J (1995) AFLP^{TM} : a novel PCR-based assay for plant and bacteria DNA fingerprinting. Focus 17:66-70

Liu ZW, Wang RR, Carman JG (1994) Hybridization and backcross progenies between wheat (*Triticum aestivum* L) and apomictic australian wheatgrass [*Elymus rectisetus* (Nees in Lehm) a-love and conner]-karyotypic and genomic analyses. Theor Appl Genet 89:599-605

Litt M, Luty JA (1989) A hypervariable microsatellite revealed by in vitro amplification of a dinucleotide repeat within the cardiac muscle actin gene. Amer J Hum Genet 44:397-401

Lubbers EL, Arthur L, Hanna WW, Ozias-Akins P (1994) Molecular marker shared by diverse apomictic *Pennisetum* species. Theor Appl Genet 89:636-642

Ma YZ, Tomita M, Nakata N, Yasumuro Y, (1994) Repeated DNA sequence-350 by family clond from *Agropyron intermedium* for identfication of the *Agropyron* chromosomes added to common wheat lines. Breed Sci 44:183-189

McCouch SR (2001) Rice molecular map. In Phillips, R. L. and Vasil, I. K. (ed) DNA-baseed markers in plants (2nd ed.). Kluwer Academic Publishers, Dordrecht, pp 337-346

Marsan PA, Gorni C, Chitto A, Redaelli R, Van Vijk R, Stam P, Motto M. (2001) Identification of QTLs for grain yield and grain-related traits of maize (*Zea mays* L.) using an AFLP map, different testers, and cofactor analysis. Theor Appl Genet 102:230-243

McGregor CE, Lambert CA, Greyling MM, Louw JH,Warnich, L (2000) A comparative assessment of DNA fingerprinting techniques (RAPD, ISSR, AFLP and SSR) in tetraploid potato (*Solanum tuberosum* L.) germplasm. Euphytica 113:135-144

Mehra PN, Remanandan P (1973) Cytological investigation in west *Himalayan Panicoideae*. Cytologia 38:259-270

Mengoni A, Gori A, Bazzicalupo M. (2000) Use of RAPD and microsatellite (SSR) variation to assess genetic relationships among populations of tetraploid alfalfa, *Medicago sativa.* Plant Breed 119:311-317

Mogie M (1992) The evolution of asexual reproduction in plants. Chapman and Hall. London. pp 276

Morgan R, Ozias-Akins P, Hanna WW (1998) Seed set in an apomictic BC3 pearl millet. International J Plant Sci 159:89-97

Mudge J, Andersen WR, Kehrer RL, Fairbanks DJ (1996) A RAPD genetic map of *Saccharum officinarum*. Crop Sci 36:1362-1366

Mukai Y, Nakahara Y, Yamamoto M (1993) Simultaneous discrimination of the three genomes in hexploid wheat with multicolor fluorescence *in situ* hybridization using total genomic and highly repeated DNA probes. Genome 36:489-496

Muntzing A (1940) Further studies on apomixis and sexuality in *Poa.* Hereditas 26:115-140

Nachit MM, Elouafi I, Pagnotta MA, El Saleh A, Iacono E, Labhilili M, Asbati A, Azrak M, Hazzam H, Benscher D, Khairallah M, Ribaut JM, Tanzarella OA, Porceddu E, Sorrells ME (2001) Molecular linkage map for an intraspecific recombinant

inbred population of durum wheat (*Triticum turgidum* L. var. durum). Theor Appl Genet 102:177-186

Nakamura R, Kitamura S, Inoue M, Ohmido N, Fukui K (2001) Karyotype analysis of *Nicotiana Kawakamii* Y. Ohashi using DAPI banding and rDNA FISH. Theor Appl Genet 102:810-814

Nassar NMA, Vieira MA, Vieira C, Grattapaglia D (1998) Molecular and embryonic evidence of apomixis in cassava interspecific hybrids (*Manihot* spp.) Canadian J Plant Sci 78:349-352

Nassar NMA, DosSantos E, David SRO (2000) The transference of apomixis genes from *Manihot neusana* Nassar to cassava, *M. sculenta* Crantz. Hereditas 132:167-170

Naumova TN, Hayward MD, Wagenvoort M (1999) Apomixis and sexuality in diploid and tetraploid accessions of *Brachiaria decumbens*. Sexual Plant Reprod 12:43-52.

Nogler GA (1984a) Gametophytic apomixis. In Embryology of angiosperms. ed. by Johri BM, Springer-Verlag

Nogler GA (1984b) Genetics of apospory in apomictic *Ranuncullus auricomus* V. Conclusion. Bot. Helvetica 94:411-422

Noyes RD (2000) Diplospory and parthenogenesis in sexual x agamospermous (apomictic) *Erigeron* (*Asteraceae*) hybrids. International J Plant Sci 161:1-12

Noyes RD, Rieseberg LH (2000) Two independent loci control agamospermy (apomixis) in the triploid flowering plant *Erigeron annuus.* Genetics 155:379-390

Nunome T, Ishiguro K, Yoshida T, Hirai M (2001)Mapping of fruit shape and color development traits in eggplant (*Solanum melongena* L.) based on RAPD and AFLP markers. Breed Sci 51:19-26

O'Hanlon PC, Peakall R, Briese DT (2000) A review of new PCR-based genetic markers and their utility to weed ecology. Weed Sci 40:239-254

Ozias-Akins P, Lubbers EL, Hanna WW, Mcnay JW (1993) Transmission of the apomictic mode of reproduction in *Pennisetum*: Co-inheritance of the trait and molecular markers. Theor Appl Genet 85:632-642

Ozias-Akins P, Roche D, Hanna WW (1998) Tight clustering and hemizygosity of apomixis-linked molecular markers in *Pennistetum squamulatum* implies genetic control of aporpory by a divergent locus that may have no allelic form in sexual genotypes. Proc Natl Acad Sci USA 95:5127-5132

Parra I, Windle B (1993) High resolution visual mapping of stretched DNA by fluorescent hybridization. Nature Genet 5:17-21

Patil BD, Hardas MW, Joshi AB (1961) Auto-alloploid nature of *Pennisetum squamulatum* Fresen, Nature 189:419-420

Peng Y, Schertz KF, Cartinhour S, Hart GE (1999) Comparative genome mapping of Sorghum bicolor (L.) Moench using an RFLP map constructed in a population of recombinant inbred lines. Plant Breed 118:225-235

Pessino SC, Ortiz JPA, Leblanc O, do Valle CB, Evans C, Hayward MD (1997) Identification of a maize linage group-related to apomixis in *Brachiaria*. Theor Appl Genet 94:439-444

Pessino SC, Evans C, Ortiz JPA, Armstead I, do Valle CB, Hayward MD (1998) A genetic map of the apospory-region in *Brachiaria* hybrids: identification of two markers closely associated with the trait. Hereditas 128:153-158

Pessino SC, Ortiz JPA, Hayward MD, Quarin CL (1999) The molecular genetics of gametophytic apomixis. Hereditas 130:1-11

Petrov DF, Belousova NI, Fokina ES (1979) Inheritance of apomixis and its elements in con-*Tripsacum* hybrids. Genetica 15:1827-1836

Pinkel D, Straume T, Gray JW (1986) Cytogenetic analysis using quantitative, high-sensitivity, fluorescence hybridizatio. Proc Natl Acad Sci USA 83:2934-2938

Powers L. (1945) Fertilization without reduction in guayule (*Parthenium argentatum* Gray) and a hypothesis as to the evolution of apomixis and polyploidy. Genetics 30:323-346

Pupilli F, Caceres ME, Quarin CL, Arcioni S (1997) Segregation analysis of RFLP markers reveals a tetrasomic inheritance in apomictic *Paspalum simplex*. Genome 40:822-828

Pupilli F, Labombarda P, Caceres ME, Quarin CL, Arcioni S (2001) The chromosome segment related to apomixis in *Paspalum simplex* is homoeologous to the telomeric region of the long arm of rice chromosome 12. Mol Breed 8:53-61

Quarin CL, Espinoza F, Martinez EJ, Pessino SC, Bovo OA (2001) A rise of ploidy level induces the expression of apomixis in *Paspalum notatum*. Sexual Plant Reprod 13: 243-249

Raap AK, van de Corput M, Vervenne R, van Gijlswijk R, Tanke H, Wiegant J (1995) Ultro-sansitive FISH using peroxidase-mediated deposition of biotin- or fluorochrome-tyramides. Hum Mol Gent 4:529-534

Ramulu KS, Sharma VK, Naumova TN, Dijkhuis P, Campagne MMV (1999) Apomixis for crop improvement. Protoplasma. 208:196-205

Ran YD, Hammett KRW, Murray BG (2001) Hybrid identification in *Clivia* (*Amaryllidaceae*) using chromosome banding and genomic *in situ* hybridization. Annals of Botany 87:457-462

Rayburn AL, Gill BS (1985) Use of biotin-labeled probe to map specific DNA sequences on wheat chromosomes. J Heredity 76:78-81

Refoufi A, Jahier J, Esnault MA (2001) Genome analysis of *Elytrigia pycnantha* and *Thinopyrum junceiforme* and of their putative natural hybrid using the GISH technique. Genome 44:708-715

Richards AJ (1986) Plant breeding systems. London, George Allen & Unwin

Riera-Lizarazu O, Vales MI, Phillips RL (2001) A compilation of molecular genetic maps of cultivated plants. In Phillips RL, Vasil IK (ed) DNA-baseed markers in plants (2^{nd} ed.). Kluwer Academic Publishers, Dordrecht, pp 463-497

Roche D, Cong PS, Chen ZB, Hanna WW, Gustine DL, Sherwood RT, Ozias-Akins P (1999) An apospory-specific genomic region is conserved between Buffelgrass (*Cenchrus ciliaris* L.) and *Pennisetum squamulatum* Fresen. Plant J 19:203-208

Roche D, Chen ZB, Hanna WW, Ozias-Akins P (2001a) Non-Mendelian transmission of an apospory-specific genomic region in a reciprocal cross between sexual pearl millet (*Pennisetum glaucum*) and an apomictic F1 (*P. glaucum* x *P. squamulatum*). Sexual Plant Reprod 13:217-223

Ronen G, CarmelGoren L, Zamir D, Hirschberg J (2000) An alternative pathway to beta-carotene formation in plant chromoplasts discovered by map-based cloning of Beta and old-gold color mutations in tomato. Proc Natl Acad Sci USA 97:11102-11107

Russell JR, Fuller JD, Macaulay M, Hatz BG, Jahoor A, Powell W, Waugh R (1997) Direct comparison of levels of genetic-variation among barley accessions detected by RFLPS, AFLPS, SSRS and RAPDS. Theor Appl Genet 95:714-722

Sadder M T, Weber G (2001) Karyotype of maize (*Zea mays* L.) mitotic metaphase chromosomes as revealed by fluorescence *in situ* hybridization (FISH) with cytogenetic DNA markers. Plant Mol Biology Reporter 19:117-123

Savidan Y, Pernes J (1982) Diploid-tetroploid-dihaploid cycles and the evolution of *Panicum maximum* Jacq. Evolution 36:596-600

Savidan Y (1990) The genetic control of apomixis. Apomixis Newsl 2 (24)

Savidan Y, Grimanell D, Leblanc O (1995) Widecross transfer of apomixis to maize: to dream an impossible dream? In Harnessing Apomixis. An international conference to address a topic of far-reaching significance. A New Frontier in Plant Sci, September 25-27. College Station, Taxas. p.20

Savidan Y (2000) Apomixis: Genetics and Breeding. Plant Breed Review 18:14-86

Schmelzer GH (1997) Review of *Pennisetum* section Brevivalvula (*Poaceae*). Euphytica 97:1-20

Schwarzacher T, Heslop-Harrison P (2000) Practical *in situ* hybridization. Springer-Verlag. New York.

Schulz-Schaeffer J (1980) Cytogenetics. Plants, Animals, Humans. Springer-Verlag. New York, Heidelberg, Berlin. pp. 203-218

Shah MM, Yen Y, Gill KS, Baenziger PS (2000) Comparisons of RFLP and PCR-based markers to detect polymorphism between wheat cultivars. Euphytica 114:135-142

Sherwood RT, Berg CC, Young BA (1994) Inheritance of apospory in Buffelgrass. Crop Sci 34:1490-1494

Simpson CE, Bashaw EC (1969) Cytology and reproductive characteristics of *Pennisetum setaceum*. Amer J Bot 56: 31-36

Simpson PR, Newman MA, Davies DR (1988) Detection of legumin gene DNA sequences in pea by *in situ* hybridization. Chromosoma 96:454-458

Singh BN, Singh AK (1989) The suppression of crossing-over between heterozygous inversions of *Drosophila ananassae.* Genetika Beograd 21:155-163

Smith JSC, Senior ML (2000) The utility of Simple Sequence Repeat (SSR) data to preferentially identify progeny lines of maize (*Zea mays* L.) that are bred from known inbred parents. Maydica. 45:205-213

Spiegelman JI, Mindrinos MN, Fankhauser C, Richards D, Lutes J, Chory J, Oefner PJ (2000) Cloning of the arabidopsis RSF1 gene by using a mapping strategy based on high-density DNA arrays and denaturing high-performance liquid chromatography. Plant Cell 12:2485-2498

Srivastava A, Gupta V, Pental D, Pradhan AK (2001) AFLP-based genetic diversity assessment amongst agronomically important natural and some newly synthesized lines of *Brassica juncea*. Theor Appl Genet 102:193-199

Suto Y, Ishikawa Y, Hyodo H, Uchikawa M, Juji T (2000) Gene organization and rearrangements at the human Rhesus blood group locus revealed by fiber-FISH analysis. Hum Genet 106:164-171

Tagashira N, Kondo K (2001) Chromosome phylogeny of *Zamia* and *Ceratozamia* by means of Robertsonian changes detected by fluorescence *in situ* hybridization (FISH) technique of rDNA. Plant Systematics And Evolution 227:145-155

Taliaferro CM, Bashaw EC (1966) Inheritance and control of obligate apomixis in breeding buffelgrass, *Pennisetum ciliare*. Crop Sci 6:473-476

Tan YF, Zhang QF (2001) Correlation of simple sequence repeat (SSR) variants in the leader sequence of the waxy gene with amylose content of the grain in rice. Acta Botanica Sinica 43:146-150

Tanksley SD, Ganal MW, Prince JP, Vicente MC. de Bonierbale MW, Broun P, Fulton TM, Giovannoni JJ, Grandillo S, Martin GB (1992) High density molecular linkage maps of the tomato and potato genomes. Genetics 132:1141-1160

Tanksley SD, Ganal MW, Martin GB (1995) Chromosome landing–a paradigm for map-based gene cloning in plants with large genomes. Trends in Genetics 11:63-68

Torabinejad J, Mueller RJ (1993) Genome analysis of intergeneric hybrids of apomictic and sexual Australian *Elymus* species with wheat, barley and eye-implication for the transfer of apomixis to cereals. Theor Appl Genet 86:288-294

Van DerNest MA, Steenkamp ET, Wingfield BD, Wingfield MJ (2000) Development of simple sequence repeat (SSR) markers in *Eucalyptus* from amplified inter-simple sequence repeats (ISSR). Plant Breed 119:433-436

Van Dijk PJ, Tas ICQ, Flaque M, BakxSchotman T (1999) Crosses between sexual and apomictic dandelions (*Taraxacum*), II. The breakdown of apomixis. Heredity 83:715-721

Van Dijk P, Van Damme J (2000) Apomixis technology and the paradox of sex. Trends In Plant Sci 5:81-84

Viegas-Péquignot E, Berrard S, Brice A, Apious F, Mallet J (1991) Localization of a 900-bp-long fragment of the human choline acetyltranferase gene to 10q11.2 by nonradioactive *in situ* hybridization. Genomics 9:210-212

Vielle JP, Burson BL, Bashaw EC, Hussey MA (1995) Early fertilization events in the sexual and aposporous egg apparatus of *Pennisetum ciliare* (L.) Link. Plant J 8:309-316

Voigt PW, Burson BL (1981) Breeding of apomictic *Eragrostis cupula.* Proc XIV Intl Grassland Congr, Lexington Ky 160-163

Vos P, Hogers R, Bleeker M, van de Lee T, Hornes, M, Frijters A, Pot J, Peleman J, Kuiper M, Zabeau M (1995) AFLP: a new technique for DNA fingerprinting. Nucl Acids Res 23:4407-4414

Vuylsteke M, Mank R, Antonise R, Bastiaans E, Senior ML, Stuber CW, Melchinger AE, Lubberstedt T, Xia XC, Stam P, Zabeau M, Kuiper M (1999) Two high-density AFLP(R) linkage maps of *Zea mays* L.: analysis of distribution of AFLP markers. Theor Appl Genet 99:921-935

Weier HUG (2001) DNA fiber mapping techniques for the assembly of high-resolution physical maps. J Histochemistry and Cytochemistry 49:939-948

Welsh J, McClland M (1990) Fingerprinting genomes using PCR with arbitrary primers. Nucl Acid Res 18:7213-7218

Wen XS, Ye XL, Li YQ, Chen ZL, Xu SX (1998) Embryological studies on apomixis in *Pennisetum squamulatum*. Acta Botanica Sinica. 40:598-605

Wiegant J, Ried T, Nederlof PM, van der Ploeg M, Tanke HJ, Raap AK (1991) *In situ* hybridization with fluoresceinated DNA. Nucl. Acids Res 19:3237-3241

Williams JGK, Kubelik AR, Livak KJ, Rafalski JA, Tingey SV (1990) DNA polymorphisms amplified by arbitrary primers are useful as genetic markers. Nucl Acids Res 18:7213-7218

Winter P, Kahl G (1995) Molecular markers technologies for plant improvement. World J Microbiology and Biotechnology 11:438-448

Wu KK, Burnquist W, Sorrells ME, Tew TL, Moore PH, Tanksley SD (1992) The detection and estimation of linkage in polyploids using single-dose restriction fragments. Theor Appl Genet 83:294-300

Yamanaka N, Ninomiya S, Hoshi M, Tsubokura Y, Yano M, Nagamura Y, Sasaki T, Harada K (2001) An informative linkage map of soybean reveals QTLs for flowering time, leaflet morphology and regions of segregation distortion. DNA Res 8:61-72

Young WP, Schupp JM, Keim P (1999) DNA methylation and AFLP marker distribution in the soybean genome. Theor Appl Genet 99:785-792

Zhang JY, Li XM, Wang RRC, Cortes A, Rosas V, Mujeeb-Kazi A (2002). Molecular cytogenetic characterization of E-b-genome chromosomes in Thinopyrum bessarabicum disomic addition lines of bread wheat. International J. Plant Sci 163:167-174

Zhong X, Fransz PF, Eden JW, Zabel P, Kammen A, de Jong JH (1996) High-resolution mapping on pachytene chromosomes and extended DNA fibres by fluorescence in-situ hybridization. Plant Mol Biology Report 14:232-242

Table 2.1. Summary of different genetic regulation models postulated with different species.

Taxonomic unit	Type	Genetic model	Reference
Beta lomatogona	aposporous	dominant (major gene)	Cleiji et al. 1976
Bothriochloa	aposporous	dominant(dosage effect)	Harlan et al. 1964
dichanthium		dominant	d'Cruz and Reddy 1971
Erigeron annuus	diplosporous	dominant (independent from parthenogenesis	Noyes and Rieseberg 2000
Cenchrus ciliaris	aposporous	dominant (1 gene with hypopistasis to sexual gene)	Taliaferro et al. 1966
		dominant (1)	Sherwood 1994
Eragrostis curvula	diplosporous	dominant (major genes)	Voigt and Burson 1981
Potentilla	aposporous	dominant (1)	Muntzing 1958
Poa	diplospory	dominant(1 or more)	Muntzing 1940
		recessive	Akerberg and Bingefors 1953
Panicum maximum	aposporous	dominant (1 supergene)	Savidan and Pernes (1982)
		recessive	Hanna et al. 1973
Pennisetum orientale	aposporous	dominant	Dujardin and Hanna 1983 (b)
			Dujardin and Hanna 1984 (b)
Pennisetum squamulatum	aposporous	dominant	Hanna and Bashaw 1987
		dominant (1gene,tetrasomic inheritance with gamete lethal)	Ozias-Akins et al.1998
Poa pratensis	aposporous	dominant (major gene)	Akerberg and Bingefors 1953
Ranunculas auricomus	aposporous	dominant (major gene)	Savidan 1990
		dominant, lethal ( )	Nogler 1984b
Rubus	aposporous	dosage effects	Nogler 1984a
		dominant	Gustafsson 1946
Taraxacum	diplosporous	multigene	Van Dijk et al. 1999

Table 2.2. Summary of the characteristics of the RFLP, RAPD, SSR and AFLP markers. [adapted from O'Hanlon et al.(2000)]

Marker characteristics	RFLP	RAPD	SSR	AFLP
Abundance[1]	+	++	++	+++
Reproducibility[2]	++	++	+++	+++
Technical ease[3]	+	+++	+	++
Precision[4]	++	+	++	++
Cross comparability[5]	+++		+++	
Development time[6]	Medium	Short	Long	Short
Lab.Equipment costs	Med-High	Low	Med-High	High
Ongoing assay costs[7]	Med	Low	Med-High	Med-High

[1] Number of DNA fragments that can be sampled in one assay.

[2] Refers to the ability to obtain the same genetic result for the same sample in repeated assays.

[3] The level of skill required to obtain accurate genetic data once an assay has been developed.

[4] Differs from reproducibility in that a reproducible genetic result may not accurately reflect the diversity present within the sample because of effects, such as mismatch because the annealing temperature is too low for RAPDs.

[5] Cross comparability refers to the comparability of the same markers a cross different species.

[6] The time required to prepare a laboratory to a satisfactory level for such analysis.

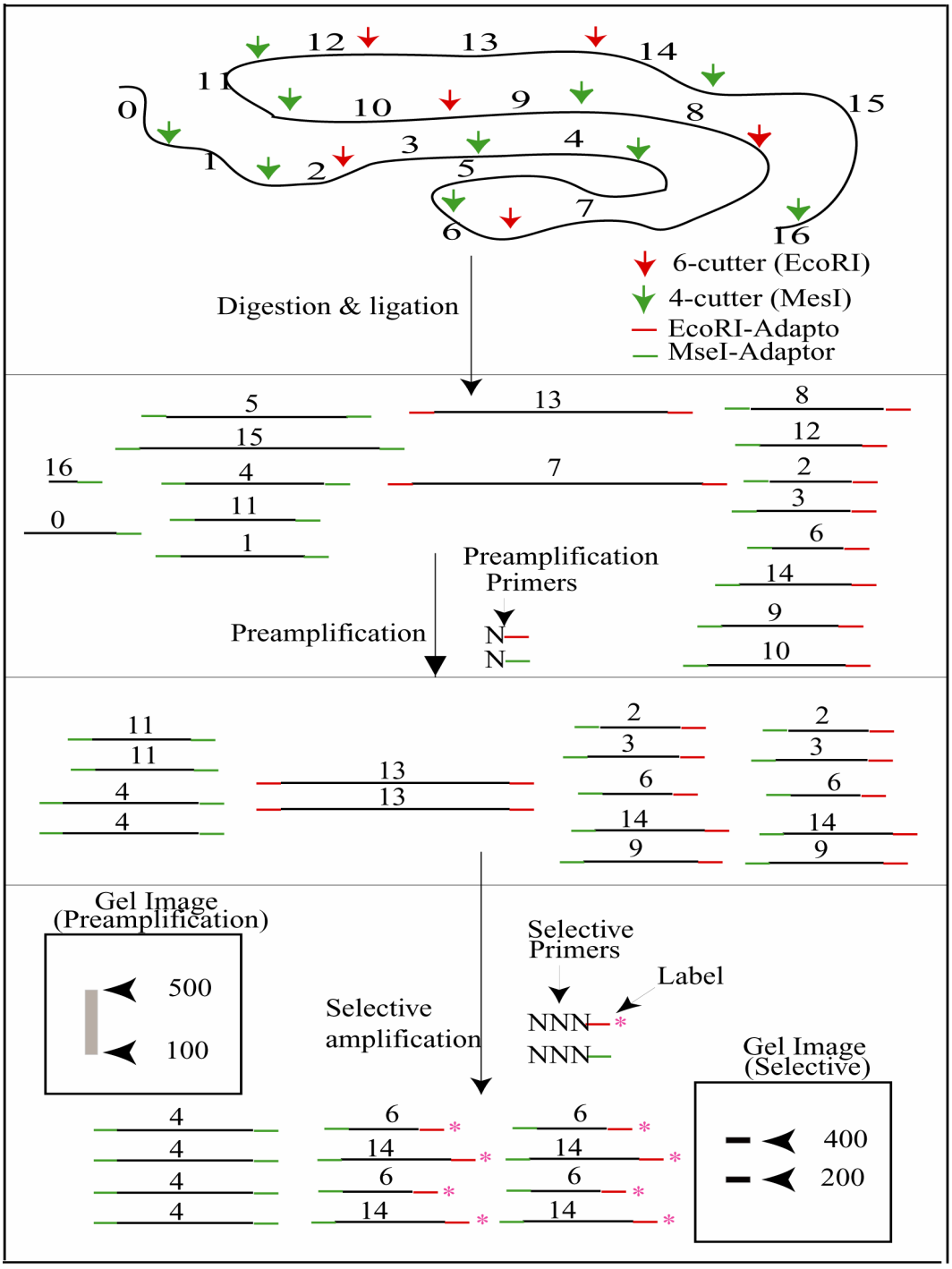
[7] The financial requirement to obtain results after laboratory estabilishment.

Table 2.3. Molecular markers associated with apomixis in different species.

Taxanomic unit	Marker (type)	Reference
P.squamulatum	UGT-197(STS)	Ozias-Akins et al.1993
	C4600 (SCAR) A10 (SCAR)	Lubbers et al.1994*
	O7 (SCAR) X18 (SCAR)	Ozias-Akins et al.1998
	Q8M (SCAR) U12 (SCAR)	
	W10M (SCAR) A14 (SCAR)	
	P16 (SCAR) R13 (SCAR)	
	V4 (SCAR)	
B .brizantha	cDNA differential display	Leblanc et al.1997
	C4 (RAPD)	Pessino et al. 1997
	csu137 (RFLP) umc72 (RFLP)	
	umc147 (RFLP) csu134(RFLP)	
	csu149 (RFLP) umc90 (RFLP)	
P. ciliaris	UGT-197 (STS) X18 (SCAR)	Roche et al. 1999
	Q8M (SCAR) U12 (SCAR)	Lubbers et al 1994
	P16 (SCAR) V4 (SCAR)	Gustine et al.1997
	C4600 (SCAR) A20 (RAPD)	
	J16 (RAPD) N15 (RAPD)	
	B14 (RAPD) M02 (RAPD)	

* Lubbers et al (1994) reported that apomixis markers such as UGT197, $C4\text{-}_{600}$ developed with *P. squamulatum* also shared by most other apomictic species in the same genus.

Fig. 2.1 Diagram illustrating the basic concept for amplified fragment length polymorphism (AFLP). Red arrow refers to *EcoR*I restriction enzyme, green arrow to *Mse*I restriction enzymes, red bar to *EcoR*I adaptor, green bar to *Mse*I adaptor, and N to extension nucleotide.



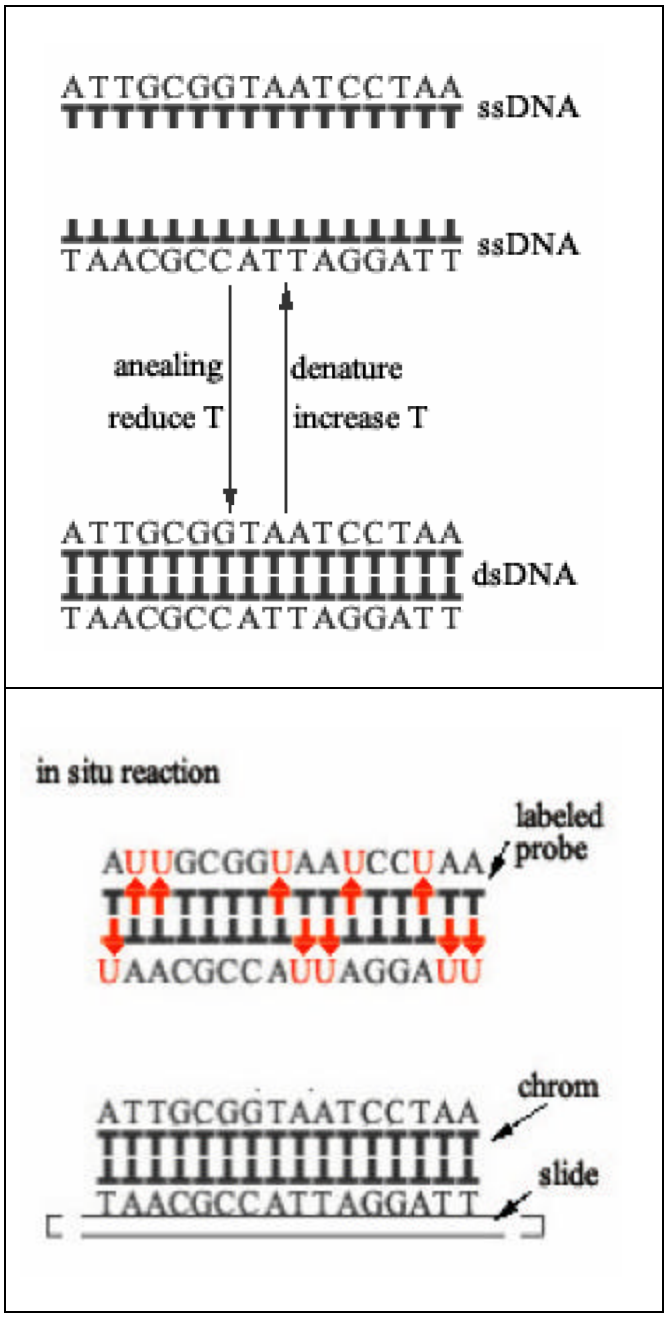


Fig. 2.2A. The dynamic process of DNA denaturation and annealing.

Fig. 2.2B. Fix the chromosomes on the surface of a slide. Apply labeled probes to chromosome preparations.

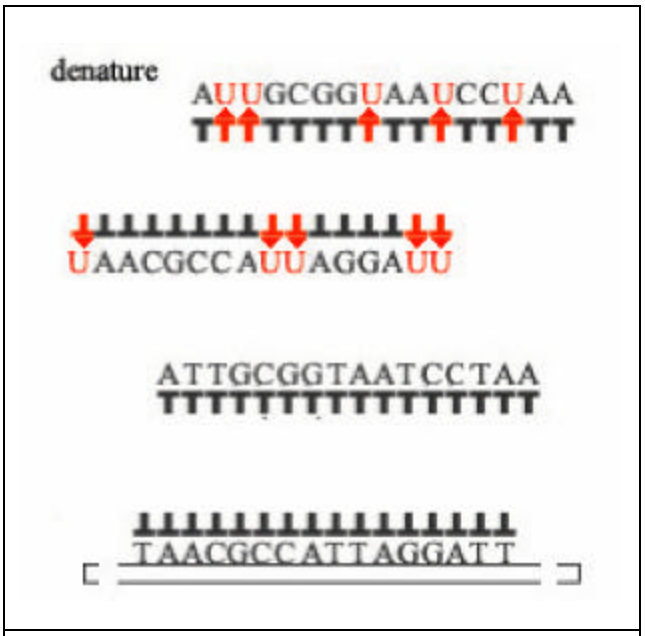


Fig. 2.2C. Both chromosome DNA and probe DNA are subjected to denaturation under high temperature, usually at 70-80 ^{o}C for 3-5min.

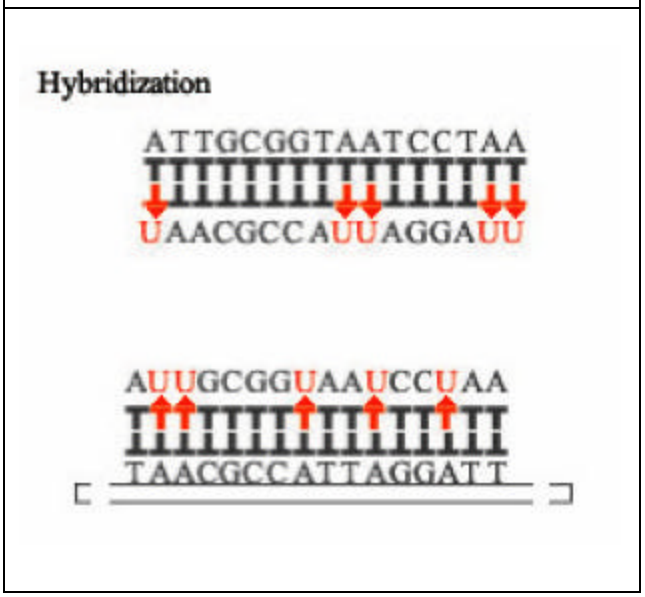


Fig. 2.2D. After denaturation of the mixture, incubate at lower temperature to let the DNA anneal or hybridize. This is a dynamic process and in order to reach a high percentage of match, the annealing temperature needs to be adjusted for each different probe. Also, this process needs to occur overnight even though most annealing is completed in about 4 hours.

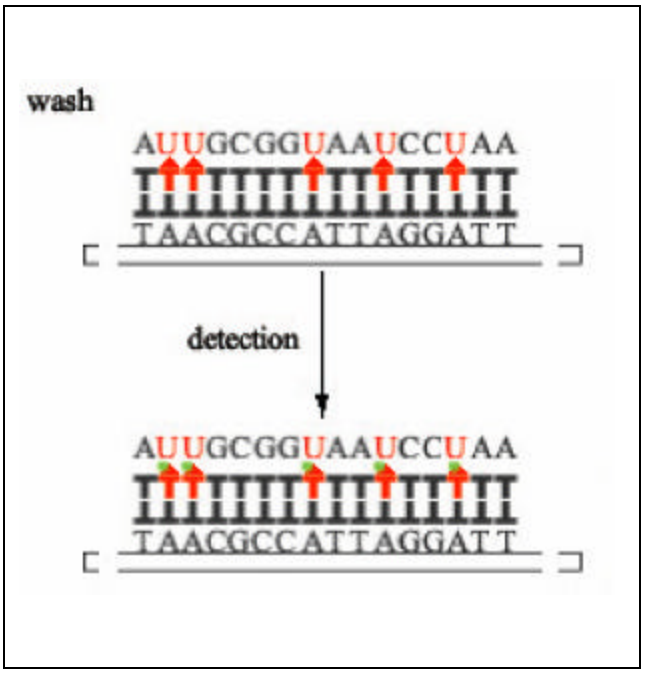


Fig. 2.2E. This step is to remove the mismatched hybridizations and the residues of unused DNA and other reagents for hybridization. The wash can reduce false positive signals and the background. Detection is a process to apply the antibody conjugated fluorochromes, which emit certain wavelength light under appropriate excitation wavelengths and can be detected under a fluorescence microscope.

CHAPTER 3

AN AFLP GENETIC LINKAGE MAP OF AN APOMICTIC POLYPLOID *PENNISETUM SQUAMULATUM*

Chen, Z. B., W. W. Hanna and P. Ozias-Akins. To be submitted to Theor. Appl. Genet.

ABSTRACT

Pennisetum squamulatum Fresen is a perennial grass from east Africa that produces seeds asexually through apospory, a kind of apomixis. It has been considered as an excellent donor of gene(s) for apomixis because the species exhibits (i) nearly obligate apomixis and (ii) crossing compatibility with its domesticated relative, pearl millet [*P. glaucum* (L.) R. Br.], an important crop widely grown in Africa and Asia. A pseudo-testcross mapping population was generated from a cross of tetraploid pearl millet x *P. squamulatum* where alleles from only the male parent were mapped. By combining AFLP markers and a single-dose allele strategy, the first genetic linkage map of *P. squamulatum* was constructed with 182 markers distributed across 44 linkage groups covering 2600 cM with an average distance of 14.3 cM between markers. The estimated genome size is 4632 cM, with an expected genome coverage of 73.7%. All markers were distributed randomly over the genome except for the apospory-specific genomic region (ASGR), in which 4 AFLP markers clustered. Repulsion analysis did not support preferential pairing of the ASGR with a single homolog during meiosis.

Key words: *Pennisetum squamulatum*, polyploid, AFLP, apospory, genetic mapping

Introduction

Pennisetum squamulatum Fresen is a hexaploid and apomictic member of the grass family. One of its relatives, pearl millet [*P. glaucum* (L.) R. Br.], is an important forage and grain crop widely grown in Africa and Asia. Pearl millet reproduces sexually while *P. squamulatum* produces seeds through apospory, a form of gametophytic apomixis, and has been considered as an excellent donor of gene(s) underlying apomixis (Dujardin and Hanna 1989). Gametophytic apomixis in angiosperms refers to asexual reproduction by which a plant produces seeds without meiosis or gametic fusion of a sperm with an egg cell of an unreduced embryo sac (Nogler 1984). The egg cell develops parthenogenetically into an embryo whose genetic make-up is thus identical to the seed-bearing plant. The genotype of an apomictic plant can be passed on through multiple seed generations regardless of how heterozygous the genetic background might be. Transfer of apomixis and manipulation of the reproductive systems of economically important crops promises great potential for application in agriculture and has drawn increasing attention for the last three decades (Ozias-Akins et al. 1993; Hanna 1995; Koltunow et al. 1995; Pessino et al. 1999; Savidan 2000; Grossniklaus et al. 2001).

There have been at least 212 molecular marker-based maps established for over 78 economically important species from 66 different genera including monocots, dicots and gymnosperms. More than 30 maps are published for maize alone, 8 for tomato, and 7 for wheat (Riera-Lizarazu et al. 2001). For diploid pearl millet, a RFLP-based map of sexual, diploid pearl millet was constructed with a F_2 population from an intervarietal cross. The map comprised 181 RFLP markers and covered 303 cM of the genome with an average distance of 2 cM between markers (Liu et al. 1994). No significant difference

was observed in the total length of the linkage maps from reciprocal crosses between pearl millet and two wild subspecies indicating that recombination frequencies were similar in male and female meiosis (Liu et al. 1996). In later studies, quantitative traits associated with the domestication of pearl millet , such as spike size and non-shedding seeds, could be attributed mainly to loci with large effects (Poncet et al. 1998, 2000).

Pennisetum squamulatum has been described as an auto-allopolyploid, based on cytogenetic analysis, with 2n = 6x = 54 chromosomes (Raman et al. 1959; Patil et al. 1961) plus 2 acro- or submeta-centric B-chromosomes in some cytotypes (Sindhe 1976). Genetic mapping of *P. squamulatum*, as for other polyploid plants, has lagged behind that of diploid species for several reasons, including the complexity of mapping multi-allelic loci in segregating polyploid populations (Wu et al. 1992), and the occurrence of irregular pairing of chromosomal homologs during meiosis (Dujardin and Hanna 1984). Mapping with single-dose restriction fragments has been implemented for polyploid species (Wu et al. 1992). Another method that has facilitated molecular mapping in polyploid as well as diploid species is amplified fragment length polymorphism (AFLP), a polymerase chain reaction (PCR)-based molecular marker technique that allows the simultaneous sampling of large numbers of locus-specific markers without prior sequence information (Vos et al. 1995). Genetic maps of polysomic polyploid plants based largely on AFLP-based markers have been constructed for sugarcane (Hoarau et al. 2001) and potato (Bradshaw 1998).

For *P. squamulatum*, although no whole genome map has been constructed, twelve PCR-based molecular markers linked with aposporous apomixis were isolated using bulked-segregant analysis in a population segregating for the mode of reproduction

(Ozias-Akins et al. 1998). An apospory-specific genome region (ASGR) was postulated because all twelve molecular markers cosegregated with the trait and several of the markers were hemizygous, i.e., they hybridized only with the apomictic plants of the population (Ozias-Akins et al. 1998). Some of the ASGR linked markers also were shared with other species of the *Pennisetum* genus and with buffelgrass, *Cenchrus ciliaris* L. (Lubbers et al. 1994; Roche et al. 1999). However, the ASGR-linked markers sampled only a small portion of the *P. squamulatum* genome, and this region, with its low rate of recombination, probably was not representative of the recombination behavior of the entire genome. In order to test the distribution of genetic recombination in *P. squamulatum*, we constructed a genetic linkage map using AFLP markers, which allowed rapid screening for single-dose alleles. This map has provided an essential overview of the genetic structure of *P. squamulatum* and more extensive characterization of the linkage group related to the ASGR.

3.1 Materials and methods

3.1.1 Mapping population and trait screening

A mapping population of 94 individuals was selected from the F_1 progeny of a cross of *P. glaucum* (induced tetraploid $2n = 4x = 28$) x *P. squamulatum* (PS26, PI 319196, $2n = 6x = 56$). All 94 F_1 plants (46 sexuals and 48 apomicts), which represented a subset of the mapping population used by Ozias-Akins et al. (1998), had been characterized previously for mode of reproduction by observation of cleared ovules and tested by PCR for all ASGR-linked molecular markers.

3.1.2 AFLP analysis

DNA samples which were used in the previous work (Ozias-Akins et al. 1998) were also utilized for AFLP analysis following the protocol of the ABI PRISM™ fluorescent dye-labeled AFLP kit for large genomes (ABI PRISM™, PE Applied Biosystems, Foster, CA, USA) with some modifications as described below. Digestion of genomic DNA and ligation of adaptors were carried out in a reaction volume of 11 μl, including 500 ng genomic DNA, 50 mM NaCl, 0.1 mg/ml of BSA, 8 U *Eco*RI, 1 U *Mse*I (New England Biolabs, Beverly, MA, USA), 0.1 μM of *Eco*RI adapter and 0.5 μM of *Mse*I adapter, 1x T4 DNA ligase buffer and 0.3 U of T4 DNA ligase (Promega, Madison, WI, USA). Digestion and ligation were carried out in a single reaction for 3 h at 37 C. Following digestion/ligation, adenine (A) and cytosine (C) were used as pre-selective nucleotides for *Eco*RI ends (*Eco*RI+A) and *Mse*I ends (*Mse*I+C), respectively. For subsequent selective amplifications, a 40x dilution of pre-amplified products was found to reduce non-specific background on gel images. Selective amplifications were carried out with three nucleotide extensions (*Eco*RI+3/*Mse*I+3) of each adaptor under a stringent touch-down temperature profile as described in the protocol of ABI PRISM™ fluorescent dye-labeled AFLP kit for large genomes. The PCR reactions were loaded on 4% denaturing polyacrylamide sequencing gels and run on an ABI PRISM™ 377 DNA sequencer. Gel images were generated with GeneScan 3.1 software (PE Applied Biosystems).

Initially, ten F_1 plants (five apomicts and five sexual plants) plus the two parents were used to test all 64 primer combinations for amplification of informative AFLPs. Primers that produced the largest number of informative fragments were later synthesized by Life Technologies and 5' end-labeled with the flourescent dye, FAM. Twenty-six

selected primer combinations were used for screening the mapping population of 94 F_1 plants plus two parents.

3.1.3 Data collection and linkage analysis

Bands on the gel image were scored manually as 1 (band present) or 0 (band absent). Only the loci, which were present in *P. squamulatum* and absent in pearl millet as well as those segregating among the progeny were considered for linkage analysis. Each informative polymorphic AFLP fragment was identified by the combination of a two-letter prefix designating the primer combination and a number indicating the estimated molecular weight of the fragment (Table 3.1). For each locus, the ratio of the number of individuals scored as positive for a locus to those scored as negative at the same locus was tested for a fit to 1:1 with χ^2 at $P < 0.05$ and $df = 1$. Loci that fit a 1:1 segregation ratio were selected as single-dose AFLP markers since a 1:1 ratio would be expected in a test cross (Wu et al.1992).

Linkage maps were generated with Mapmaker/Exp version 3.0 (Lander et al. 1987). Markers were first sorted by using the group command at a LOD score threshold of 5 and a recombination fraction threshold of 0.37. The LOD score subsequently was reduced to 4 and 3 while maintaining a maximum recombination fraction of 0.37 to find the possible additional, but statistically less well supported, linkages. Orders of marker loci were determined with multi-point comparisons using the standard commands. When no starting point could be found, two-point comparisons were used to determine the order.

Possible linkage in repulsion was analyzed using reversion of the original data set (Wu et al.1992). After grouping the original and reversed data with a LOD score of 5, 4 and 3 at a recombination fraction of 0.37, two-point joint analysis was employed to find

the markers most closely linked in repulsion. Linkage in repulsion would indicate that preferential pairing could occur between two chromosomes since such linkages would be undetectable in the present population size of 94 individuals if polysomic inheritance were occurring (Wu et al. 1992).

3.1.4 Estimated genome length and expected genome coverage

The genome length was estimated according to the equation $Y_x = [n(n-1)/2]2x/G$ given by Hulbert et al. (1988), where the Y_x is the number of two-point linkages at a given distance of equal to or less than x at a certain LOD, n is the number of mapped markers at the same LOD, x is a given distance in cM, and G is the estimated genome length. Genome coverage was estimated with a formula $E(C_n) = 1 - p_{1,n}$, where $E(C_n)$ is the estimated genome coverage with n mapped makers and $p_{1,n}$ is the probability for a random point to not be covered by n randomly placed markers. $p_{1,n}$ was calculated according to $p_{1,n} = 2r/(n+1)[(1-x/2G)^{(n+1)} - (1-x/G)^{(n+1)}] + (1-rx/G)(1-x/G)^n$, where r is the haploid chromosome number, n is the number of mapped markers at a certain LOD, x is a given distance in cM, and G is the estimated genome length in cM at the same LOD (Bishop et al. 1983).

3.2 Results

3.2.1 Efficiency of marker detection with AFLP

Sixty-four primer combinations (8 *Eco*RI + 3 x 8 *Mse*I + 3) were screened to determine the most informative combinations. Reactions with thirty-eight primer combinations resulted in either no amplified bands or a smear. These primer combinations were not further tested in this study. Twenty-six primer combinations produced reproducible and polymorphic bands between the two parents as well as among the 10 sample F_1 progeny

(Table 3.1). The number of polymorphic bands between the parents generated with each informative primer combination varied considerably, ranging from a low of 34 (ACG/CAT) up to 108 (ACT/CTA) with an average of 71.6 bands. Polymorphic bands comprised up to 90% of the total amplified bands (Table 3.1). The segregation ratio of each AFLP locus was tested with χ^2 for a goodness of fit to a 1:1 ratio ($P < 0.05$, $df = 1$). Fragments that fit the 1:1 ratio were considered to be single-dose AFLP markers and were included in the dataset used for mapping. Among these polymorphic bands, 1 to 30 loci per AFLP primer combination were qualified as single-dose AFLP markers, which was only 14% of the total number of polymorphic bands. The number of polymorphic fragments that could be used for mapping was comparatively low because 86% of the polymorphic bands were in multiplex form. Bands shared by both *P. squamulatum* and *P. glaucum* made up 9% of the total number of amplified bands indicating that the two species shared some similar sequence. Multiple dose markers were not included for mapping because pairing relationships among *P. squamulatum* chromosomes have not been fully determined.

3.2.2 Genetic map of *P. squamulatum*

Linkage groups generated with Mapmaker/exp 3.0 are shown in Fig. 3.1. Two hundred thirty markers were first grouped, at a LOD score of 5, into 48 linkage blocks with 73 unlinked markers. When the LOD score was reduced to 3, 23 previously unlinked markers were added to the map, and the number of linkage groups was reduced from 48 to 44. Two additional markers (those indicated with R following the marker name) were added to the map as a result of repulsion analysis. The final linkage map was constructed at LOD 3 and a maximum recombination fraction of 0.37. This genetic map comprised

182 single-dose AFLP markers with 48 markers, or 21% of the total, left unlinked. The total length of the map was 2600 cM with an average distance of 14.3 cM between markers. The estimated genome length is 4632 cM. Our linkage map covered approximately 56% of the total estimated genome, although the expected genome coverage calculated according to Bishop et al. (1983) was approximately 73%.

Repulsion linkage analysis found only 15 markers linked in repulsion at a LOD score of 3 while 185 markers remained linked in coupling. The ratio of repulsion linked markers to coupling linked markers was significantly lower than a 1:1 ratio expected for allopolyploids and more closely fit the ratio of 0:1 expected for autopolyploids. All pairs of repulsion-linked markers showed relatively large genetic distances (28-39 cM; Fig. 3.1); therefore, extensive preferential pairing was not strongly supported by these data.

3.2.3 Distribution of AFLP markers across the *P. squamulatum* genome

The AFLP markers showed primarily random but a few clustered distributions. Two markers clustered together on linkage group 6. The largest cluster contained four markers and was positioned on linkage group 1. Notably, the cluster on linkage group 1 cosegregated with the trait for aposporous embryo sac development and the previously characterized SCAR, ugt197. No other clusters of more than two markers were found on the remaining linkage groups.

3.2.4 Linkages with apospory in *P. squamulatum*

Four AFLP markers, pu70, pa502, pv600, and pw298, were totally linked with apospory. They comprised 1.7% of the total single-dose AFLP markers. Another 4 AFLP fragments were linked to apospory within a distance of 10.8 cM. Two each of the markers flanked the target trait on opposite sides although the order of the tightly linked

marker cluster and px299 was ambiguous since the best order was less than tenfold more likely than the second best order (Fig. 3.1). These eight markers accounted for 3.5% of the total single-dose AFLP markers screened but only 1.2% of the total map length, and define the boundary of the apospory-specific genomic region.

3.3 Discussion

3.3.1 AFLP markers in *P. squamulatum*

An average of 52 AFLP fragments was amplified with each *Eco*RI/*Mse*I primer combination in this study, which is similar to the results with European and Japanese larch (Arcade et al. 2000), pepper (Kang et al. 2001), and *Zea mays* L. (Vuylsteke et al. 1999). However, the number of informative single-dose AFLP markers is the bottleneck in polyploid mapping versus the diploid studies cited above. The auto-allopolyploid nature of *P. squamulatum*, as described by Patil et al. (1961), likely resulted in a large proportion of multiplex or monomorphic fragments that were eliminated from our study. The percentage of single-dose AFLP markers in *P. squamulatum* (13%) was much lower than that reported for sugarcane [94.5% (887/939); Hoarau et al. 2001]. Low numbers of informative AFLP bands also were obtained with the genetic mapping of tef [*Eragrostis tef* (Zucc.) Trotter], where with 50 primer combinations, only 211 markers could be mapped to 25 linkage groups, covering 2149 cM of the genome (Bai et al. 1999). However, by changing the restriction enzymes and the combination of primer extension nucleotides in the AFLP reaction, the abundance of AFLPs theoretically could be unlimited. For example, 189 more AFLPs were generated with *Pst*I/*Mse*I in soybean, and these markers were more evenly distributed in the genome than *EcoR*I/*Mse*I AFLP markers (Young et al. 1999).

We began this study with the assumption that AFLPs would be randomly distributed across the genome, at least with regard to physical distances. Barring one exception at the ASGR, we did not encounter substantial clusters of markers, even near centromeric regions where recombination often is suppressed (Tanksley et al. 1992; Sherman and Stack 1995). The centromeric clustering of AFLP markers from *Eco*RI/*Mse*I primer combinations has been observed in other plant AFLP linkage maps, such as potato (van Eck et al. 1995), pepper (Kang et al. 2001), and maize (Vuylsteke et al. 1999). However, the low density of the *P. squamulatum* map may have precluded sampling of such clusters.

3.3.2 Genetic map of *P. squamulatum*

We present the first comprehensive genetic map for the genome of *P. squamulatum* in this study. This map comprises 182 single-dose AFLP markers and covers 2600 cM with an average distance of 14 cM between markers. This map covers over half (56.13%) of the estimated genome size of 4632 cM although the genome size of *P. squamulatum* is approximately 5000 Mbp (1C) (Roche et al. 2002; Bennett and Leitch 2001). However, this linkage map is far from saturation as the markers are distributed over the genome with a large average distance. The relatively low density of this map is due in part to the polyploid nature of this species where single-dose markers may be more abundant in some regions of the genome. A similar mapping strategy with pseudotestcross progeny frequently has been used to establish genetic maps for polyploid out-crossing plants such as sweet potato (*Ipomoea batatas* (L.) Lam.) (Ukoskit 1997) and sugarcane *(Saccharum officinarum* L.) (Mudge et al. 1996). We observed that less than 17% of the total scored AFLP fragments were single-dose polymorphisms. This implies that much of the

genome in *P. squamulatum* might not be highly heterozygous, perhaps due to autopolyploidy. Cytologically, trivalents (1-2/cell) and quadrivalents (1-8/cell) have been observed during meiosis of *P. squamulatum*, an indication that random pairing occurs among at least some of the homologous groups (Dujardin and Hanna 1984). An allopolyploid *P. squamulatum* was suggested because of the chromosome behavior observed in a 41-chromosome hybrid of *P. glaucum* x *P. squamulatum* where 16 bivalents were interpreted as 7 pairs of pearl millet chromosomes and 9 pairs of *P. squamulatum* chromosomes. In addition to the bivalents, 9 univalents were observed which supported the conclusion that one diploid and one tetraploid genome were combined in *P. squamulatum* (Patil et al.1961). Autopolyploidy, or at least the lack of strong preferential pairing, also was supported by repulsion analysis in our study which showed that the ratio of markers linked in repulsion phase to those linked in coupling phase did not approach 1:1 and was even much lower than the ratio of 0.25:1 expected for an autotetraploid. However, repulsion phase linkages in polysomic polyploids, where homologous chromosomes pair randomly during meiosis, are difficult or impossible to detect in small populations (Wu et al. 1992). Repulsion analysis in our study, however, did show fifteen markers linked in repulsion at LOD 3, but only two markers (pu363 and pr225) on one linkage group remained linked in repulsion to another linkage group at LOD 5. Limited preferential pairing may occur during microsporogenesis though random pairing among the homologous chromosomes appears to dominate. It is also possible that preferential pairing may be limited to certain chromosomal regions, not entire chromosomes. Since no markers were found to be linked in repulsion with the ASGR, our results also agree with the autotetraploid inheritance of apospory proposed by

Ozias-Akins et al. (1998). Although polyploidy may have resulted in a lower frequency of polymorphic fragments, and hence a lower-density map containing many isolated, minor linkage groups, the chromosome segment harboring the ASGR seems to be highly heterozygous.

Regarding genome coverage, Hoarau et al. (2001) reported an AFLP linkage map of 5,849 cM with 939 simplex markers for sugarcane cultivar R570 and estimated that it covered only one-third of the total map length. The genome of *Saccharum* spp. is 3724 Mbp (1C) and smaller than that of *P. squamulatum* (5000 Mbp, 1C). According to Hoarau=s estimation method, the complete map length could be calculated using the chromosome number and the mean length of the longest linkage groups. The genome of *P. squamulatum*, accession PS26, contains 56 chromosomes. The longest linkage groups in this study were 1 through 6 with an average length of about 200 cM, thus the total estimated map length could be as much as 56 x 200 = 11,200 cM. The present linkage map would have covered only 23% (2600/11,200) of the estimated global map length, or less than one-fouth of the genome. With methods of Hulbert et al. (1988) and Bishop et al. (1983), the estimated genome size of *P. squamulatum* was 4632 cM which resulted in an genome coverage of 56% although the expected coverage was higher (73%). Different methods resulted in quite different estimates. These differences may reflect an uneven distribution of markers in some regions of the genome. Significant size differences exist among different chromosomes in *P. squamulatum* (Chen et al. chapter 4, Fig. 3.2); therefore, genome size may be overestimated by using the average length of the longest linkage groups. Additional single-dose AFLP markers perhaps could be generated with different restriction enzyme sites, such as *Pst*I (Young et al. 1999).

Additional markers would be expected to reduce the percentage of unlinked markers and to converge the number of linkage groups and chromosomes. The ASGR also would be more easily saturated because of its greater probability to contain simplex alleles.

3.3.3 Genetic mapping of aposporous apomixis

In *P. squamulatum*, twelve molecular markers were screened against a segregating population of 397 F1 plants, and no recombination was detected between these markers and the trait for apospory (Ozias-Akins et al. 1998). This genomic region was shown to be conserved between buffelgrass (*C. ciliaris* L.) and *P. squamulatum* (Roche et al. 1999) (Fig.3.2). In our whole-genome genetic linkage map of *P. squamulatum*, four AFLP markers also were shown to cluster with the trait. One major outcome of the whole genome map is that we now have generated AFLP markers that flank the apomixis locus, which would help to define the size of the ASGR. The cluster of apomixis-associated markers appears to be at the distal end of a linkage group, and we have indeed observed that the location of these markers, shown by fluorescence in situ hybridization, is on the subtelomeric region of a single chromosome of *P. squamulatum* (data not shown). The substantial cluster of markers at the ASGR reinforces our previous conclusion that recombination is repressed in this region of the genome and the ratio of physical to genetic distance may be large. It also is possible that the hemizygosity we previously observed with some ASGR-linked sequences may be extensive throughout the region which might contribute to the low recombination rate, but would increase the probability of finding simplex alleles.

The repression of recombination around the genomic region associated with apomixis has been observed not only in *P. squamulatum*, but also in aposporous

Paspalum simplex (Pupilli et al. 2001). Low recombination associated with diplospory also was shown in the maize relative, *Tripsacum dactyloides* (Grimanelli et al. 1998) and in the dicot, *Erigeron annuus* (Noyes and Rieseberg 2000). Based on comparative mapping, the region of synteny between aposporous grasses and model sexual species has not been consistent. For example, five rice markers from the telomeric regon of rice chromosome 12 mapped to the apospory locus in *Paspalum simplex* (Pupilli et al. 2001), but in *Brachiaria*, several maize and rice clones shown to be associated with apomixis mapped to maize chromosome 5 and rice chromosome 2 (Pessino et al. 1998). Given the low recombination in regions of several genomes containing apomixis gene(s) and the complexity of syntenic relationships emerging, fine-scale genetic and physical mapping within apomictic species (Roche et al. 2002) will be necessary to fully elucidate the effect that genome rearrangements may have had on expression of the trait for apomixis.

Acknowledgments

We thank Drs. Kelly Dawe and Gary Kochert, Department of Botany, for providing laboratory space in Athens, and Mark Mackiewitz, Anne Bell and Evelyn Perry for technical assistance. Comprehensive support of our work was provided by the USDANRI Plant Genome Program, award no. 99-35300-7691. Experiments have been conducted in compliance with current US laws.

References

Arcade A, Anselin K, Faivre Rampant P, Lesage MC, Pâques LE, Prat D (2000) Application of AFLP, RAPD and ISSR markers to genetic mapping of European and Japanese larch. Theor Appl Genet 100:299-307

Bai G, Tefera H, Ayele M, Nguyen HT (1999) A genetic linkage map of tef [*Eragrostis tef* (Zucc.) Trotter] based on amplified fragment length polymorphism. Theor Appl Genet 99:599-604

Bennett MD, Leitch IJ (2001) Angiosperm DNA C-values database (release 3.1, Sept. 2001). http://www.rbgkew.org.uk/cval/homepage.html.

Bishop DT, Cannings C, Skonick M (1983) The number of polymorphic DNA clones required to map the human genome. In: Statistical Analysis of DNA Sequence Data, Weir BS, ed. New York: Marcel Dekker

Bradshaw JE, Hackett CA, Meyer RC, Milbourne D, McNicol WJ, Phillips MS, Waugh R (1998) Identification of AFLP and SSR markers associated with quantitative resistance to *Globodera pallida* (Stone) in tetraploid potato (*Solanum tuberosum* subsp. tuberosum) with a view to marker-assisted selection. Theor Appl Genet 97:202-210

Dujardin M, Hanna WW (1984) Microsporogenesis, reprodutivebehavio, and fertility in five *Pennisetum* species. Theor Appl Genet 67:197-201

Dujardin M, Hanna WW (1989) Developing apomictic pearl millet - characterization of a BC_3 plant. J Genet Breed 43:145-151

Grimanelli D, Leblanc O, Espinosa E, Perotti E, DeLeon DG, Savidan Y (1998) Mapping diplosporous apomixis in tetraploid *Tripsacum*: one gene or several genes? Heredity 80:33-39

Grossniklaus U, Nogler GA, van Dijk PJ (2001) How to avoid sex: The genetic control of gametophytic apomixis. Plant cell 13:1491-1497

Hanna WW (1995) Use of apomixis in cultivar development. Adv Agron 54: 333-350

Hoarau JY, Offmann R, D=Hont A, Risterrucci AM, Roques D, Glaszmann JC, Grivet L (2001) Genetic dissection of a moden sugarcane cultivar (*Saccharum* spp.). 1. Genome mapping with AFLP markers. Theor Appl Genet 103:84-97

Hulbert SH, Ilott TW, Legg EJ, Lincoln SE, Lander ES, Michelmore RW (1988) Genetic analysis of the fungus, *Bremia lactucae*, using restriction fragment length polymorphisms. Genetics 120:947-958

Kang BC, Nahm SH, Hul JH, Yoo HS, Yu JW, Lee MH, Kim BD (2001) An interspecific (*Capsicum annuum* x C-chinese) F-2 linkage map in pepper using RFLP and AFLP markers. Theor Appl Genet 102:531-539

Koltunow AM, Bicknell RA, Chaudhury AM (1995) Apomixis: Molecular strategies for the generation of genetically identical seed without fertilization. Plant Physiol 108:1345-1352

Lander ES, Green P, Abrahmson J, Barlow A, Daly MJ, Lincoln SE, Newburg L (1987) MAPMAKER: an interactive computer package for constructing primary genetic linkage maps of experimental and natural populations. Genomics 1:174-181

Liu CJ, Devos K, Witcombe JR, Pittaway TS, Gale MD (1996) The effect of genome and sex on recombination rates in *Pennisetum* species. Theor Appl Genet 93:902-908

Liu CJ, Witcombe JR, Pittaway TS, Nash M, Hash CT, Busso CS, Gale MD (1994) An RFLP-based genetic map of pearl millet (*Pennisetum glaucum*). Theor Appl Genet 89:481-487

Lubbers EL, Arthur L, Hanna WW, Ozias-Akins P (1994) Molecular markers shared by diverse apomictic *Pennisetum* species. Theor Appl Genet 89:636-642

Mudge J, Andersen WR, Kehrer RL, Fairbanks DJ (1996) A RAPD genetic map of *Saccharum officinarum*. Crop Sci 36:1362-1366

Nogler GA (1984) Gametophytic apomixis. In: Johri, B. M. (ed) Embryology of Angiosperms. Springer, Berlin Heidelberg New York, pp 475-518

Noyes RD, Rieseberg LH (2000) Two independent loci control agamospermy (apomixis) in the triploid flowering plant *Erigeron annuus*. Genetics 155:379-390

Ozias-Akins P, Lubbers EL, Hanna WW, McNay JW (1993) Transmission of the apomictic mode of reproduction in *Pennisetum*: Co-inheritance of the trait and molecular markers. Theor Appl Genet 85:632-638

Ozias-Akins P, Roche D, Hanna WW (1998) Tight clustering and hemizygosity of apomixis-linked molecular markers in *Pennisetum squamulatum* implies genetic control of apospory by a divergent locus that may have no allelic form in sexual genotypes. Proc Natl Acad Sci USA 95:5127-5132

Patil BD, Hardas MW, Joshi AB (1961) Auto-alloploid nature of *Pennisetum squamulatum* Fresen. Nature 189:419-420

Pessino SC, Evans C, Ortiz JPA, Armstead I, do Valle CB, Hayward MD (1998) A genetic map of the apospory-region in *Brachiaria* hybrids: identification of two markers closely associated with the trait. Hereditas 128:153-158

Pessino SC, Ortiz JPA, Hayward MD, Quarin CL (1999) The molecular genetics of gametophytic apomixis. Heteriditas 130:1-11

Poncet V, Lamy F, Enjalbert J, Joly H, Sarr A, Robert T (1998) Genetic analysis of the domestication syndrome in pearl millet (*Pennisetum glaucum* L., Poaceae): inheritance of the major characters. Heredity 81:648-658

Poncet V, Lamy F, Devos KM, Gale MD, Sarr A, Robert T (2000) Genetic control of domestication traits in pearl millet (*Pennisetum glaucum* L., Poaceae). Theor Appl Genet 100:147-159

Pupilli F, Labombarda P, Caceres ME, Quarin CL, Arcioni S (2001) The chromosome segment related to apomixis in *Paspalum simplex* is homoeologous to the telomeric region of the long arm of rice chromosome 12. Mol Breed 8:53-61

Raman VS, Chandrasekharan P, Krishnaswami D (1959) A note on some chromosome numbers in Gramineae. Curr Sci 29:127-128

Riera-Lizarazu O, Vales MI, Phillips RL. (2001) A compilation of molecular genetic maps of cultivated plants. In Phillips RL, Vasil IK (ed) DNA-based Markers in Plants (2nd ed.). Kluwer Academic Publishers, Dordrecht, pp 463-497

Roche D, Cong PS, Chen ZB, Hanna WW, Gustine D, Sherwood RR, Ozias-Akins P (1999) An apospory-specific genomic region is conserved between buffelgrass (*Cenchrus ciliaris* L.) and *Pennisetum squamulatum* Fresen. Plant J. 19:203-208

Roche D, Conner JA, Budiman MA, Frisch D, Wing R, Hanna WW, Ozias-Akins P (2002) Construction of BAC libraries from two apomictic grasses to study the microcolinearity of their apospory-specific genomic regions. Theor Appl Genet (in press)

Sherman JD, Stack SM (1995) 2-Dimensional spreads of synaptionemal complexes from solanaceous plants. 6. high-resolution recombination nodule map for tomato (*Lycopersicon esculentum*). Genetics 141:683-708

Sindhe ANR (1976) Supernumerary chromosomes in *Pennisetum squamulatum* Fresen. Curr Sci 45:526

Savidan Y (2000) Apomixis: Genetics and Breeding. Plant Breed Rev 18:13-86

Tanksley SD, Ganal MW, Prince JP, Vicente MC de, Bonierbale MW, Broun P, Fulton TM, Giovannoni JJ, Grandillo S, Martin GB (1992) High density molecular linkage maps of the tomato and potato genomes. Genetics 132:1141-1160

Ukoskit K, Thompson PG (1997) Autopolyploidy versus alloploidy and low-density randomly amplified polymorphic DNA linkage maps of sweet-potato. J Amer Soc Hort Sci 122:822-828

Vaneck HJ, Vandervoort JR, Draaistra J, Vanzandvoort P, Vanenckevort E, Segers B, Peleman J, Jacobsen E, Helder J, Bakker J (1995) The inheritance and chromosomal localization of AFLP markers in a noninbred potato offspring. Mol Breed 1:397-410

Vos P, Hogers R, Bleeker M, Reijans M, Van der Lee T, Hornes M, Frijters A, Pot J, Peleman J, Kuiper M, Zabeau M (1995) AFLP: a new concept for DNA fingerprinting. Nucl Acids Res 23:4407-4414

Vuylsteke M, Mank R, Antonise R, Bastiaans E, Senior ML, Stuber CW, Melchinger AE, Lübberstebt T, Xia XC, Stam P, Zabeau M (1999) Two high-density AFLP linkage maps of *Zea mays* L.: analysis of distribution of AFLP markers. Theor Appl Genet 99:921-935

Wu KK, Burnquist W, Sorrells ME, Tew TL, Moore PH, Tanksley SD (1992) The detection and estimation of linkage in polyploids using single-dose restriction fragments. Theor Appl Genet 83:294-300

Young WP, Schupp JM and Keim P (1999) DNA methylation and AFLP marker distribution in the soybean genome. Theor Appl Genet 99:785-790

Table 3.1. List of primer combinations and summary of data generated with each combination.

ID[1]	Primer Combination	Ampli Bands[2]	Shared Bands[3]	M-D Bands[4]	S-D Bands[5]	L-Band[6]	% of L/A[7]
pa	AAC/CTA	91	6	69	16	13	14.29
pb	ACA/CAT	71	12	52	7	7	9.85
pc	ACC/CAA	68	4	55	9	8	11.76
pd	ACG/CAG	93	20	63	10	9	9.68
pe	AGG/CAT	85	22	52	11	8	9.41
pf	ACT/CAT	85	12	64	9	7	8.24
pg	ACC/CAT	65	3	58	4	2	3.08
ph	AGG/CAG	88	2	81	5	1	1.14
pi	AGG/CTA	91	4	79	8	6	6.59
pj	AGG/CTC	79	6	62	11	7	8.86
pl	ACG/CAT	36	2	31	3	3	8.33
pm	AAC/CAG	55	7	44	4	3	5.36
pn	ACG/CTC	54	7	43	4	3	5.56
po	ACA/CTC	78	11	61	6	4	5.13
pp	ACT/CAA	74	7	66	1	1	1.35
pq	ACA/CAA	95	13	66	16	15	15.79
pr	AGG/CAA	88	2	78	8	6	6.82
ps	ACT/CAG	74	1	65	8	7	9.46
pt	ACT/CTC	60	3	47	10	7	11.67
pu	ACT/CTA	116	8	78	30	24	20.79
pv	ACA/CAG	86	11	64	11	8	9.3
pw	ACG/CTA	88	8	65	15	14	15.91
px	ACG/CAA	62	6	45	11	9	14.52
py	AGC/CTA	90	1	85	4	4	4.44
pz	AGC/CAG	89	3	78	8	5	5.62
pA	AGC/CAA	89	6	82	1	1	1.12
Total	26	2050	187	1633	230	182	
Average		78.8	7.2(9.13)[8]	62.8(79.7)	8.8(11.2)	7	8.88

[1] Primer code;
[2] Total amplified and recordable bands with each primer combination;
[3] Bands shared by both *P. squamulatum* and *P. glaucum*;
[4] Multiple-dose bands segregating at a ratio greater than 1:1 statistically;
[5] Single-dose bands segregating at a ratio of 1:1 statistically;
[6] Bands included in the linkage map;
[7] The percentage of mapped bands over the total number of amplified bands;
[8] The number in parentheses refers to the percentage out of the total number of amplified bands.

Fig. 3.1. AFLP map of *P. squamulatum* generated with a F_1 population of 94 individuals from a cross of pearl millet (*P. glaucum* x *P. squamulatum*). This linkage map contains 182 single-dose AFLP markers amplified from 26 primer combinations. AFLP markers were grouped at the threshold of LOD 3 and cM $\leq$ 37. The numbers on the left side of the map are the distances between markers. Marker names are on the right side of the linkage map. The number (1-44) located on top of each linkage group was assigned in descending order according the linkage map length in cM. The total length of each linkage group is given under each group. Markers linked in repulsion are connected with gray lines and genetic distance is given along the line. Markers that remained linked at LOD 5 are connected by a thick line. The linkage groups inside the same box are supported by the data as hom(e)ologous.

Fig. 3.2. Summary of genetic map information for aposporous apomixis in *Pennisetum/Cenchrus* (Ozias-Akins et al. 1998; Roche et al. 1999; Gustine et al.1997). Apo refers to the linkage group transmitting aposporous apomixis. A large block of markers co-segregating with apomixis has been identified in both species.

Fig. 3.1

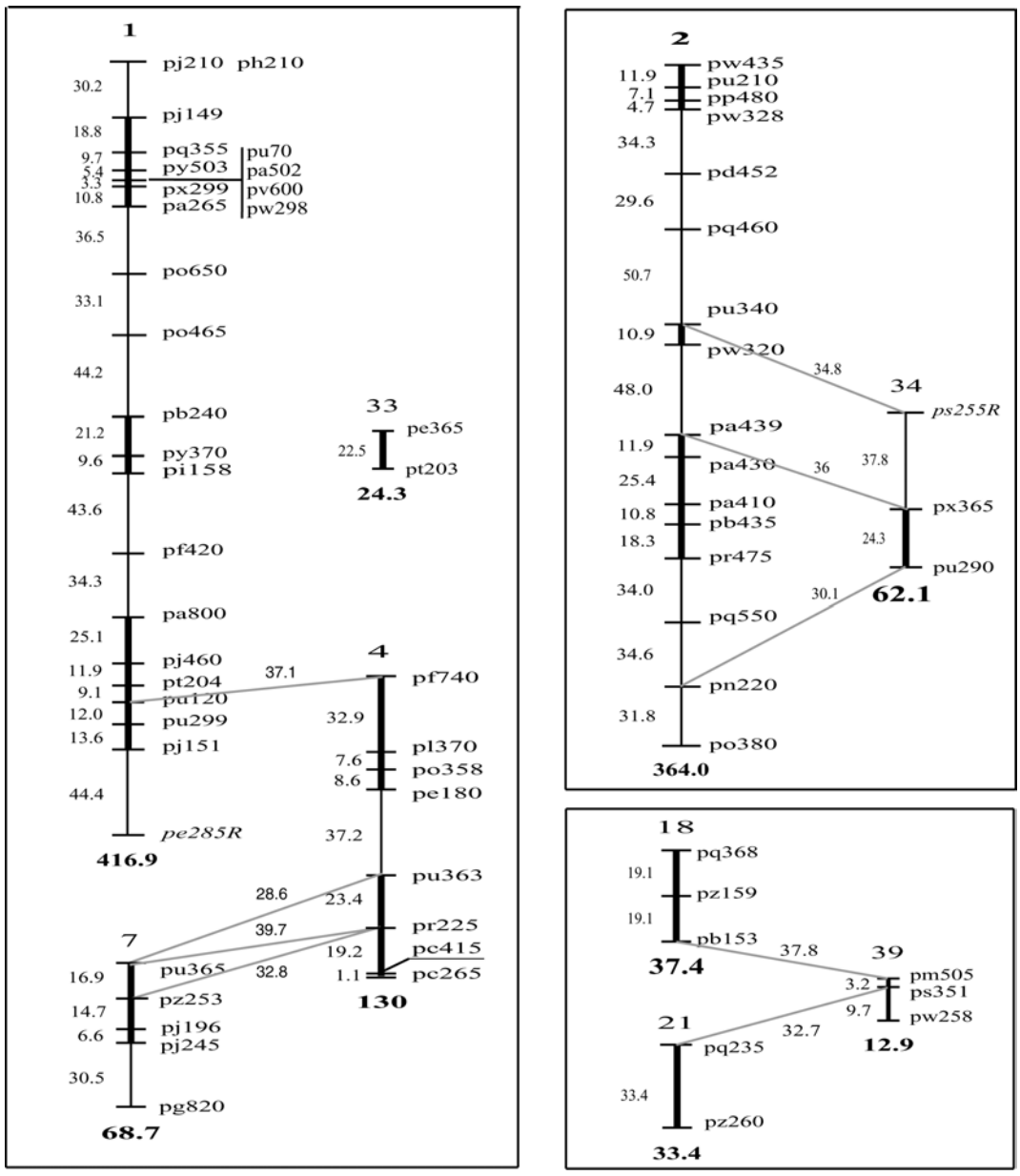

1
pj210 ph210
30.2
pj149
18.8
pq355 pu70
9.7
py503 pa502
5.4
px299 pv600
3.3
pa265 pw298
10.8
36.5
po650
33.1
po465
44.2
pb240
21.2
py370
9.6
pi158
43.6
pf420
34.3
pa800
25.1
pj460
11.9
pt204
9.1
pu120
12.0
pu299
13.6
pj151
44.4
pe285R
416.9
33
pe365
22.5
pt203
24.3
4
37.1
pf740
32.9
pl370
7.6
po358
8.6
pe180
37.2
pu363
28.6
23.4
39.7
pr225
19.2
pc415
32.8
1.1
pc265
130
7
16.9
pu365
14.7
pz253
6.6
pj196
pj245
30.5
pg820
68.7
2
pw435
11.9
pu210
7.1
pp480
4.7
pw328
34.3
pd452
29.6
pq460
50.7
pu340
10.9
pw320
34.8
34
ps255R
48.0
pa439
11.9
pa430
36
37.8
25.4
pa410
10.8
pb435
18.3
pr475
24.3
px365
pu290
34.0
30.1
62.1
pq550
34.6
pn220
31.8
po380
364.0
18
pq368
19.1
pz159
19.1
pb153
37.4
37.8
39
pm505
3.2
ps351
9.7
pw258
12.9
21
pq235
32.7
33.4
pz260
33.4

Fig. 3.1 continue

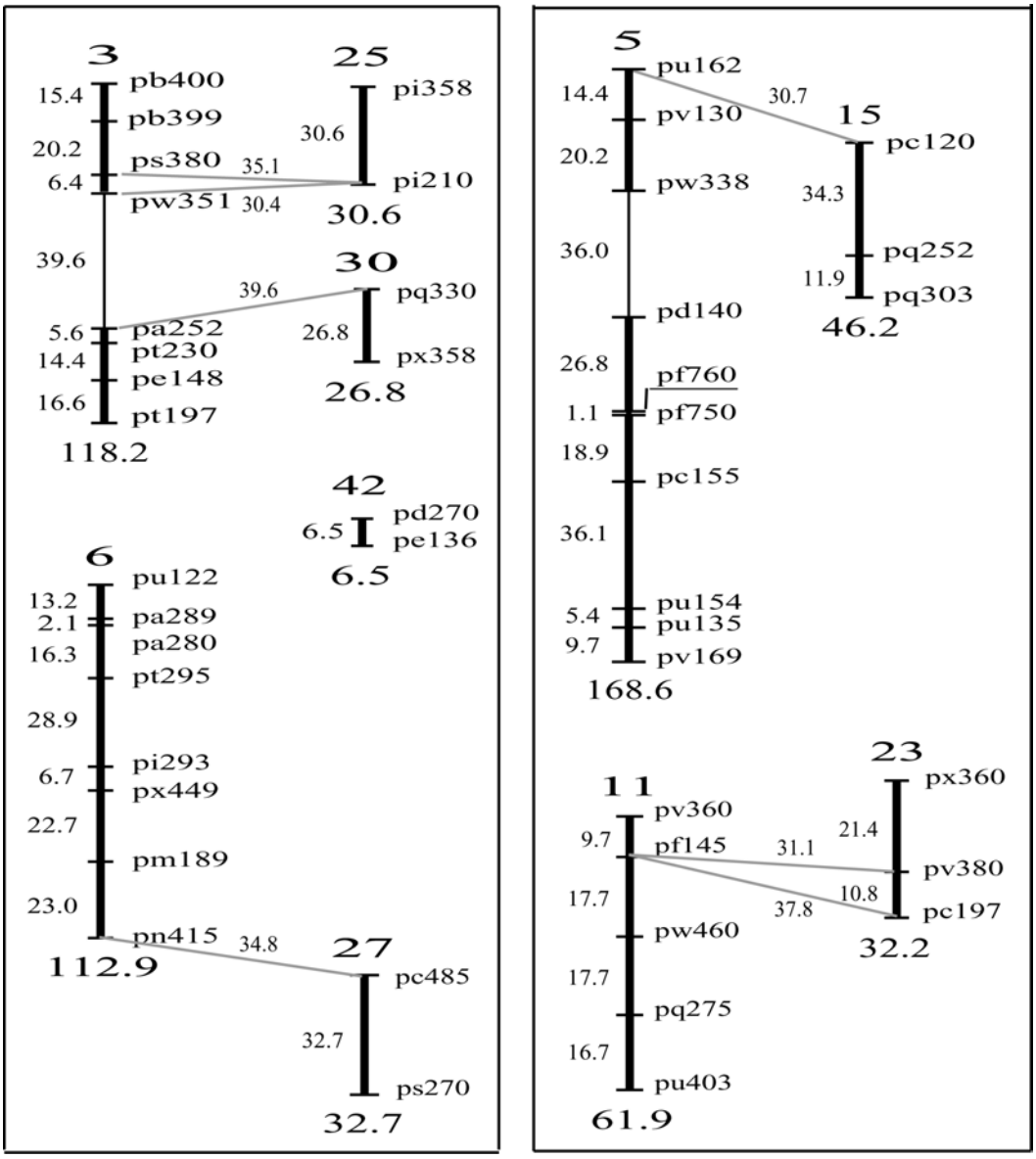


Fig. 3.1 continue

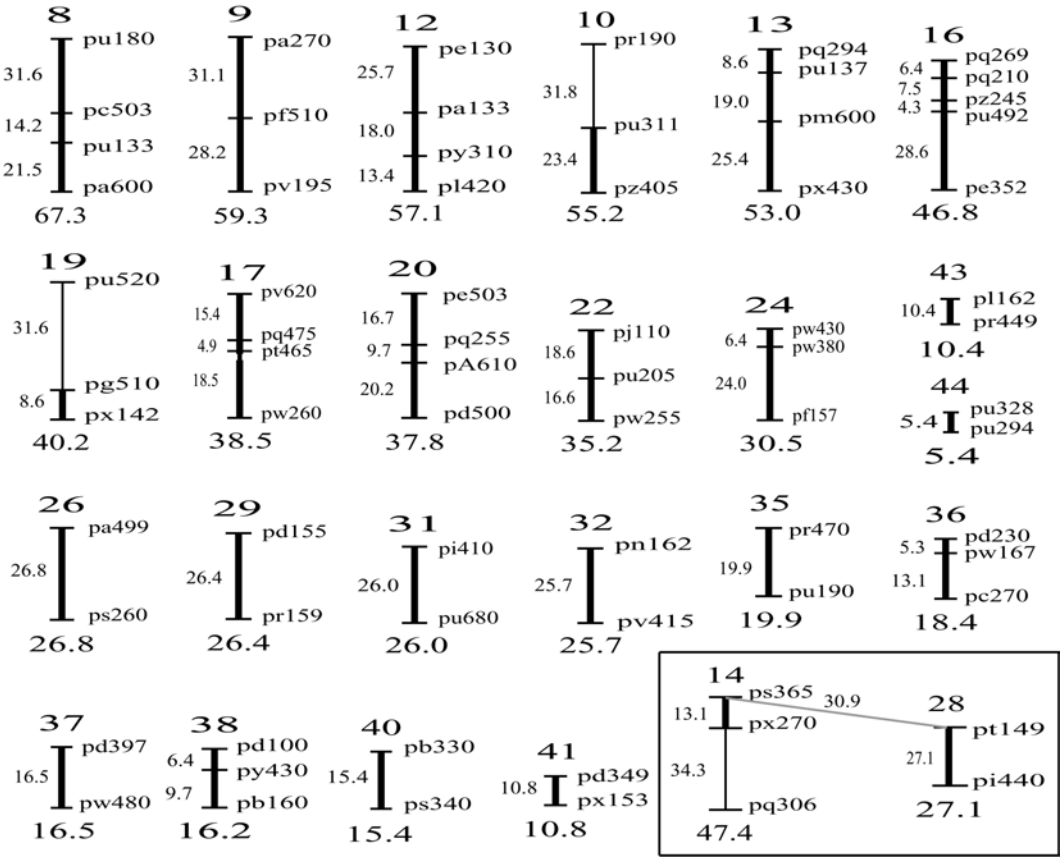
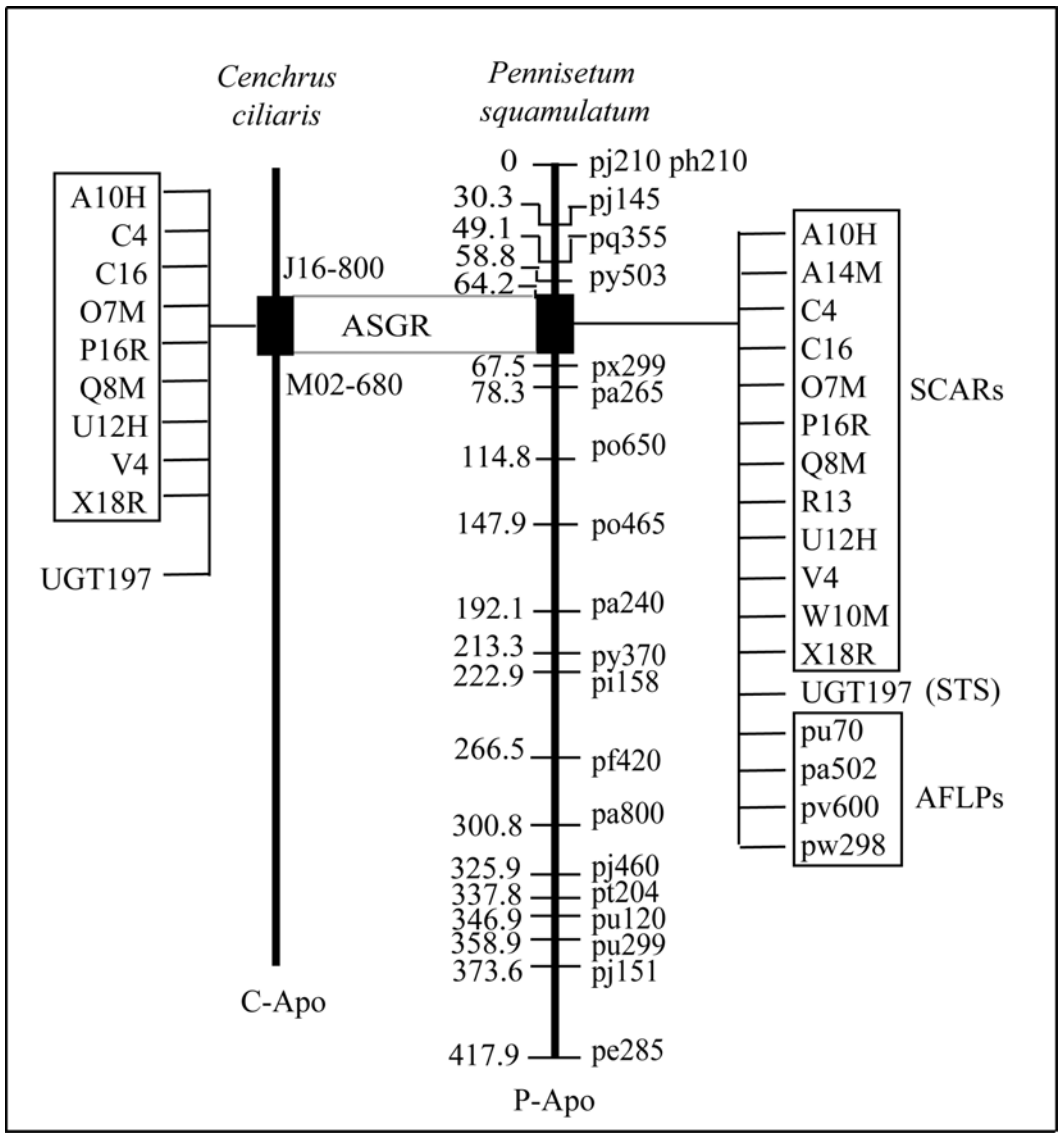


Fig. 3.2


Cenchrus ciliaris
Pennisetum squamulatum
A10H
C4
C16
O7M
P16R
Q8M
U12H
V4
X18R
UGT197
J16-800
ASGR
M02-680
C-Apo
0 pj210 ph210
30.3 pj145
49.1 pq355
58.8 py503
64.2
67.5 px299
78.3 pa265
114.8 po650
147.9 po465
192.1 pa240
213.3 py370
222.9 pi158
266.5 pf420
300.8 pa800
325.9 pj460
337.8 pt204
346.9 pu120
358.9 pu299
373.6 pj151
417.9 pe285
P-Apo
A10H
A14M
C4
C16
O7M
P16R
Q8M
R13
U12H
V4
W10M
X18R
SCARs
UGT197 (STS)
pu70
pa502
pv600
pw298
AFLPs

CHAPTER 4

TRANSMISSION OF A SINGLE CHROMOSOME CARRYING THE APOSPORY SPECIFIC GENOMIC REGION (ASGR) FROM *PENNISETUM SQUAMULATUM* IS REQUIRED FOR EXPRESSION OF THE APOMICTIC PHENOTYPE IN BACKCROSS HYBRIDS

Z. B. Chen, Y. Akiyama, W. W. Hanna and P. Ozias-Akins. To be sumitted to Genetics

ABSTRACT

Apomixis is a system of asexual reproduction through which plants produce embryos without meiosis and fertilization. Progenies of apomictic plants are genetically identical to their maternal parents even when their genetic backgrounds are highly heterozygous. *Pennisetum squamulatum*, a distant relative of pearl millet (*P. glaucum*), produces seeds by obligate apospory. This tertiary gene pool species was used as the donor of apomixis in the backcross breeding program for transferring apomixis into pearl millet (*P. glaucum*). Here we document chromosome transmission from *P. squamulatum* in a series of backcrosses (BC) with *P. glaucum* by a combination of fluorescence *in situ* hybridization (FISH) and genomic *in situ* hybridization (GISH). By probing root-tip metaphase spreads with a mixture of 12 apospory-linked molecular markers, we found a single chromosome carrying the apospory-specific genomic region (ASGR) in *P. squamulatum*. The ASGR was located to the distal region of the long arm of this chromosome. With *P. squamulatum* genomic DNA as a probe, one to three *P. squamulatum* chromosomes were detected in BC6, but only one of the three hybridized with the ASGR-linked markers. The three chromosomes behaved as univalents during meiosis and segregated randomly in BC_3 and later BC generations. Comparison of the chromosome number and reproductive mode in different backcross generations led to the conclusion that the ASGR-carrier chromosome alone was sufficient for apospory.

Key words: Apomixis, Hemizygous, *Pennisetum*, FISH, GISH

Introduction

Gametophytic apomixis is a system of asexual reproduction that occurs in a number of flowering plant families. Apomictic plants produce unreduced embryo sacs from megaspore mother cells through mitosis or a modified meiosis (in the case of diplospory) or from nucellar cells through mitosis (in the case of apospory). Unreduced egg cells within these embryo sacs develop into embryos without fertilization by pollen (Nogler 1984). Progenies from apomictic plants inherit all genetic information from their maternal parent and fixed gene combinations are passed on from generation to generation. Apomictically derived progeny are genetically identical, morphologically uniform, and stable between generations. These features are the same objectives that plant breeders can attain only by selecting the progeny over many years or generations. Employment of apomixis in plant breeding would revolutionize traditional breeding programs as well as hybrid seed production systems (Hanna and Bashaw 1987).

Unfortunately, no cultivated crop displays a sufficient degree of apomixis for practical application, and only a few crop plants have wild relatives that are near-obligate apomicts. *Pennisetum squamulatum* Fresen is one such species that is related to the domesticated plant, pearl millet, [*P. glaucum* (L.) R.Br.]. Pearl millet is grown for its grain primarily in Africa and India and as a forage crop in tropical and subtropical regions including the southern US. The transfer of apomixis from *P. squamulatum* to *P. glaucum* has been pursued for the last two decades, and the crossing scheme that led to the recovery of a backcross 3 (BC_3) individual showing obligate apomixis has been described (Dujardin and Hanna 1989). Subsequently, additional backcross generations (BC_4-BC_7) were produced. The transfer of apomixis from *P. squamulatum* to pearl millet

has been hindered by linkage drag of undesired characteristics, among them low seed set and high pollen sterility. Genetic and cytogenetic analyses of these apomictic backcross lines have been carried out to study their chromosome behavior and the inheritance of apomixis (Dujardin and Hanna 1989; Hanna et al. 1993). All of these backcross lines have 27 to 29 chromosomes, but traditional cytogenetic investigation has not been able to distinguish the alien chromosomes from the pearl millet chromosomes.

Since introgression of apomixis into pearl millet from a closely related species has been problematic, an alternative approach for transferring this trait to crop plants might be to introduce well characterized genes by transformation. Such genes controlling apomictic reproduction have yet to be cloned, although recent molecular genetic analysis has shed light on the inheritance of the trait from *P. squamulatum* (Ozias-Akins et al. 1998), *Cenchrus ciliaris* (Roche et al. 1999), *Paspalum simplex* (Pupilli et al. 2001), *Tripsacum dactyloides* (Grimanelli et al. 1998), *Brachiaria brizantha* (Pessino et al. 1998) and *Erigeron annuus* (Noyes and Rieseberg 2000). In *P. squamulatum*, bulked segregant analysis of phenotypic classes (apomictic and sexual) from a mapping population (*P. glaucum* x *P. squamulatum*) led to the isolation of 12 molecular markers that were screened in the total population of 397 individuals. No recombination between apomixis and any marker could be detected. Furthermore, several markers showed a hemizygous segregation pattern when hybridized to genomic DNAs (Ozias-Akins et al. 1998). Based on these observations (no recombination and sequence divergence), an apospory-specific genomic region (ASGR) was defined. Although transmission of the ASGR from *P. squamulatum* to F_1 hybrids appeared to be sufficient for the expression of apomixis, the marker content of a BC_4 population had previously determined that at least

two independent linkage groups from *P. squamulatum* were transmitted from BC_3 to BC_4 (Ozias-Akins et al. 1993). A whole genome map was not constructed in either of these studies; therefore, the extent of recombination around the apomixis locus could not be estimated. This question recently has been addressed with an AFLP map (Chen et al. Chapter 3).

Molecular marker data strongly supported the transmission of apomixis by a single linkage block (Ozias-Akins et al. 1993, 1998), although the size of the linkage block has not been determined. Since it has been difficult to reconcile total chromosome numbers in apomictic backcrosses with numbers of alien chromosomes, we sought to combine molecular markers with cytogenetic studies in order to visually identify the *P. squamulatum* chromosomes and specifically the chromosome transmitting the apomixis locus. Molecular cytogenetics exploits fluorescence *in situ* hybridization (FISH) to probe chromosome spreads with fluorescently tagged DNA molecules (Langer-Safer et al. 1982). FISH was first introduced to plant research by Rayburn and Gill (1985). When a 120 bp repeated sequence of rye was used as a probe onto hexploid wheat (Chinese Spring), the B genome differentially hybridized with this sequence and chromosomes 1A, 2D, 3D, and 5D also showed major hybridization signals in telomeric regions (Rayburn and Gill 1985). Instead of a single repetitive sequence, the hybridization procedure can be modified to include labeled total genomic DNA as the probe (referred to as genomic *in situ* hybridization or GISH), which results in discrimination between different genomes, alien chromosomes, or chromosome fragments that may be present in an interspecific hybrid or its backcrosses (Schwarzacher et al.1989; Heslop-Harrison et al. 1990; Mukai and Gill 1991; Vega et al. 1994; Chen et al. 2001).

We report here the results of molecular cytogenetic analyses of F_1 hybrids and backcrosses of *P. squamulatum* x *P. glaucum* and related hybrids. By using a combination of FISH and GISH in conjunction with 4', 6-diamidino-2-phenylindole dihydrochloride (DAPI) staining of whole chromosomes, we (i) studied the transmission of *P. squamulatum* chromosomes to different backcross generations and (ii) determined that a single chromosome was sufficient for the transmission of apomixis and molecular markers linked to the trait.

4.1 Materials and Methods

4.1.1 Genetic stocks

The source of apomixis was *P. squamulatum* (PS26; PI 319196; $2n = 56$), which was used as the male parent in all crosses. The recurrent maternal parent was an induced tetraploid pearl millet (*P. glaucum*, $2n = 4x = 28$). Backcross generations one to seven were generated and screened according to the methods outlined in Dujardin and Hanna (1989). Since a double cross hybrid of (*P. glaucum* x *P. purpureum*) x (*P. glaucum* x *P. squamulatum*) was used early in the crossing scheme to increase male fertility in the backcrosses (Dujardin and Hanna 1985, 1987; Hanna et al. 1993), the apomictic double cross also was included in the present study. All progenies were screened for embryo sac development by microscopically examining ovules cleared in methyl salicylate (Young et al. 1979) and for apomictic or sexual reproduction by scoring the uniformity of progeny in the field or greenhouse. Progenies from apomictic and sexual lines used in the present study (Fig. 4.1; Table 4.1) were grown in the field in the summers of 1999 and 2000 (for collecting inflorescences containing various stages of meiosis) and in the greenhouse in the winter of 2000 (for collecting root-tips).

4.1.2 Chromosome preparation:

4.1.2.1 Meiotic chromosome spreads.

Inflorescences protruding about 1/3 of their length from the boot were collected from *P. squamulatum, P. glaucum*, and BC_3, BC_4, BC_5, BC_6 and BC_7 in the field in the fall of 1999 and 2000. After checking the stage of meiosis in each inflorescence by squashing anthers in acetocarmine, inflorescence sections containing meiotic cells at metaphase I were fixed in ethanol : acetic acid (3:1) and stored at 4^{o}C. Up to one to two months after fixation, florets were removed from the fixative and soaked in 30 mM citrate buffer (pH 4.5) for 5 to 10 min. Dissected anthers were cut at the apex and squeezed with a surgical knife to push the pollen mother cells (PMCs) out into a 10 x 35 mm petri dish containing 1 ml of 30 mM citrate buffer (pH 4.5). PMCs were pipetted into a 1.5 ml microcentrifuge tube where digestion was carried out in 50 µl enzyme mixture (0.3% cellulase RS, 0.3% pectolyase Y23 and 0.3% cytohelicase in 30 mM citrate buffer, pH 4.5) (Zhong et al.1996) at 37^{o}C for 30-45 min. Digestion time was dependent on the length of time materials had been stored in fixative: longer fixation times required shorter digestion times. Digested PMCs were collected by centrifugation at 600 x g for 5 min at room temperature. The supernatant was removed with a pipetter, and PMCs were resuspended in a volume of 60% acetic acid equal to 3 times the volume of the digestion solution and incubated for 10 min on ice. PMCs then were collected by centrifugation as described above and resuspended in 4 µl 60 % acetic acid for each slide. Usually one slide was made from each anther by applying the 4 µl of PMC suspension to a precleaned slide and covering it immediately with a 22 x 22 mm coverglass. The slide was dipped into liquid nitrogen for approximately 30 sec and the coverglass was subsequently removed using a

razor blade. The slide was dehydrated for 3 min each in a 70%, 90% and 100% ethanol series and air-dried. After dehydration, slides were examined under a microscope to select those with the most metaphase I spreads and the least background. The selected slides could be stored at -20 C for later use.

4.1.2.2 Mitotic chromosome spreads.

Seeds were germinated in trays containing autoclaved sand and then transplanted individually into 5-cm clay pots. After 2-3 weeks in pots, the secondary roots appeared around the inner surface of the pot. Healthy roots were excised, washed in water, and transferred to a saturated mono-bromonaphthalene solution (Sigma Chemical Co., St Louis, MO, USA) for a 2 hr pretreatment on ice. Pre-treated root tips were fixed in ethanol : acetic acid (3:1) for at least 4 d at room temperature. Shorter fixation times resulted in higher background after hybridization. The fixed root tips were dissected under a stereomicroscope to collect the apical meristem, and the meristem was digested in an enzyme mixture (0.3% cellulase RS, 0.3% pectolyase Y23 and 0.3% cytohelicase in 30 mM citrate buffer, pH 4.5) at 37^{o}C for 80-90 min. The digested tissue was transferred to a clean slide. After removing as much digestion solution as possible, 2.5 µl of 60% acetic acid was immediately added to the digested root-tip. Any undigested clumps of tissue were removed from the slide. Chromosomes were spread by covering the cell suspension with a 22 x 22 mm coverglass. Subsequent processing was as described above for meiotic chromosome preparations.

Before addition of probe DNAs, both mitotic and meiotic chromosome preparations were pretreated with 100 µl of 100 µg/ml RNase A (Sigma) in 2x SSC for 60 min at 37^{o}C and 100 µl of 5 µg/ml pepsin (Sigma) in 0.01 M HCl for 7 to 9 min at

37°C (Zhong et al. 1996). Slides were rinsed with deionized water and fixed in 1% formaldehyde solution (Fisher Scientific, Fair Lawn, USA) for 10 min. After fixation, the slides were rinsed once with 1x PBS buffer and twice in 2x SSC for 5 min each. The chromosomes were denatured in 70% formamide for 2 min at 70°C and dehydrated in a 70%, 90 % and 100% ethanol series.

4.1.3 Probe preparation:

Genomic DNAs were isolated using the method described in Ozias-Akins et al. (1993) and purified with phenol : chloroform : isoamyl alcohol (25:24:1) extraction. Plasmid DNAs containing 12 molecular markers mapped to the ASGR (Ozias-Akins et al. 1998) were isolated by alkaline lysis minipreps (Sambrook et al.1989). Equal amounts (by weight) of DNA from each marker were mixed together and labeled as an ASGR-specific probe. A total of 1.5 μg of the combined DNAs was labeled by nick translation following the manufacturer's instructions (Roche Diagnostics Corp., Indianapolis, IN, USA), but dTTP was replaced with either digoxigenin-11-dUTP (Roche) or biotin-11-dUTP (Roche). Labeled DNA was ethanol precipitated to remove unincorporated label, and resuspended in 20 μl of 100% formamide (Sigma).

4.1.4 *In situ* hybridization, post-wash and fluorochrome detection:

The hybridization mixture was composed of 50% formamide, 10% dextran sulphate, 2xSSC, 0.01% salmon sperm DNA, 100 ng/μl sheared *P. glaucum* DNA (as blocking DNA), 1.5 ng/μl labeled genomic DNA and 1.5 ng/μl labeled ASGR-specific marker mixture. The hybridization mixture was denatured in boiling water for 10 min and cooled on ice for 5 min. After cooling, 10 to 20 μl of the mixture was applied to each slide. The slide was covered with a 24 x 30 mm coverglass and sealed with rubber

cement. After denaturing the chromosomes by heating the slides for 3 min at 80°C on a heatblock, hybridization was carried out at 39°C to 42°C overnight in a humid chamber. After hybridization, the coverslips were removed and the slides were washed one time in 2x SSC at 37°C for 5 min, 15 min in 50% formamide in 2x SSC at 42°C, 15 min in 1x SSC at 37°C, and 5 min in 4x SSC at 37°C.

To detect the labeled DNAs, 60 µl of FITC-labeled anti-dig antibody (10 µg/ml) (Roche) and 5 µg/ml Avidin-Texas Red (Vector Laboratories, Burlingame, CA, USA) in 4x SSC with 3% BSA (bovine serum albumin) were applied to each slide. The slides were incubated at 37°C for 60 min, and then washed one time in 4x SSC for 10 min, three times in 4x SSC plus 0.1% Triton-X100 (Sigma) for 10 min each, and 10 min in 4x SSC. Slides then were rinsed in 2x SSC for 5 min and dehydrated in 70%, 90% and 100% ethanol. After air-drying the slides, coverglasses were mounted with 3-5 µl of Vectashield mounting medium containing DAPI (1.5 µg/ml) (Vector Laboratories).

4.1.5 Image acquisition and processing:

At least two slides and five spreads for each genotype were viewed under an Olympus fluorescence microscope (BX50). Fluorescent signals were detected for DAPI (λ_{ex} = 360, λ_{em} = 420), FITC (λ_{ex} = 480, λ_{em} = 515) and Texas Red (λ_{ex} = 560, λ_{em} = 645), and monochrome digital images were captured with a charge-coupled device (CCD) camera (SenSys, Photometrics, Tucson, AZ, USA). Images were pseudocolored with blue for DAPI, green for FITC and red for Texas Red. Images were compiled with Image Pro Plus, version 4 for Win 95/98 (Media Cybernetics, Silver Spring, MD, USA).

DAPI images of five chromosome spreads from different F_1 apomictic plants were selected to investigate the main characteristics of chromosomes from both *P.*

glaucum and *P. squamulatum*. Chromosome lengths were obtained with the image analysis program, CHIAS III (Kato and Fukui 1998).

4.2 Results

4.2.1 Characteristics of chromosomes of *P. glaucum*.

There were 7 pairs of chromosomes from *P. glaucum* in the F_1 hybrid with *P. squamulatum* (Fig. 4.2, top row). Chromosome lengths ranged from 9.44 to 5.48 μm (Table 4.2). Only three chromosome pairs easily could be distinguished from others using morphological features. Chromosome 1 is the largest among the 7 pairs of chromosomes and typical of metacentric chromosomes. Chromosome 7 is the shortest chromosome with a long arm to short arm ratio greater than two, which is typical of a subtelocentric chromosome. The other distinguishing feature of chromosome 7 is a satellite on the short arm, which often is visible on only two of the four homologs in tetraploid millet (Fig. 4.3e and 4.3k). Chromosome 6 is also one of the shortest chromosomes but without the satellite. Chromosome pairs 2, 3, 4 and 5 do not have any distinguishing features since all are submetacentric and similar in size.

4.2.2 Characteristics of chromosomes of *P. squamulatum*.

We observed 56 chromosomes in *P. squamulatum*, accession PS26 (Fig. 4.3a). In the F_1 plants of *P. glaucum* x *P. squamulatum* that we examined, there were 28 *P. squamulatum* chromosomes. We used F_1 plants to investigate the chromosome characteristics of *P. squamulatum* since each F_1 contained half of the chromosomes of *P. squamulatum***,** and chromosomes from both species could be directly compared in F_1 hybrids. Fig. 4.2 (2^{nd} and 3^{rd} rows) shows the characteristics of chromosomes from *P. squamulatum*. The lengths of these chromosomes ranged from 9.45 to 3.89 μm and the length changes

formed almost a continuous gradient from the largest to the smallest chromosome (Table 4.2). Most of the chromosomes were metacentric or submetacentric (Fig. 4.2). It was not possible to identify the potential homo(eo)logous chromosomes based on morphology. The calculated average DNA density (Mbp/μm) was not significantly different between the genomes of the two species (Table 4.2) indicating that the degree of condensation was similar.

4.2.3 Transfer of *P. squamulatum* chromosomes into *P. glaucum* through backcrossing. Seven different generations (including *P. squamulatum*, F_1, BC_1, BC_3, BC_5, BC_6 and BC_7), 20 different lines, and 2 to 22 plants from each line were sampled for chromosome preparations (Table 4.1). Both metaphase I of meiosis and mitotic metaphase plates were investigated with the combination of FISH and/or GISH. Genomic *in situ* hybridization with biotinylated genomic DNA of *P. squamulatum* confirmed that chromosomes from *P. squamulatum* were introduced into *P. glaucum* through crossing of the two distantly related species. Given that the accession of *P. squamulatum* used for the F_1 cross had 56 chromosomes (see above), the number of chromosomes that hybridized with labeled genomic DNA was the expected number of 28 in two different apomictic F_1 individuals (290-124 and 290-181). A total of 42 chromosomes, including 28 *P. squamulatum* chromosomes and 14 *P. glaucum* chromosomes, was observed in both apomictic and sexual F1 individuals (Fig. 4.3b and 4.3c, respectively). This number of *P. squamulatum* chromosomes was progressively reduced as backcrossing advanced with the recurrent parent, *P. glaucum*.

In BC_1, differential labeling of genomic DNAs from *P. squamulatum* and *P. purpureum* (a bridging species used as a parent prior to the backcrossing step in the

introgression program) resulted in hybridization of both probes to the same 14 chromosomes as could be observed by the yellow signal from the combined red and green fluorescence (Fig. 4.3d). This result indicates that there is strong sequence similarity among repeats in the two genomes. Also in BC_1, the number of chromosomes that hybridized with pearl millet DNA increased to 21 (Fig. 4.3d). At the BC_3 generation, three chromosomes and one segment of a fourth chromosome hybridized with *P. squamulatum* genomic DNA (Fig. 4.3e and 4.3l). Genomic DNA of *P. purpureum* also hybridized to the three alien chromosomes when it was used as one of the probes (Fig. 4.3k). Beyond the BC_3 generation, plants classified as apomictic (i.e., producing aposporous embryo sacs, including both obligate and facultative outcomes), contained one to three chromosomes that hybridized with *P. squamulatum* DNA, and the number of *P. glaucum* chromosomes ranged from 26 to 28 (Fig. 4.3f – 4.3j). In these materials, the total chromosome number was 28 or 29.

GISH of meiotic metaphase I indicated that the three chromosomes from *P. squamulatum* in BC_3 and later backcross generations did not pair and thus are presumed not to be homologous to each other. Neither were they homologous to any chromosome of *P. glaucum* because they were exclusively observed as univalents that lagged at anaphase I (Fig. 4.3l and 4.3m) or assorted to either pole randomly (Fig. 4.3n and 4.3o).

4.2.4 A single chromosome transmits the ASGR to backcross progeny.

Twelve ASGR-linked molecular markers were pooled, labeled with digoxigenin, and probed simultaneously with labeled *P. squamulatum* DNA onto the materials as shown in Table 4.1. Six of the markers were known to be low-copy-number DNAs and the others were repetitive sequences (Ozias-Akins et al. 1998), but the distribution of the repeats

across the genome had not been determined. The FISH signal from these pooled markers was consistently observed at the end of a single *P. squamulatum* chromosome (Fig. 4.3a and 4.3b; 4.3e – 4.3h). It is likely that the signal was derived mainly from the repetitive members of the pool since a probe pool that eliminated the single copy markers gave the same result (data not shown). In all advanced backcross generations, the pooled-marker hybridization signal was found at the end of the long arm on one of the three transferred *P. squamulatum* chromosomes. In the F_1 metaphase spread shown in Fig. 4.2, this chromosome was metacentric and similar in morphology and size to some of the pearl millet chromosomes. Plants with the terminal ASGR signal were later classified as either obligate or facultative apomicts. Plants with only meiotically derived embryo sacs did not show the terminal ASGR signal on any chromosome, although other *P. squamulatum* chromosomes were sometimes present as shown by GISH.

4.2.5 The ASGR has no homologous region in *P. squamulatum*.

The result of FISH with the ASGR-linked marker mixture as probe against *P. squamulatum* and the apomictic F1s showed that a single chromosome carried the terminal ASGR signal. No homologous chromosome could be detected with these probes and hybridization conditions (Fig. 4.3a and 4.3b). Thus, among the 56 chromosomes in *P. squamulatum* (PS26), only one chromosome showed the hybridization signal, and this chromosome was transmitted to only apomictic F1 plants. No signal from the ASGR-linked markers was observed in any sexual F1 plant (Fig. 4.3c).

4.3 Discussion

4.3.1 Comparison of chromosomes between *P. glaucum* and *P. squamulatum*. Pearl millet (*P. glaucum*) is one of the two species in Section *Penicillaria* with a base

chromosome number of $x = 7$ (Stapf and Hubbard 1934; Jauhar 1981). The karyological data of Khalfallah et al. (1993) showed that relative chromosome length ranged from 16.2% for the longest chromosome to 11% for the shortest with a total length of 26.59 μm. Our data were obtained from early metaphase chromosomes that were less condensed than those studied by Khalfallah et al. (1993). Therefore, the total length of 53.94 μm was considerably greater, although the relative lengths (individual chromosome length/total *P. glaucum* chromosome length) remained in the same range (from 17.5% to 10.2%).

P. squamulatum was reported to have 54 chromosomes in PMCs (Raman et al. 1959) and has been mostly cited as a hexaploid ($2n = 6x = 54$) with a base chromosome number of $x = 9$ (Patil et al. 1961; Rangaswamy 1972; Sisodia 1970; Dujardin and Hanna 1984). Sindhe (1976) observed 2 supernumerary chromosomes in this species that were rod-like and acrocentric or sub-metacentric and that were largely eliminated during meiotic cycles. We likewise observed 56 rather than 54 chromosomes in *P. squamulatum*; however, the "supernumerary" chromosomes in accession PS26 do not fit the description of those in Sindhe (1976). None of the 28 chromosomes we analyzed for morphology could be described as acrocentric. Furthermore, although we examined the chromosome number in only four F_1 individuals, all showed 28 *P. squamulatum* chromosomes (differentiated by GISH), which indicates that regular disjunction occurred during meiosis and subsequent transmission to progeny was normal.

By examining metaphase plates from apomictic F_1 hybrids of *P. glaucum* x *P. squamulatum*, chromosomes from both parents could be compared. The lengths of *P. squamulatum* chromosomes ranged from 9.45 μm to 3.89 μm. The length of *P. glaucum*

chromosomes ranged from 9.44 to 5.48 μm. Based on the lengths, it was not possible to distinguish the chromosomes of *P. glaucum* from the larger chromosomes of *P. squamulatum*, except for the satellited chromosomes from *P. glaucum*. *P. squamulatum* apparently shares similar DNA sequence with *P. glaucum* at telomeric and centromeric regions since these regions on the chromosomes of *P. squamulatum* were blocked with *P. glaucum* genomic DNA (Fig. 4.3a and 4.3b). It previously has been shown that an abundant cloned repeat from *P. glaucum* (140 bp) shares significant sequence similarity with *P. squamulatum* repeats (140 bp and 160 bp), even though there is an additional repeat unit structure in *P. squamulatum* (Ingham et al. 1993). The smaller repeat family likely diverged from the larger repeat unit by a deletion. These repeats localize to the centromeres of pearl millet and *P. squamulatum* (Goel et al. submitted). In spite of the similarities among these localized regions, GISH could unambiguously distinguish between the chromosomes of the two species.

4.3.2 The ASGR is inherited as a single genetic unit.

In our investigation, the third, sixth, and seventh backcross generations were largely autotetraploid pearl millet with only one to three chromosomes that hybridized with *P. squamulatum* genomic DNA. Only individuals with the ASGR-carrier chromosome from *P. squamulatum* were apomictic, and plants with other alien chromosomes but not the ASGR-carrier chromosome demonstrated sexual reproduction. Furthermore, only one of 56 *P. squamulatum* chromosomes showed a major hybridization signal when probed with a mixture of 12 molecular markers mapped to the ASGR. The ASGR does not appear to have a strictly colinear region on the "sexual" homologs in *P. squamulatum*, thus it would behave genetically like a dominant, single gene. Given the absolute correlation between

the presence of the ASGR-carrier chromosome and apomictic reproduction, it seems likely that there are specific genes in the ASGR, which underlie certain developmental pathways that allow nucellar cells to form aposporous embryo sacs.

Since apomicts typically are polyploid, a model postulated by Carman (1997) proposed that apomixis was the result of asynchronous expression of reproductive genes from different genomes in a polyploid angiosperm. According to this hypothesis, each genome has its own temporal control over the expression of reproductive genes, which may be brought into conflict upon hybridization, thus leading to apomixis. Such a hypothesis is difficult to test experimentally, therefore, we have focused our research on plants where a highly penetrant form of apomixis already exists. Our results continue to support the conclusion that apomixis is controlled by specific gene(s) in a discrete region of the *P. squamulatum* genome. This conclusion also is consistent with other recent molecular genetic studies, which have contributed to the emergence of a predominant hypothesis that apomixis is regulated by one or two, usually dominant loci. Investigations of several members of the grass family, *Tripsacum dactyloides* (Leblanc et al. 1995), *C. ciliaris* (*P. ciliare*) (Sherwood et al.1994; Roche et al. 1999), *P. squamulatum* (Ozias-Akins et al. 1998), *Brachiaria brizantha* cv Marandu (Pessino et al. 1997), and *Paspalum simplex* (Caceres et al. 2001), are consistent with dominant inheritance of a single gene. As for dicots, univalent inheritance of diplospory independently from pathenogenesis was reported in triploid *Erigeron annuus* (Noyes and Rieseberg 2000). In *Hieracium* species, aposporous apomixis also is regulated by a single dominant gene that can be transferred through both haploid and diploid gametes (Bicknell et al. 2000).

Both of the apomictic F_1 plants we investigated with FISH/GISH had one ASGR-carrier chromosome. This observation is what we would have predicted based on our previous model of tetrasomic inheritance with random chromatid segregation in *P.squamulatum* where gametes with two copies of the ASGR-carrier chromosome would be inviable (Ozias-Akins et al. 1998). This model was proposed because of the segregation ratio observed for apomictic and sexual reproduction among 397 individuals from the F_1 population. Further support for polysomic, though not specifically tetrasomic, inheritance has been obtained in related work where a genome-wide AFLP map provided little evidence for preferential pairing among homologous chromosomes, particularly in the region known to transmit apomixis (Chen et al., unpublished). In the current study, localization of the pooled, mapped markers to the terminus of a single chromosome further supports the likelihood of random chromatid segregation where a recombination event between the ASGR and the centromere would be expected. In tetrasomic inheritance with random chromatid segregation, two chromatids carrying the ASGR could end up in one out of 13 apomictic F_1s (theoretically, this model applied to our testcross would predict the following ratio of tetrasomic genotypes: 1AAaa:12Aaaa:15aaaa if the ASGR (A) is single dose in the apomictic parent). It now should be possible to use FISH to empirically test for double reduction and the recovery of AAaa genotypes in the F_1 population, although a large number of individuals would have to be surveyed to have a high probability of detecting an event that should occur in only one out of 28 F_1s. The occurrence of exclusively a single copy of the ASGR in any F_1 still would not address the question of gamete inviability vs. zygotic lethality.

4.3.3 Hemizygosity might contribute to repressed recombination within the ASGR of *P. squamulatum*.

A prerequisite for recombination is pairing and synapsis of homologous chromosomes. If certain regions of homologous chromosomes diverge greatly in sequence or structure, due to perhaps invasion of mobile elements or chromosomal rearrangements, pairing is likely to be diminished. From the results of our study, the end of a single chromosome showed very strong hybridization signal with a mixture of the ASGR-linked molecular markers while no similar region on any other chromosome could be detected in *P. squamulatum*. Most of this signal originated with one or more of the repetitive sequences in the pool. The limited distribution of FISH signal to the end of a single chromosome in this species could be a consequence of introgression of a divergent chromosomal fragment through hybridization or, perhaps, the localized amplification and degeneration of particular repetitive sequences. Either mechanism could lead to sequence divergence among homologs and interfere with recombination. None of our current results detract from our previous conclusion that the ASGR may be a complex locus (Ozias-Akins et al. 1998). On the contrary, the distinction of a substantial portion of a chromosome arm by FISH reinforces the earlier conclusion that hemizygosity may play a major role in reducing recombination, thus allowing multiple genes to remain linked in coupling. Hemizygosity of the ASGR may keep this region asynapsed during meiosis in microsporogenesis and avoid recombination-based disturbance of the genes regulating the apomixis pathway. Recently, a report of meiotic silencing by unpaired DNA in *Neurospora* (Shiu et al. 2001) raises the intriguing possibility that in the ASGR, unpaired gene(s) required for meiosis

could silence orthologs and paralogs at other loci, thereby creating a permissive condition to allow an embryo sac developmental program to function ectopically in somatic cells.

Hemizygosity is characteristic of idiomorphic loci that may contain multiple genes, which do not share significant sequence similarity between "alleles". Such idiomorphs have been described for *Neurospora crassa* mating-type loci (Glass et al. 1990; Staben and Yanofsky 1990). Even more extensive haplotypes characterize the mating-type locus in pseudohomothallic *Neurospora tetrasperma* (Merino et al. 1996) where suppressed recombination is correlated with a lack of pairing in this chromosomal region during meiosis (Gallegos et al. 2000). Mammalian sex chromosomes have similar impediments to homology recognition that prevent synapsis outside of the pseudoautosomal region of the X and Y chromosomes (Handel and Hunt 1992; Amaral 1994). Whether multiple linked genes at the complex apomixis locus may be required for the expression of apomixis still awaits experimental evidence. More than 50 BAC clones that have been assigned to the locus (Roche et al. 2002) currently are being surveyed for gene content, and a subset of these BAC clones has been used for FISH. BAC-FISH confirms that hemizygosity of low-copy sequences extends for several kb (Goel et al. submitted) and provides further evidence that sequence divergence between homologs has occurred.

4.3.4 One chromosome with the ASGR is sufficient for the expression of apomixis.

Plants with the ASGR-carrier chromosome from *P. squamulatum* were observed to be apomictic (either near-obligate or facultative), no matter how many other alien chromosomes were introduced in the hybrids or backcross plants. We have noted considerable variation in the degree of apomixis (as measured by progeny analysis)

among various aposporous backcross lines ranging in chromosome number from 27 to 29 (Hanna et al. 1993). These exact materials were not available for FISH analysis; therefore, it remains inconclusive whether there is any correlation between one, two, or three alien chromosomes and the penetrance of the trait. Facultativeness also could be affected by environment, although there is little evidence for such effects in *Pennisetum* (Hussey et al. 1991). Genetic modifiers of apomixis have been reported to exist in *Hieracium* (Koltunow et al. 2000), and it may eventually be necessary to understand the role of such modifiers in the penetrance of the trait if it is to be utilized in crop plant breeding and genotype maintenance. The more immediate goal, however, will be analysis of the essential locus for aposporous apomixis mapped to a single ASGR-carrier chromosome, which is sufficient for the expression of apomixis in the background of tetraploid pearl millet. Further study of this chromosome and the detailed structure of the ASGR may eventually unveil the regulatory mechanism underlying aposporous reproduction that resides in the ASGR.

Acknowledgments

This work was supported by the USDANRI Plant Genome Program, award no. 99-35300-7691. We are grateful for the technical training provided by Hans de Jong and Jannie Wennekes, Wageningen Agricultural University, for the technical advice of Shailendra Goel, and for the technical assistance of Anne Bell, Jacolyn Merriman, and Freddie Cheek.

References

Amaral, M. E., 1994 Synaptonemal complex-analyses of the sex bivalent in goats. Genet. Selec. Evol. 26: 369-375.

Bicknell, R. A., N. K. Borst and A. M. Koltunow, 2000 Monogenic inheritance of apomixis in two *Hieracium* species with distinct developmental mechanisms. Heredity 84: 228-237.

Caceres, M. E., F. Matzk, A. Busti, F. Pupilli and S. Arcioni, 2001 Apomixis and sexuality in *Paspalum simplex*: characterization of the mode of reproduction in segregating progenies by different methods. Sex. Plant Reprod. 14: 201-206.

Carman, J. G., 1997 Asynchronous expression of duplicate genes in angiosperms may cause apomixis, bispory, tetrospory, and polyembryony. Biol. J. Linn. Soc. 61: 51-94.

Chen, Q., F. Eudes, R. L. Conner, R. Graf, A. Comeau, J. Collin, F. Ahmad, R. Zhou, H. Li, Y. Zhao and A. Laroche, 2001 Molecular cytogenetic analysis of a durum wheat x *Thinopyrum distichum* hybrid used as a new source of resistance to *Fusarium* head blight in the greenhouse. Plant Breed. 120: 375-380.

Chen, Z. B., W. W. Hanna and P. Ozias-Akins, n.d. An AFLP genetic linkage map of an apomictic polyploid, *Pennisetum squamulatum*. In preparation.

Dujardin, M. and W. W. Hanna, 1984 Microsporogenesis, reproductive behavior, and fertility in five *Pennisetum* species. Theor. Appl. Genet. 67: 197-201.

Dujardin, M. and W. W. Hanna, 1985 Cytology and reproduction of reciprocal backcrosses between pearl millet and sexual and apomictic hybrids of pearl millet x *Pennisetum squamulatum*. Crop Sci. 25: 59-62.

Dujardin, M. and W. W. Hanna, 1987 Cytotaxonomy and evolutionary significance of two offtype millet plants. J. Hered. 78:21-23.

Dujardin, M. and W. W. Hanna, 1989 Developing apomictic pearl millet – characterization of a BC3 plant. J. Genet. Breed. 43: 145-151.

Gallegos, A., D. J. Jacobson, N. B. Raju, M. P. Skupski and D. O. Natvig, 2000 Suppressed recombination and a pairing anomaly on the mating-type chromosome of *Neurospora tetrasperma*. Genetics 154:623-633.

Glass, N. L., J. Grotelueschen and R. L. Metzenberg, 1990 *Neurospora crassa A* mating-type region. Proc. Natl. Acad. Sci. USA 87: 4912-4916.

Goel, S., J. A. Conner, W. W. Hanna and P. Ozias-Akins, n.d. Physical evidence that a single hemizygous chromosomal region is sufficient to confer aposporous embryo sac formation in *Pennisetum squamulatum* and *Cenchrus ciliaris*. submitted

Grimanelli, D., O. Leblanc, E. Espinosa, E. Perotti, D. G. DeLeon and Y. Savidan, 1998 Mapping diplosporous apomixis in tetraploid *Tripsacum*: one gene or several genes? Heredity 80: 33-39.

Handel, M. A. and P. A. Hunt, 1992 Sex-chromosome pairing and activity during mammalian meiosis. Bioessays 14:817-822.

Hanna, W. W. and E. C. Bashaw, 1987 Apomixis: its identification and use in plant breeding. Crop Sci. 27:1136-1139.

Hanna, W. W., M. Dujardin, P. Ozias-Akins, E. L. Lubbers and L. Arthur, 1993 Reproduction, cytology, and fertility of pearl millet x *Pennisetum squamulatum* BC_4 plants. J. Hered. 84: 213-216.

Heslop-Harrison, J. S., A. R. Leitch, T. Schwarzacher and K. Anamthawat-Jonsson, 1990 Detection and characterization of 1B/1R translocations in hexaploid wheat. Heredity 65: 356-362.

Hussey, M. A., E. C. Bashaw, K. W. Hignight and M. L. Dahmer, 1991 Influence of photoperiod on the frequency of sexual embryo sacs in facultative apomictic buffelgrass. Euphytica 54: 141-145.

Ingham, L. D., W. W. Hanna, J. W. Baier and L. C. Hannah, 1993 Origin of the main class of repetitive DNA within selected *Pennisetum* species. Molec. Gen. Genet. 238: 350-356.

Jauhar, P. P., 1981 Cytogenetics and breeding of pearl millet and related species. Alan R. Liss Inc., New York.

Khalfallah, N., A. Sarr and S. S. Yakovlev, 1993 Karyological study of some cultivated and wild stocks of pearl millet from *Pennisetum typhoides* Stapf et Hubb. and *P. violaceum* (Lam.) L. Rich. Caryology 46: 127-138.

Kato, S. and K. Fukui, 1998 Condensation pattern (CP) analysis of plant chromosomes by an improved chromosome image analyzing system, CHIAS III. Chromosome Res. 6: 473-479.

Koltunow, A. M., S. D. Johnson and R. A. Bicknell, 2000 Apomixis is not developmentally conserved in related, genetically characterized *Hieracium* plants of varying ploidy. Sex. Plant Reprod. 12: 253-266.

Langer-Safer, P. R., M. Levine and D. C. Ward, 1982 Immuological method for mapping genes on *Drosophila* polytene chromosomes. Proc. Natl. Acad. Sci. USA 79: 4381-4385.

Leblanc, O., D. Grimanelli and D. G. DeLeon, 1995 Detection of the apomictic mode of reproduction in maize-*Tripsacum* hybrids using RFLP makers. Theor. Appl. Genet. 90: 1198-1203.

Merino, S. T., B. L. Nelson, D. J. Jacobson and D. O. Natvig, 1996 Pseudohomothallism and evolution of the mating-type chromosome in *Neurospora tetrasperma*. Genetics 143: 789-799.

Mukai, Y. and B. S. Gill, 1991 Detection of barley chromatin added to wheat by genomic *in situ* hybridization. Genome 34: 448-452.

Nogler, G. A., 1984 Gametophytic apomixis. Pp 475-518 in *Embryology of Angiosperms*, edited by B. M. Johri. Springer-Verlag, Berlin.

Noyes, R. D. and L. H. Rieseberg, 2000 Two independent loci control agamospermy (apomixis) in the triploid flowering plant *Erigeron annuus*. Genetics 155: 379-390.

Ozias-Akins, P., E. L. Lubbers, W. W. Hanna and J. W. McNay, 1993 Transmission of the apomictic mode of reproduction in *Pennisetum*: Co-inheritance of the trait and molecular markers. Theor. Appl. Genet. 85: 632-642.

Ozias-Akins, P., D. Roche and W. W. Hanna, 1998 Tight clustering and hemizygosity of apomixis-linked molecular markers in *Pennisetum squamulatum* implies genetic control of apospory by a divergent locus that may have no allelic form in sexual genotypes. Proc. Natl. Acad. Sci. USA 95: 5127-5132.

Patil, B. D., M. W. Hardas and A. B. Joshi, 1961 Auto-alloploid nature of *Pennisetum squamulatum* Fresen. Nature 189: 419-420.

Pessino, S. C., J. P. Ortiz, O. Leblanc, C. B. DoValle, C. Evans and M. D. Hayward, 1997 Identification of a maize linkage group-related to apomixis in *Brachiaria*. Theor. Appl. Genet. 94: 439-444.

Pessino, S. C., C. Evans, J. P. A. Ortiz, I. Armstead, C. B. DoValle and M. D. Hayward, 1998 A genetic map of the apospory-region in *Brachiaria* hybrids: identification of two markers closely associated with the trait. Hereditas 128: 153-158.

Pupilli, F., P. Labombarda, M. E. Caceres, C. L. Quarin and S. Arcioni, 2001 The chromosome segment related to apomixis in *Paspalum simplex* is homoeologous to the telomeric region of the long arm of rice chromosome 12. Molec. Breed. 8: 53-61.

Rangaswamy, S. R. S., 1972 Cytological studies on diploid and polyploid taxa of the genus *Pennisetum* Rich. Genetica 43: 257-273.

Rayburn, A. L. and B. S. Gill, 1985 Use of biotin-labeled probe to map specific DNA sequences on wheat chromosomes. J. Hered. 76: 78-81.

Raman, V. S., P. Chandrasekharan and D. Krishnaswami, 1959 A note on some chromosome numbers in *Gramineae*. Current Sci. 29: 127-128.

Roche, D., P. S. Cong, Z. B. Chen, W. W. Hanna, D. L. Gustine, R. T. Sherwood and P. Ozias-Akins, 1999 An apospory-specific genomic region is conserved between buffelgrass (*Cenchrus ciliaris* L.) and *Pennisetum squamulatum* Fresen. Plant J. 19: 203-208.

Roche, D., J. A. Conner, M. A. Budiman, D. Frisch, R. Wing, W. W. Hanna and P. Ozias-Akins, 2002 Construction of BAC libraries from two apomictic grasses to study the microcolinearity of their apospory-specific genomic regions. Theor. Appl. Genet. (in press)

Sambrook, J., E. F. Fritsch and T. Maniatis, 1989 Molecular Cloning, A Laboratory Manual. Second Edition. pp. 1.25-1.28. Cold Spring Harbor Laboratory Press.

Schwarzacher, T., A. R. Leitch, M. D. Bennett and J. S. Heslop-Herrison, 1989 *In situ* localization of parental genomes in a wild hybrid. Ann. Bot. 64: 315-324.

Sherwood, R, T., C. C. Berg and B. A. Young, 1994 Inheritance of apospory in buffelgrass. Crop Sci. 34:1490-1494.

Shiu, P. K. T., N. B. Raju, D. Zickler and R. L. Metzenberg, 2001 Meiotic silencing by unpaired DNA. Cell 107: 905-916.

Sindhe, A. N. R., 1976 Supernumerary chromosomes in *Pennisetum squamulatum* Fresen. Current Sci. 45: 526.

Sisodia, K. P. S., 1970 Cytological studies on some species in genus *Pennisetum*. Theor. Appl. Genet. 40: 26-31.

Staben, C. and C. Yanofsky, 1990 *Neurospora crassa a* mating-type region. Proc. Natl. Acad. Sci. USA 87: 4917-4921.

Stapf, O. and C. E. Hubbard, 1934 *Pennisetum*. pp. 954-1070. In *Flora of Tropical Africa*. D. Prain, Ed. Vol. 6. Part 6. Reeve & Co, Ltd, Ashford, England.

Vega, J. M., S. Abbo, M. Feldman and A. A. Levy, 1994 Chromosome pairing in plants: *In situ* hybridization with a DNA probe from a specific microdissected chromosome arm of common wheat. Proc. Natl. Acad. Sci. USA. 91:12041-12045.

Young, B. A., R. T. Sherwood and E. C. Bashaw, 1979 Cleared-pistil and thick-sectioning techniques for detecting aposporous apomixis in grasses. Can. J. Bot. 57: 1668-1672.

Zhong, X. B., P. F. Fransz, J. W. V. Eden, P. Zabel, A. Kammen and J. H. de Jong, 1996 High-resolution mapping on pachytene chromosomes and extended DNA fibers by fluorescence *in situ* hybridization. Plant Mol. Biol. Rep. 14: 232-242.

Table 4.1. Summary of materials used and results of FISH/GISH.

Gene-ration	Line or plant ID		Pheno-type[1]	Tissue used	Probe-DNA[2]	No. of Pg.chrom[3]	No. of Ps.chrom[4]	ASGR Signal[5]
Parent	PS26	(1/28)[7]	A	Root	PS26+MM	0	56	+
F1[6]	124	(3/17)	A	Root	PS26+MM	14	28	+
	181	(2/13)	A	Root	PS26+MM	14	28	+
	12	(2/18)	S	Root	PS26+MM	14	28	-
	105	(2/4)	S	Root	PS26+MM	14	28	-
BC3	J34	(22/53)	A	Anther	PS26	26	3	n/a
	56-1	(1/6)	F	Root	PS26+MM	26	3	+
	56	(15/87)	A	Root	PS26+MM	26	3	+
BC5	44-1	(1/4)	A	Root	PS26	26	3	n/a
	44-2	(1/5)	A	Root	PS26	27	2	n/a
	44-3	(1/13)	A	Root	PS26+MM	27	2	+
	44-4	(1/12)	S	Root	PS26+MM	28	1	-
	46-1	(1/1)	F	Root	PS26+MM	27	2	+
	46-5	(1/8)	A	Root	PS26+MM	27	2	+
BC6	J35	(12/33)	A	Anther	PS26	26	3	n/a
	42-1	(1/5)	F	Root	PS26+MM	27	2	+
	43-1	(1/5)	F	Root	PS26	26	3	n/a
	43-3	(1/5)	F	Root	PS26+MM	26	3	+
	54	(3/13)	F	Root	PS26+MM	28	1	+
	62	(2/9)	F	Root	PS26+MM	28	1	+
BC7	J57-7	(1/5)	F	Anther	PS26		3	n/a
	J57	(2/11)	A	Anther	PS26		3	n/a
	J60-13	(1/13)	F	Anther	PS26		1	n/a
	J64-11	(1/10)	A	Anther	PS26		1	n/a
	49-15	(1/3)	S	Root	PS26+MM	28	0	-
	49	(3/9)	A	Root	PS26	26	3	n/a
	58-4	(1/8)	A	Root	PS26	27	2	n/a
	58-6	(1/16)	F	Root	PS26+MM	26	2	+
	60-4	(1/4)	A	Root	PS26	27	2	n/a
	60-9	(1/3)	A	Root	PS26	27	2	n/a
	60-12	(1/6)	A	Root	PS26+MM	27	2	+
	61-3	(1/5)	F	Root	PS26	27	2	n/a
	61-20	(1/3)	A	Root	PS26+MM	27	2	+
	61-21	(1/2)	A	Root	PS26	27	2	n/a

[1] A, F, and S refer to reproductive phenotypes, respectively, of apomixis (only aposporous embryo sacs observed), facultative apomixis (both aposporous and meiotic embryo sacs observed), and sexual reproduction (only meiotic embryo sacs observed);

[2] DNAs used in FISH and GISH as labeled probes. PS26 - genomic DNA from *P. squamulatum*, accession PS26; MM - mixture of ASGR-linked marker DNA;

[3] the number of chromosomes that hybridized with genomic DNA of *P. glaucum*;

[4] the number of chromosomes that hybridized with genomic DNA of *P. squamulatum*;

[5] the presence (+) or absence (-) of hybridization signal revealed by the labeled DNA mixture of ASGR-linked markers, n/a – not applicable;

[6] F_1 of tetraploid *P. glaucum* x *P. squamulatum*;

[7] the number in parentheses indicates the number of plant(s) (before slash) and spreads (after slash) investigated.

Table 4.2. Length statistics for chromosomes of *P. glaucum* (top row) and *P. squamulatum* (2nd and 3rd rows). The data were an average of five mitotic spreads from apomictic F_1 plants (290-124B and 181D). Each spread contained 42 chromosomes, which were assigned to their respective genomes by GISH. The chromosome lengths were obtained by the chromosome image analysis system (CHIAS III).

g-Chrom[1]	1	2	3	4	5	6	7
Length[2]	9.44	8.77	8.36	7.77	7.35	6.79	5.48
S.D.[3]	2.42	2.22	2.21	1.95	1.76	1.64	1.73
Relative length[4]	3.45	3.23	3.07	2.87	2.72	2.51	2.14
S.D.[5]	0.19	0.11	0.12	0.17	0.12	0.10	0.29
DNA density of chromosome [6]	38.15 ± 9.43 Mbp/ μm						

Ps-Chrom[7]	1	2	3	4	5	6	7	8	9	10	11	12	13	14
Length	9.45	8.10	7.63	7.13	6.83	6.72	6.58	6.46	6.31	6.17	5.97	5.87	5.81	5.73
S.D.	2.97	1.59	1.76	1.75	1.88	1.84	1.72	1.66	1.75	1.72	1.54	1.52	1.55	1.58
Relative length	3.46	3.03	2.83	2.64	2.51	2.47	2.43	2.39	2.32	2.67	2.21	2.17	2.14	2.11
S.D.	0.24	0.25	0.14	0.14	0.07	0.07	0.07	0.06	0.12	0.08	0.09	0.08	0.08	0.07
Ps-Chrom	15	16	17	18	19	20	21	22	23	24	25	26	27	28
Length	5.66	5.60	5.50	5.13	5.18	5.04	4.87	4.79	4.72	4.56	4.53	4.37	4.18	3.89
S.D.	1.53	1.54	1.51	1.39	1.33	1.22	1.17	1.12	1.10	1.13	1.14	1.04	1.06	1.00
Relative length	2.08	2.06	2.03	1.96	1.92	1.87	1.80	1.78	1.75	1.69	1.68	1.62	1.55	1.44
S.D.	0.07	0.08	0.08	0.12	0.10	0.09	0.05	0.06	0.06	0.09	0.09	0.07	0.06	0.11
DNA density of chromosome	33.24 ± 8.05 Mbp/μm													

[1] *Pg*-chrom refers to *P. glaucum* chromosomes;

[2] Length of chromosome in μm;

[3] Standard deiviation for chromosome length;

[4] Relative length = chromosome length/total chromosome length in each spread including both *P. glaucum* and *P. squamulatum* chromosomes;

[5] Standard deviation for relative length;

[6] Average DNA density of chromosome was calculated with the formula: Average DNA density = genome size (Mbp/C)/total chromosome length (μm) (haploid). Genome size of 1950 Mbp/C and 5150 Mbp/C were used for *P. glaucum* and *P. squamulatum*, respectively (Roche et al. 2002);

[7] *Ps*-chrom refers to the *P. squamulatum* chromosomes.

Fig. 4.1. Pedigree summary of hybrids and backcrosses used for FISH/GISH. Materials used for this investigation are shown in bold. The F_1 of *P. glaucum* x *P. purpureum*, BC_2 and BC_4 were not included in these experiments. Chromosome doubling was accomplished with colchicine treatment.

Fig. 4.2. Metaphase chromosomes of *P. glaucum* (top row) and *P. squamulatum* (2^{nd} and 3^{rd} rows). The average data of five mitotic spreads from apomictic F_1 plants (290-124B and 181D) containing these 42 chromosomes is given in Table 4.2. Chromosomes were assigned to their respective genomes by GISH. The arrowhead beside the chromosome of *P. squamulatum* on the 2^{nd} row indicates the chromosome arm carrying the ASGR.

Fig. 4.3. Results of GISH and FISH on chromosomes from materials in the pedigree shown in Fig. 4.1. a-c, e-h. Hybridization of mitotic chromosome preparations with dual-labeled probes of the mixture of 12 ASGR-linked markers and *P. squamulatum* genomic DNA. The images were pseudo-colored with red for *P. squamulatum* genomic DNA, green for the ASGR-linked marker DNA mixture, and blue for blocking DNA of *P. glaucum*. d, k. Dual-labeled genomic DNAs of *P. squamulatum* (red) and *P. purpureum* (green), blocked with *P. glaucum* genomic DNA (blue). Because both probes hybridized to the same chromosomes, the color of the alien chromosomes in BC_1 (d) and BC_3 (k) appeared yellow. i, j. GISH with *P. squamulatum* genomic DNA as probe (green) and *P. glaucum* DNA as blocking DNA (blue). l – o. The behavior of alien chromosomes in different backcrosses during meiosis. *P. squamulatum* genomic DNA was used as probe (green) and *P. glaucum* genomic DNA as blocking (blue). a. *P. squamulatum*; b. apomictic F_1 290-181; c. sexual F_1 290-105; d. BC_1; e. BC_3; f. BC_6 43-1; g. BC_5 44-4; h. BC_6 62-1; i. BC_7 60-9; j. BC_5 44-1; k. BC_3; l. BC_3 J34; m. BC_7 J57; n. BC_6 J35; o. BC_7 J57.

Fig. 4.1

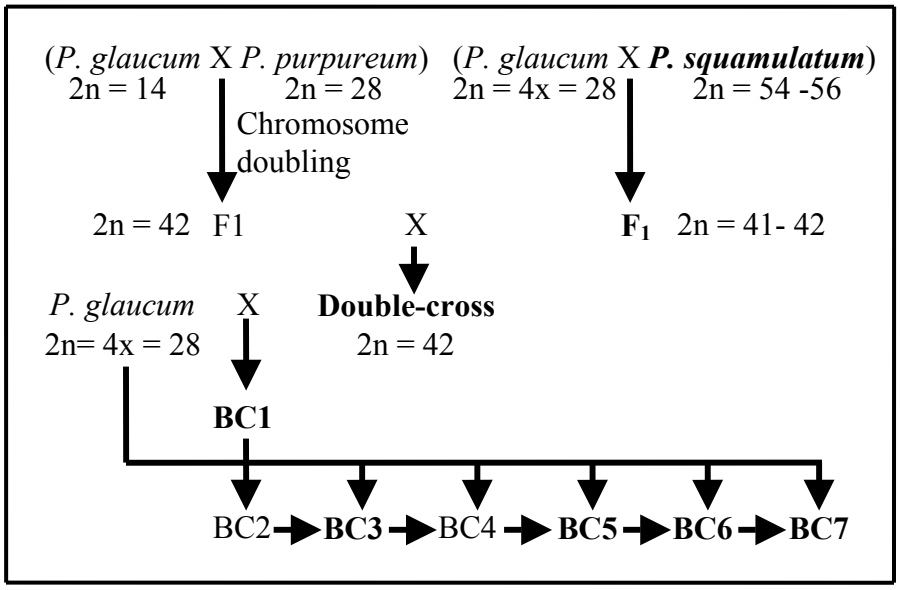
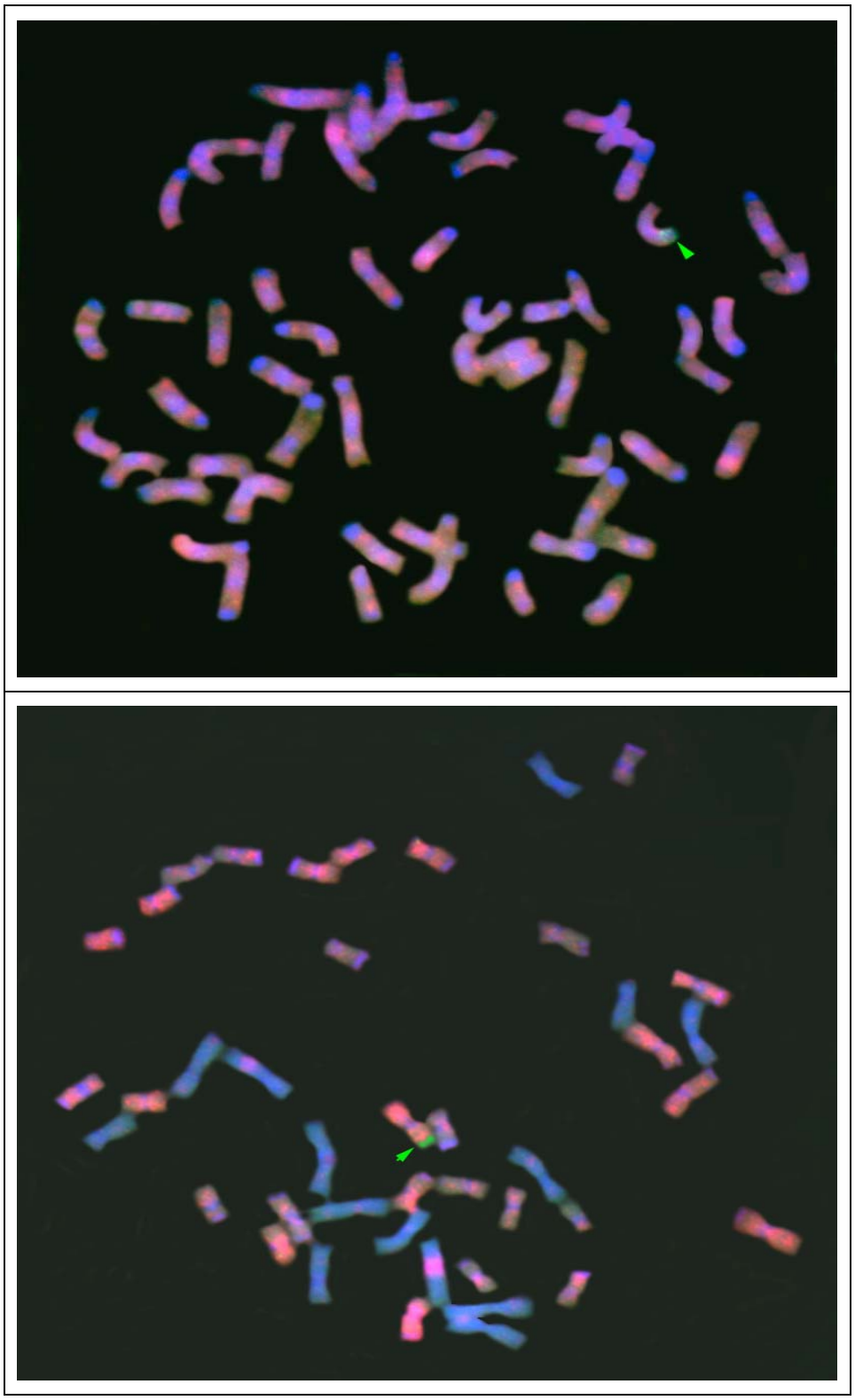

(*P. glaucum* X *P. purpureum*)
2n = 14
2n = 28
Chromosome doubling
2n = 42 F1
X
P. glaucum
2n= 4x = 28
X
BC1
BC2 → **BC3** → BC4 → **BC5** → **BC6** → **BC7**
(*P. glaucum* X ***P. squamulatum***)
2n = 4x = 28
2n = 54 -56
F_1 2n = 41- 42
Double-cross
2n = 42

Fig. 4.2

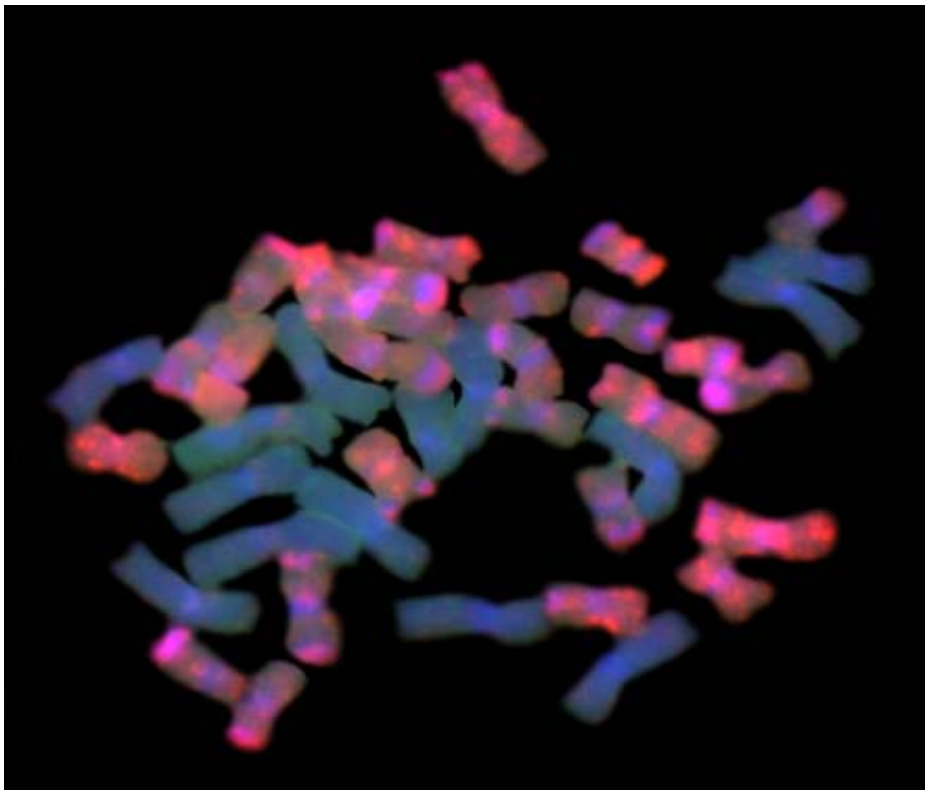
Pennistum glaucum chromosomes

Pennisetum squamulatum chromosomes

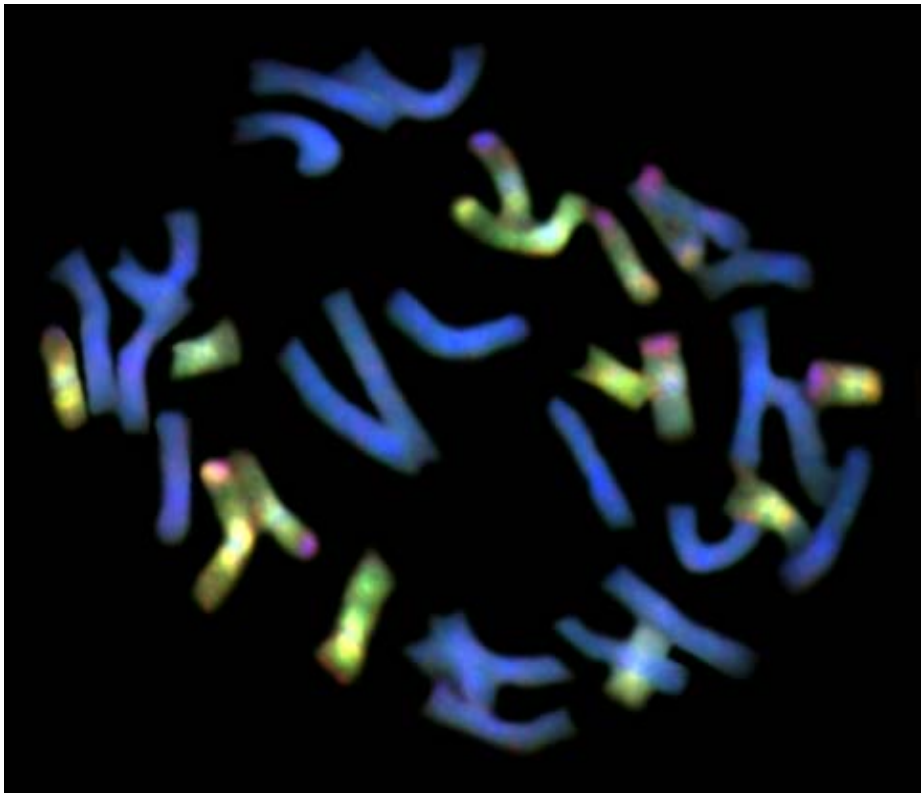
Fig. 4.3



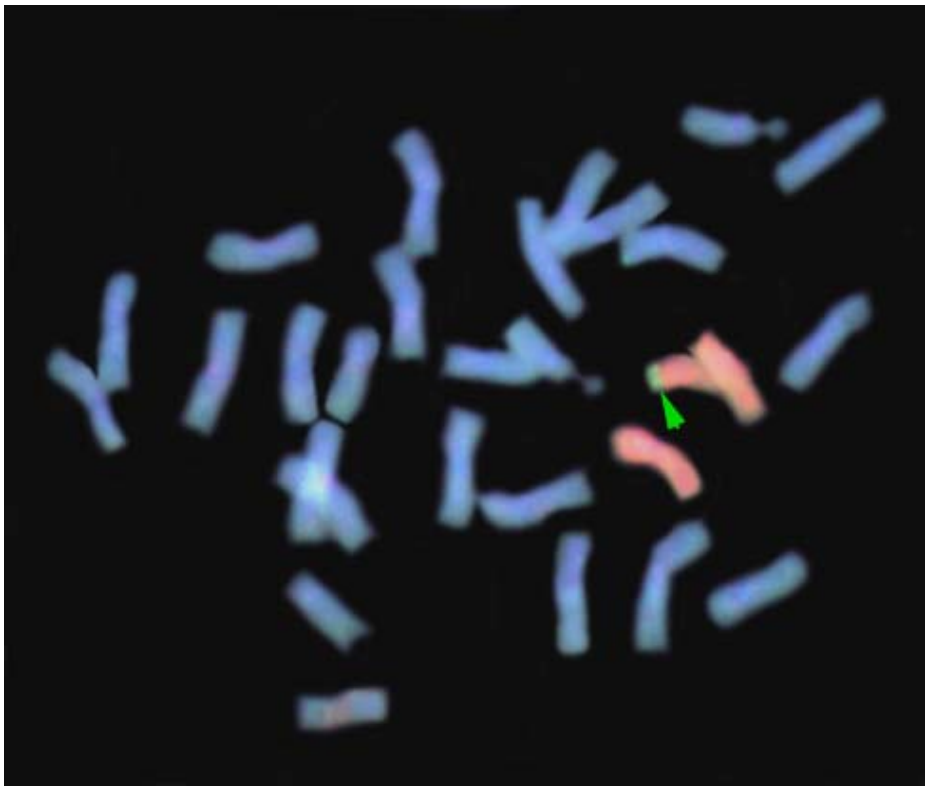
c



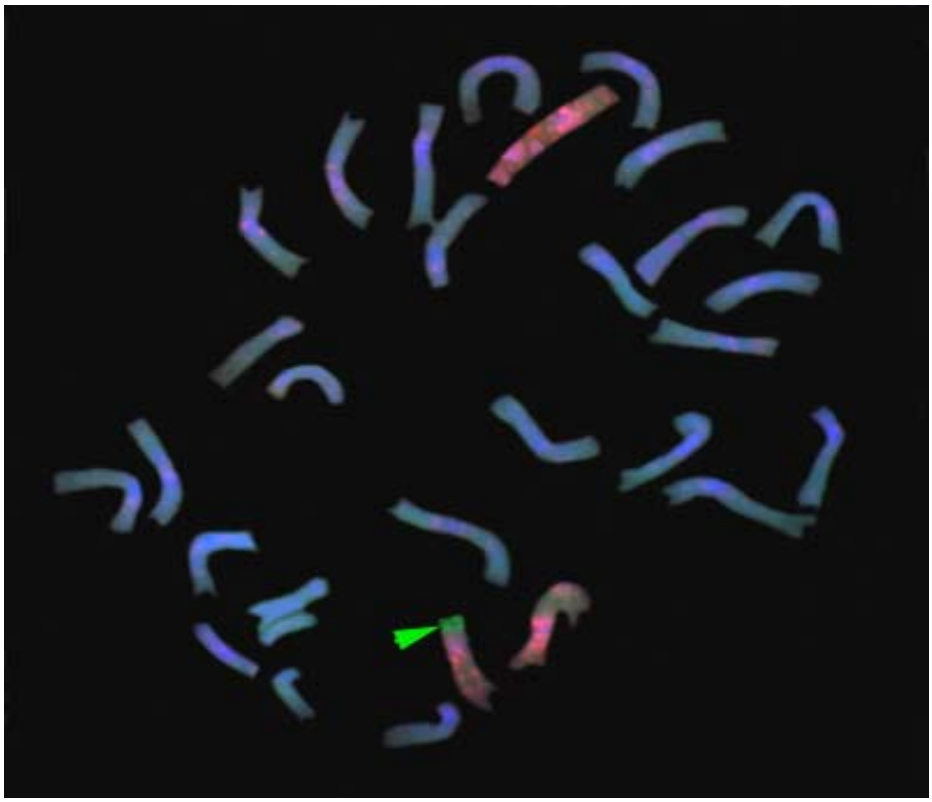
d



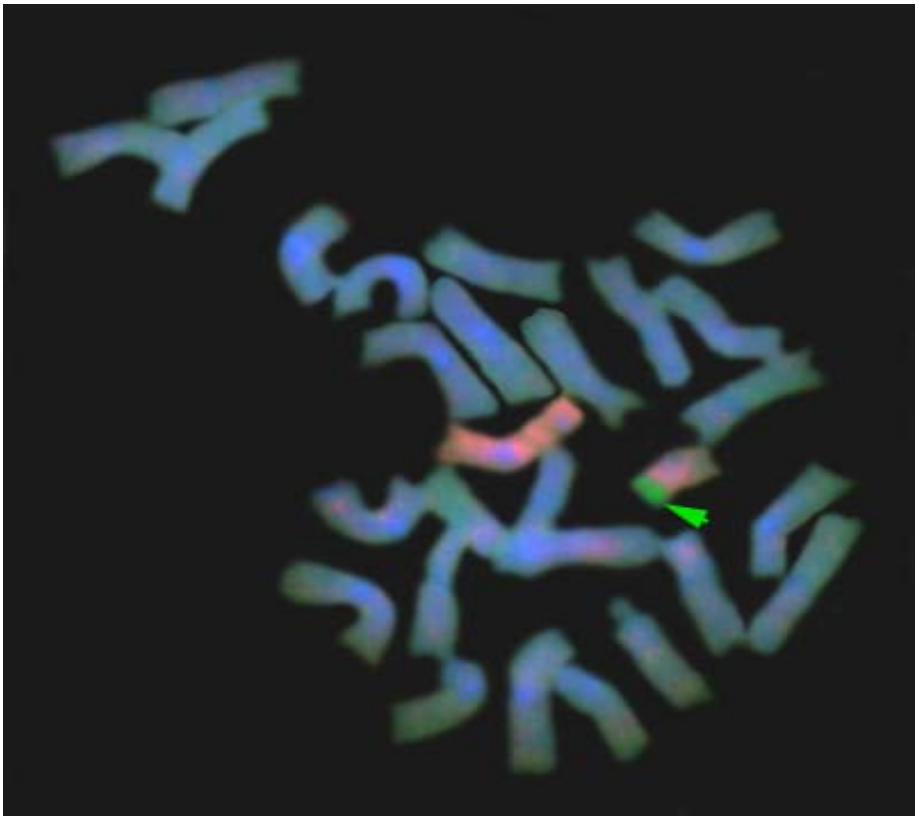
e



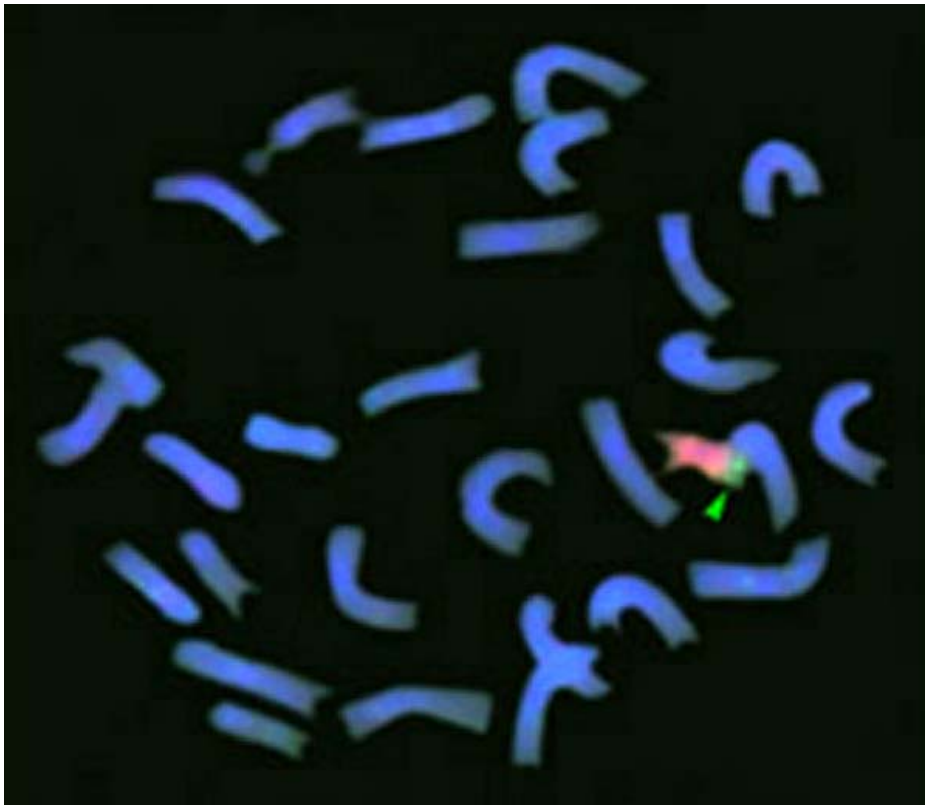
f



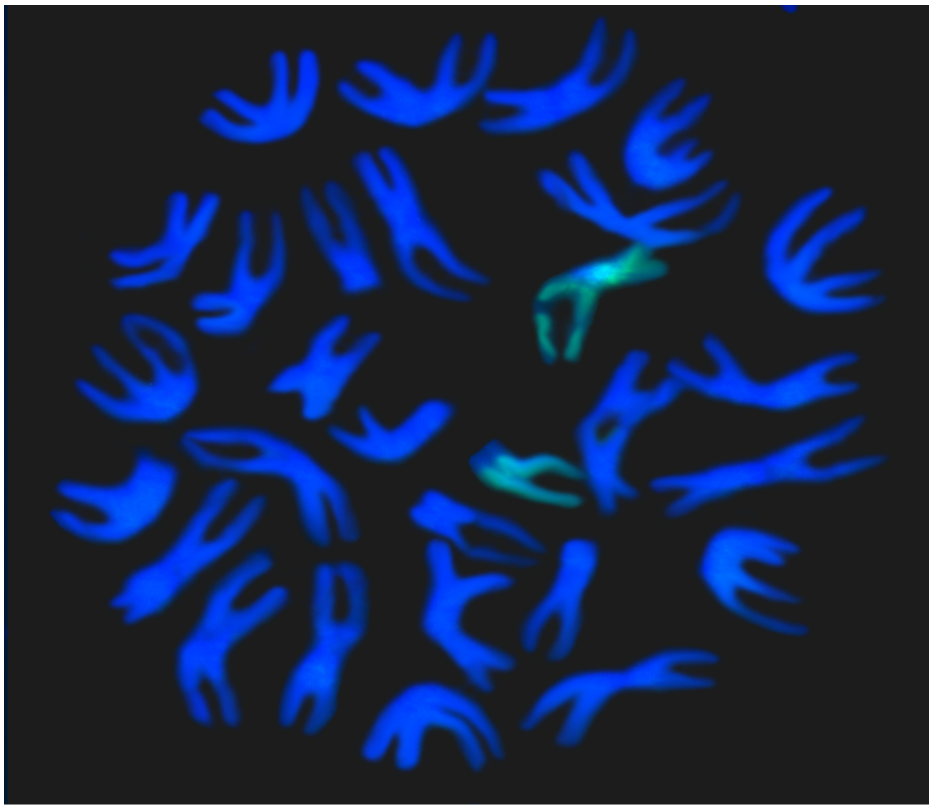
g



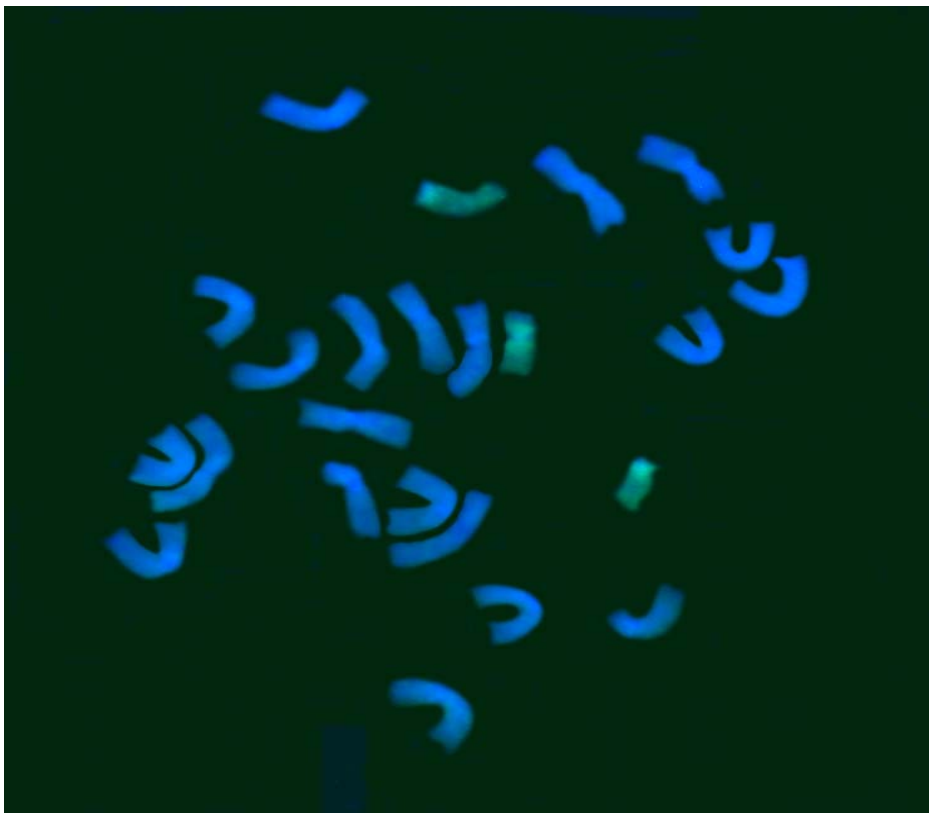
h



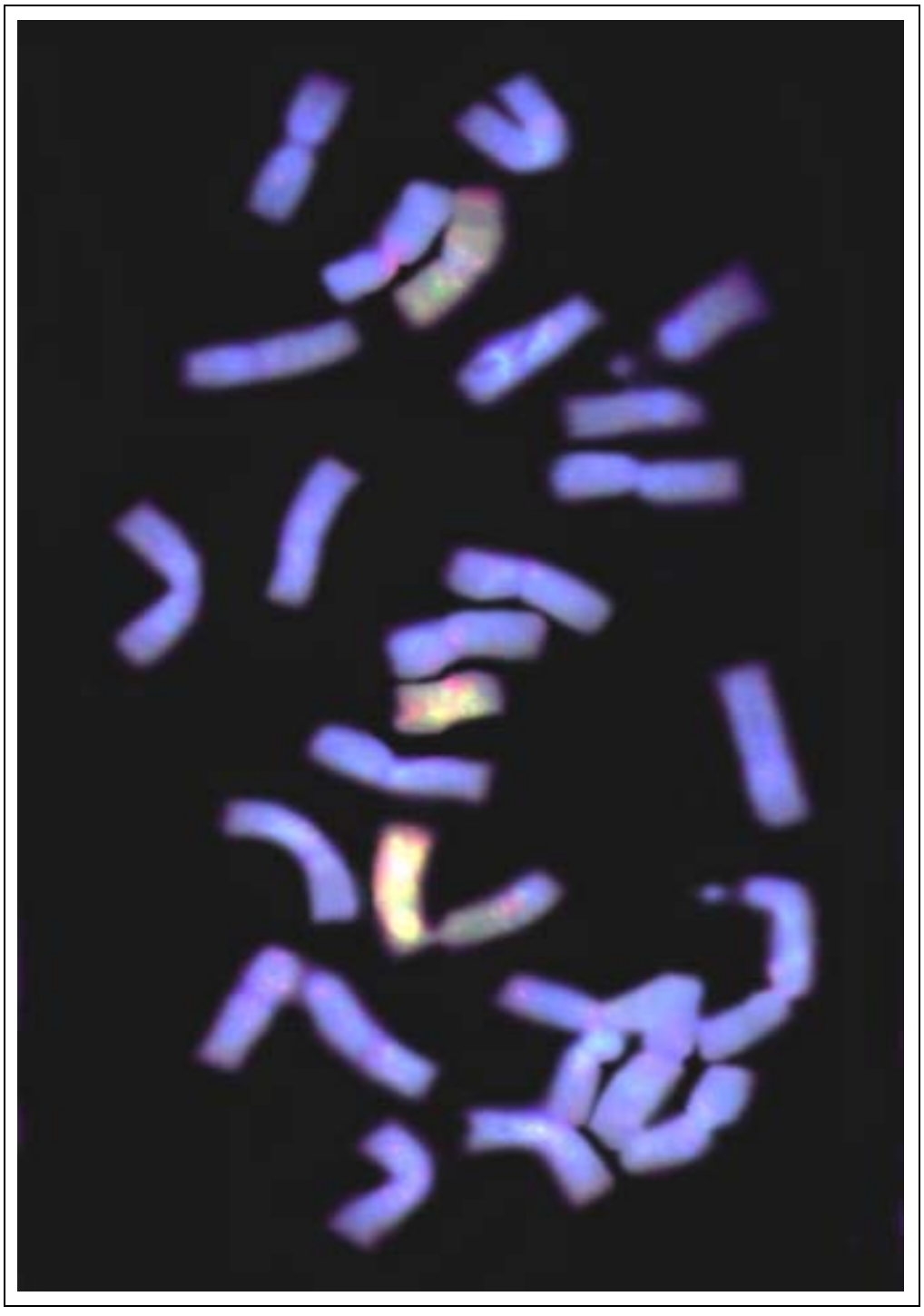
i

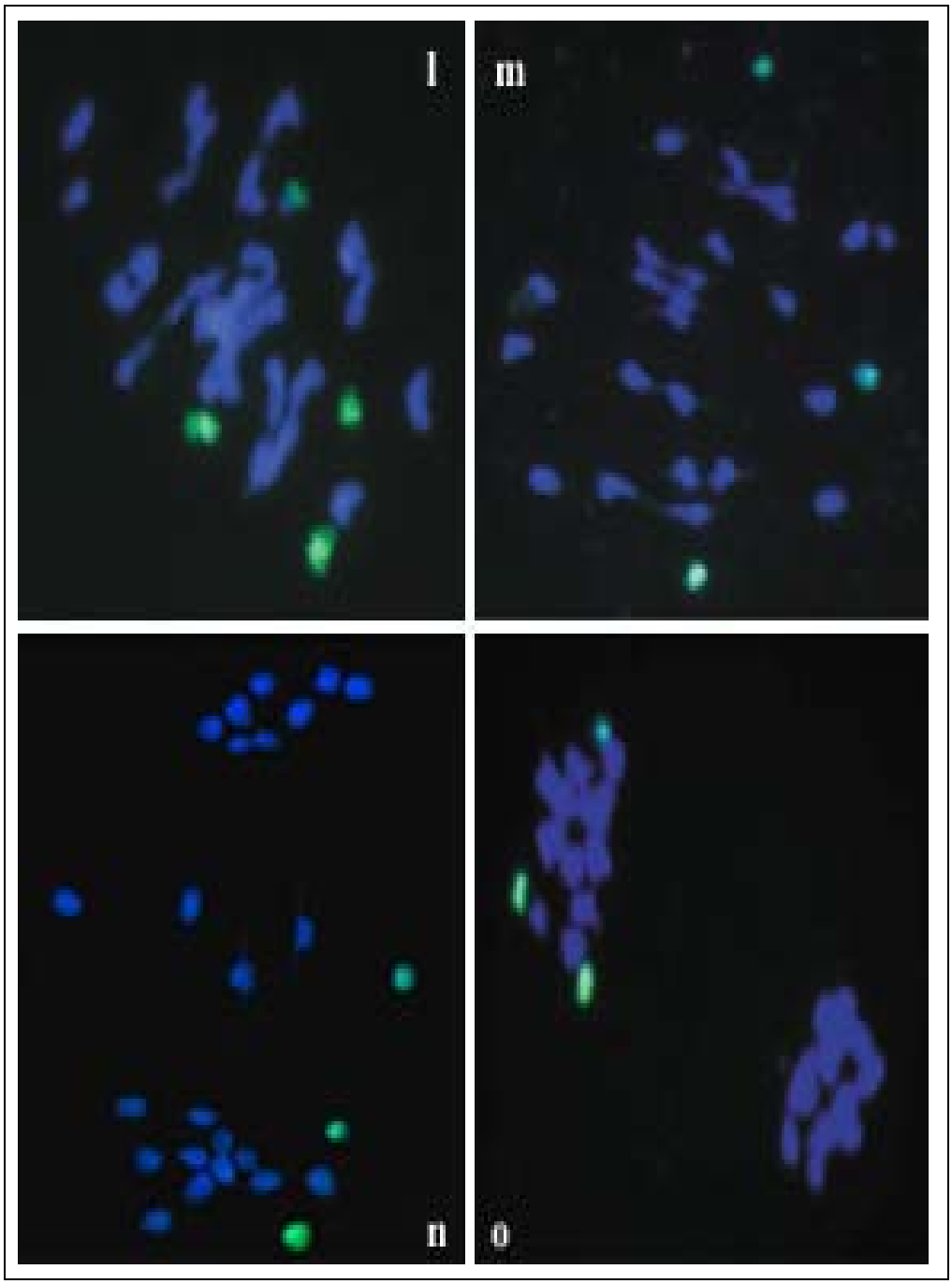


j



k




I
m
n
o

CHAPTER 5

CONCLUSIONS

1. A genetic linkage map for *P. squamulatum* was established with 182 single-dose AFLP markers. This map included 44 linkage groups covering 2600 cM with an average distance of 14.3 cM between markers. Most of the markers were distributed randomly over the genome except for the apospory-specific genomic region (ASGR), in which 4 AFLP markers clustered. Repulsion analysis revealed mainly random pairing among the homologs in the genome though preferential pairing might occasionally happen.
2. The apomixis-related linkage group included 24 AFLP markers, covering 416 cM. Four AFLP markers were completely associated with apomixis. Another four markers flanked the apomixis cluster with two on each side. The nearest flanking markers, py503 and px299, were 5.4 cM and 3.3 cM away from the cluster, which narrowed the ASGR down to a region of less than 10 cM genetic distance. No repulsion-linked marker was detected in or near the ASGR, implying that preferential pairing around the ASGR did not occur.
3. Cytogenetic investigation of *P. squamulatum* showed that PS26 has 56 chromosomes and all of the F_1 hybrids between induced tetraploid pearl millet (*P. glaucum*) and *P. squamulatum* showed 42 chromosomes, 14 from millet and 28 from *P. squamulatum*. In backcross 1 (BC_1), 14 chromosomes from *P. squamulatum* remained. Such regular segregation of sets of 7 chromosomes seems unusual for a species that has been reported to have a base chromosome

number of 9 and to exist largely as hexaploid cytotypes (Patil et al. 1961; Rangaswamy 1972; Sisodia 1970; Dujardin and Hanna 1984) but to occasionally display two B-chromosomes (Sindhe 1976). Among the 56 chromsomes of *P. squamulatum*, there was only one chromosome that showed hybridization with a probe mixture containing 12 apomixis-linked markers. This result was direct evidence to support the hemizygous nature of the ASGR (Ozias-Akins et al. 1998).

4. Apospory has been transmitted from *P. squamulatum* into pearl millet (*P. galucum*) through a backcross program (Bashaw and Hanna 1990; Hanna et al. 1993). FISH/GISH were employed to investigate the transmission of chromosomes from *P. squamulatum* through the backcross generations. There were three *P. squamulatum* chromosomes observed in BC3. Only one of them, however, showed hybridization to a probe mixture containing 12 apomixis-linked makers (the ASGR-carrier chromosome). Because the selection for aposporous embryo sacs was carried out during backcrossing, the number of *P. squamulatum* chromosomes varied from 1 to 3 among different lines in each backcross generation, BC5, BC6 or BC7. All plants with the ASGR-carrier chromosome were apomictic even when other *P. squamulatum* chromosomes were present in the backcross. Sexual reproduction was recovered in the plants that did not show the hybridization signal to the mixture of 12 apomixis-linked markers. One chromosome was sufficient to confer apomixis in BC lines. No obvious association was observed between the development of aposporous embyo sacs and the number of *P. squamulatum* chromosomes.
5. The *P. squamulatum* chromosomes in BCs all behaved as univalents during meiosis and randomly sorted toward either pole at anaphase I. The chance for crossover between *P. squamulatum* chromosomes and *P. glaucum* chromosomes

would be very small. The *P. squamulatum* chromosome should be fragmented before the ASGR can be integrated into the *P. glaucum* genome through the conventional breeding program.

6. Further investigation of meiosis of in *P. squamulatum* is needed to determine the behavior of the apomixis-related chromosome. More detailed characterization of the ASGR would help us to understand the genetic and developmental consequences of this region's unusual genomic structure.

References:

Bashaw EC, Hanna WW (1990) Apomictic reproduction. In reproductive versatility in the grasses. G.P. Chapman, ed (Cambridge University Press). pp. 100-130

Dujardin M, Hanna WW (1984) Microsporogenesis, reproductive behavior, and fertility in five *Pennisetum* species. Theoret Appl Genet 67:197-201.

Hanna WW, Dujardin M, Ozias-Akins P, Lubbers E, Arthur L (1993) Reproduction, cytology, and fertility of pearl millet X *Pennisetum squamulatum* BC_4 . J Hered 84:213-216

Ozias-Akins P, Roche D, Hanna WW (1998) Tight clustering and hemizygosity of apomixis-linked molecular markers in *Pennisetum squamulatum* implies genetic control of apospory by a divergent locus that may have no allelic form in sexual genotypes. Proc Natl Acad Sci USA 95:5127-5132

Patil BD, Hardas MW, Joshi AB (1961) Auto-alloploid nature of *Pennisetum squamulatum* Fresen. Nature 189:419-420

Rangaswamy SRS (1972) Cytological studies on diploid and polyploid taxa of the genus *Pennisetum* Rich. Genetica 43:257-273

Sindhe ANR (1976) Supernumerary chromosomes in *Pennisetum squamulatum* Fresen. Current Sci 45:526

Sisodia KPS (1970) Cytological studies on some species in genus Pennisetum. Theor Appl Genet 40:26-31

APPENDIX

METHODS FOR CHROMOSOME PREPARATION

A.1 Meiotic chromosome spreads for GISH/FISH

A.1.1. Material

Collection times were dependent on the desired stage of development. For metaphase I chromosome spreads, heads were collected when approximately half exserted from the boot. This condition, however, changed with different lines, seasons and growth rate. *Pennisetum squamulatum* was collected when the inflorescence began to exsert from the boot, while *P. glaucum* was collected as described above. When drought slows growth, heads were collected while still in the boot. The best external indicator of developmental stage was the color of the spikelets. Green was too late, white was too early and metaphase I was usually in the yellowish section. A pale yellow color of the anthers appeared to be the best stage. There are two flowers in each spikelet. The staminate flower developed slower than the hermaphroditic dominant flower and also can be used if the hermaphroditic flower is too old.

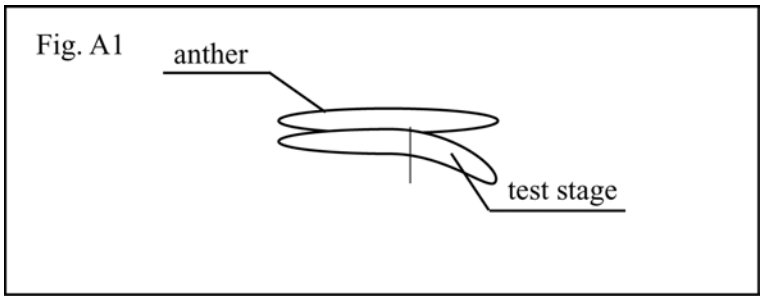
A.1.2. Fixation

Each inflorescence was checked at ~2 cm increments to select the section including metaphase I chromosomes. Tissue was fixed in 3 ethanol : 1 acetic acid (see 1.3). Chromosomes were observed when stained with 2% acetocarmine (use within 6 months of preparation). To enhance the speed of fixation, inflorescence segments immersed in fixative were placed under vacuum for about 10-15 minutes. Fixed material was stored at 4^{o}C. The fixative was changed when the color became yellowish. Materials were fixed

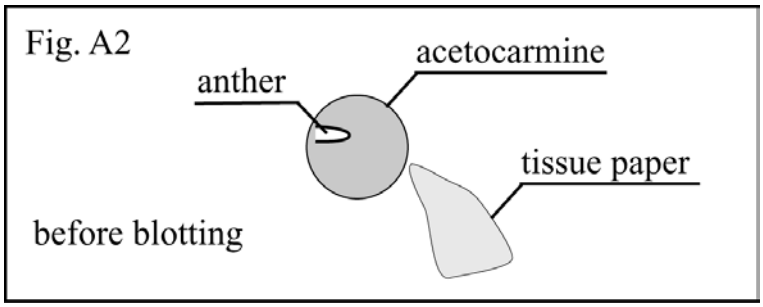
for at least one week and no longer than 6 months before being used for chromosome spreads. Chromosome spreads from material kept longer than 6 months showed poor morphological characteristics, although they can be used to collect certain types of data if no other materials are available.

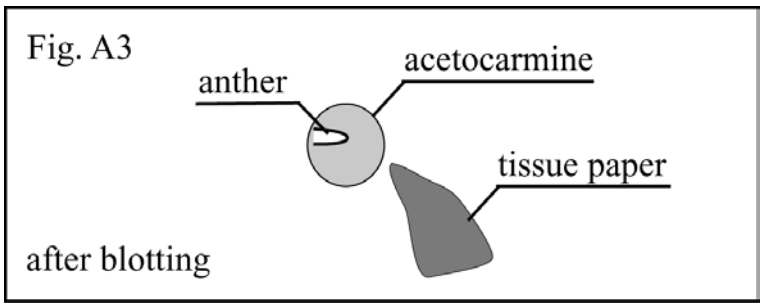
A.1.3. Collection of anthers containing the stage of meiosis I

Spikelets were excised from the fixed inflorescence and kept in water to avoid drying. As little as one locule of an anther was used for checking the stage of meiosis under the microscope (Fig.A1).



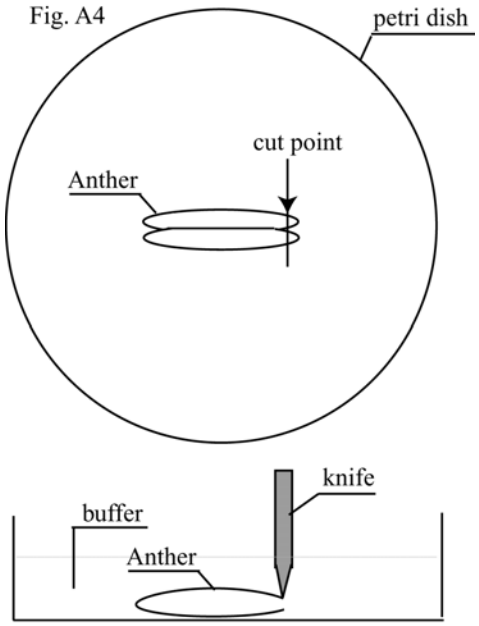
To observe the chromosomes, a small drop of acetocarmine was applied onto the slide and the anther section was pressed several (3-5) times with the flat surface of a needle. The anther wall was removed and the extruded meiocytes were covered with a coverglass. Observations were made under the microscope at 200x, bright field. If the stain was not fresh, the slide was heated slightly to enhance staining. Excess stain can dilute the density of cells, and it will take a longer time to check a predetermined number

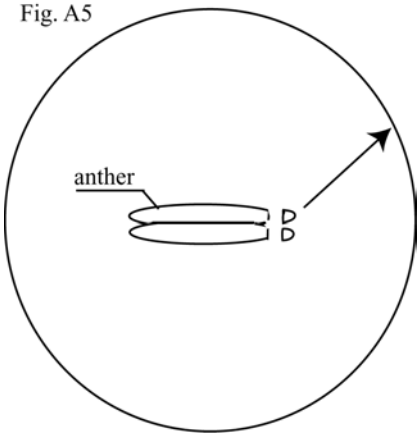




of cells. If too much stain is added initially to the slide, a piece of tissue paper can be used to blot some of the stain before the anther section is pressed (Fig.A2-A3).

If the flower was at the ideal stage, all the anthers from the same flower were placed into a petri dish containing 1ml of 10 mM citrate buffer (pH 4).





A.1.4. Collecting the pollen mother cells (PMCs)

Selected anthers were cut at one of the ends (Fig. A4) with a surgical blade (No. 11), and moved to one side of the petri dish (Fig. A5). Each anther was gently pressed from the uncut end with the edge of a knife blade (Fig. A6) and moved towards the open end. The PMCs were extruded from the anther (Fig. A7).

Fig. A6

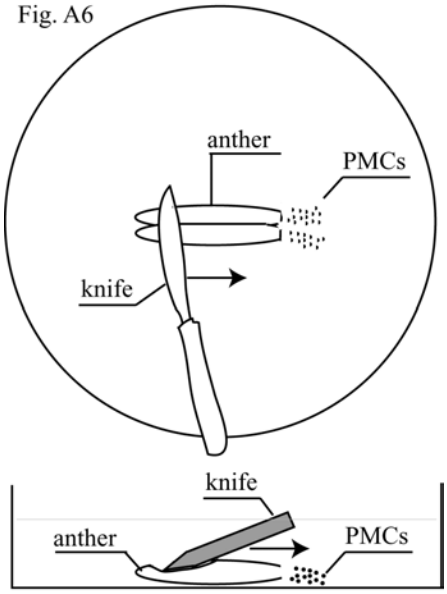
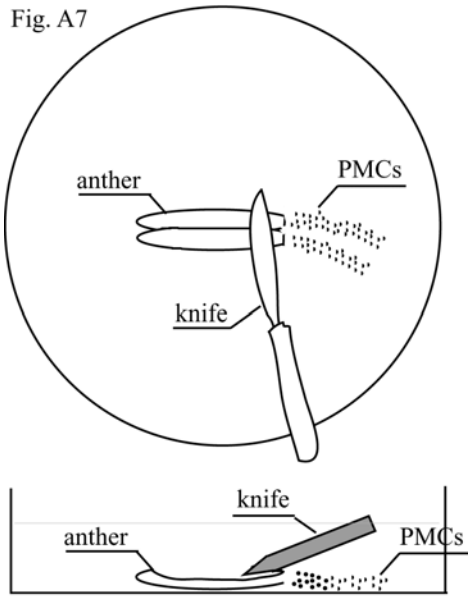


Fig. A7

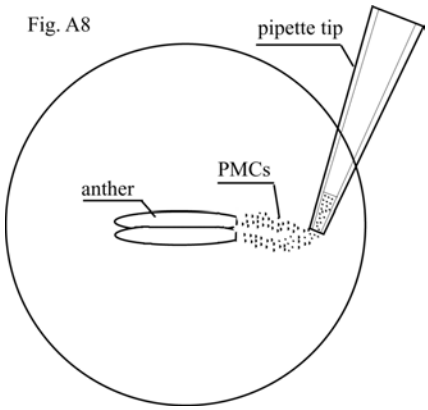


Movement of the knife or forceps should be very careful and slow. PMCs were easily dispersed, but difficult to collect. PMCs suspended in citrate buffer were transferred with a pipette into a 0.5 ml microcentrifuge tube (Fig. A8). Avoid transferring too much buffer.

A.1.5. Digestion of PMCs

Enzyme mixture (0.3% cellulase RS, 0.3% pectolyase Y23, 0.3% cytohelicase in 30 mM citrate buffer, pH 4.5) (Zhong et al.1996) (1/4 of the total PMC collection volume) was added to the microcentrifuge tube, mixed gently, and digestion was carried out at 37^{o}C for 30-45 minutes. The digested PMCs should be stored on ice when digestion is finished.

Fig. A8



A.1.6. Washing of PMCs

Tubes were spun at 600 x **g** for 3 minutes and the supernatant was removed with a pipette. Three volumes of 60% acetic acid were added to gently resuspend the PMCs which were placed on ice for 10-15 minutes. This step clears the cytoplasm. Spin again at 600 x **g** for 3 minutes, and resuspend in the volume of 5 µl of 3 ethanol : 1 acetic acid times the number of slides to be prepared with the dropping method. Each slide is prepared with 5µl of digested PMC suspension. If chromosomes are spread by surface tension rather than by dropping, resuspend in the volume of 4 µl 60% acetic acid times the number of slides.

A.1.7. Slide preparation

Boil new glass slides for 10 minutes (Dr. Honggou Yu, personal communication) in deionized H_2O and then store in 100% ethanol. Dry in the laminar flow hood at least 10 minutes before use. I never compared the effect of boiling slides with no treatment. The reason for boiling is to clean the slides thoroughly.

A.1.8. Making chromosome spreads

A.1.8.1 Dropping

The method I used is modified from the dropping method of Schwarzacher and Heslop-Harrison (2000). A volume of 5 µl did not form a sufficiently large droplet to fall unaided from the tip of the pipette. Therefore, a repetitive pipette (Distriman, Gilson, France) was used to eject 5 µl of the PMC suspension onto a slide from a distance of about 4 cm. This method was very effective for spreading and much easier to use than to drop from a distance of 50 cm, which makes it difficult to control where the droplet

lands. The slides were then dried in a laminar flow hood and were ready for pretreatment for ISH.

A.1.8.2. Surface tension

Apply 3.5 µl PMC suspension to the slide and cover the drop with a 22 x 22 mm coverglass. The liquid spread between the slide and coverglass by capillary action. Since the gap between coverglass and slide gets thinner as the liquid spreads out, the objective is to apply only enough suspension to allow the liquid to spread almost to the edge of the coverglass. Applying an excess amount of suspension will cause the cells to overlap. If there is any obvious bubble, gently tap on the top of the coverglass with the tips of the forceps to force it out. The slide was then dipped into liquid nitrogen for 30 seconds to freeze. The coverglass was quickly removed with a razor blade, and the slide was placed into a slide jar with 100% ethanol for a few minutes. The slide was dried in the laminar flow hood. The slides are now ready for pretreatment.

A.1.9. For fixed materials stored more than one year

After collecting the staged anthers, PMCs were digested directly on a slide. Apply 5 µl of enzyme mixture (0.3% cellulase RS, 0.3% pectolyase Y23, 0.3% cytohelicase in 30 mM citrate buffer, pH 4.5) (Zhong et al.1996) on the slide and put one half of the anther on it. Cut one end of the anther and press gently with a needle several times. Remove the anther wall and place a coverglass (22 x 22 mm) over the PMC suspension. Keep the slide in a humid chamber at 37^{o}C for 10-15 minutes. Remove slide, cover the slide with a piece of tissue paper and hold the cover slip with one hand. Gently press the coverglass straight down with thumb of the other hand. Dip the slide into liquid nitrogen for 30 seconds, then remove the coverglass with a razor blade, keep the slide in 60% acetic acid

for over 30 minutes, and then transfer to 100% ethanol for 3 minutes. Air-dry the slides and hold for pretreatment.

A.1.10. Checking the chromosome spreads

It will save time if slides are checked for quality of spreads before using them for ISH. First, those slides that do not pass the criteria set for quality can be discarded. Secondly, the positions of the best chromosome spreads on each slide can be recorded and these spreads can be easily relocated.

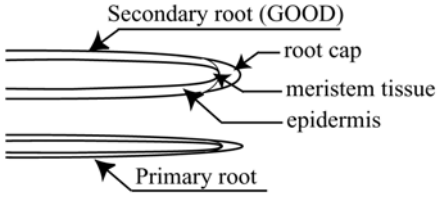
A.2. Mitotic chromosome preparation from root tips for GISH/FISH

The main goal of this method is to try to remove as much debris and undesired tissue as possible, leaving only the meristem tissue. This will be achieved in two steps.

A.2.1. Collecting root tips

If a successful FISH were rated as a 10, a good chromosome preparation can be rated at least 60% of it. To make a good preparation from root tips, select healthy, actively growing root tips. As for *Pennisetum*, root tips collected from plants grown in clay pots are better than seedlings in a petri dish, and secondary roots are better than the primary root. A good root should possess these morphological characteristics: growing straight forward, thick or fat with a smooth surface and no root hairs visible around the whitish growing portion (Fig. A9). Collected root tips should be washed with tap water before fixation.

Fig. A9



A.2.2. Fixation

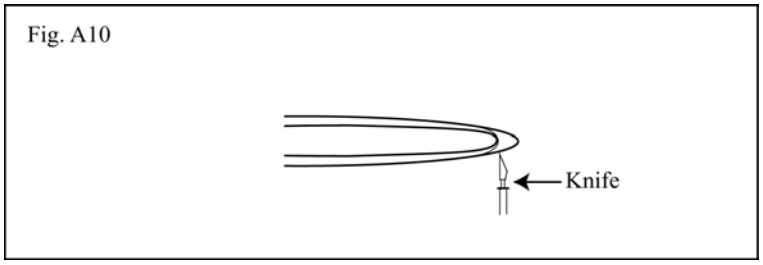
After a wash in water, root tips were transferred into saturated mono-bromonaphthalene in H_2O for 1.5-2 hours on ice to induce condensation of chromosomes. Subsequently, root tips were fixed in 3 ethanol : 1 acetic acid at room temperature. The fixation time should be at least 4 days at room temperature. The fixed material can be stored at room temperature and used for two months or longer.

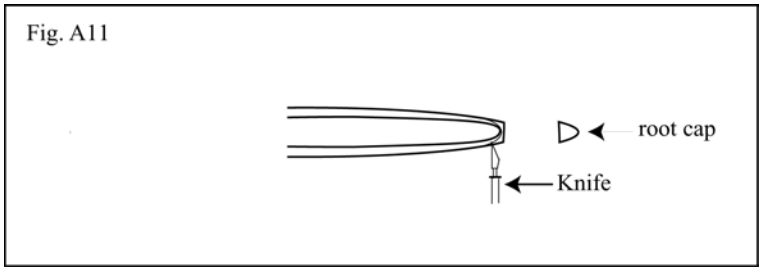
A.2.3. Selecting root tips for digestion

Some root tips became transparent in the meristematic region with a certain amount of shrink after fixation. These root tips typically had very few or no dividing cells and were discarded.

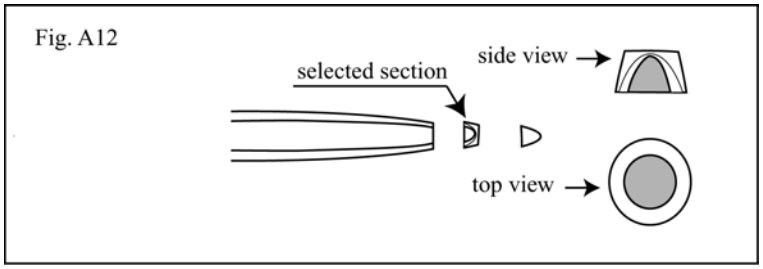
A.2.4. Cut the root tips

Dividing cells are found primarily in the meristem of the root apex, which is only a very small fraction of the root tip. Root cap, epidermis, cortex and stele tissue composed the main body of a root tip. Therefore, root tips must be cut on a slide under a dissection



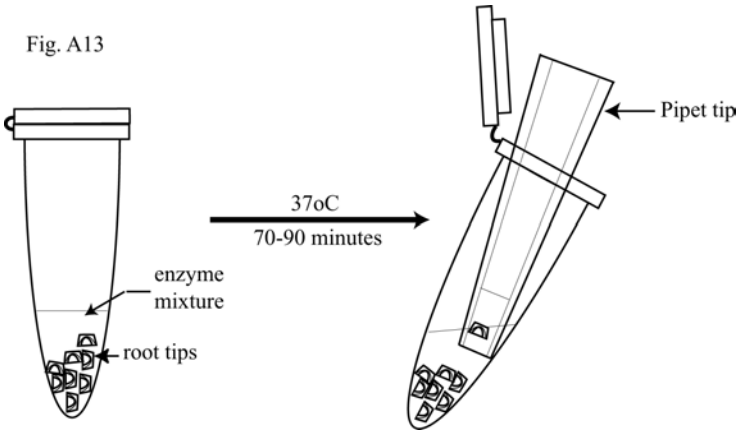


microscope to remove all but the meristem tissue (Fig. A10-A12). Attention should be paid here that the first cut should not be too deep, otherwise the meristem will be removed with the rootcap. If in doubt, it is better to cut less because there is another step that will allow the undesired part to be removed (notice in Fig. A11-A12 that some root cap remains).



A.2.5. Digestion of the selected sections of root tips

Transfer the selected sections into a 1.5 ml microcentrifuge tube containing enzyme mixture (0.3% cellulase RS, 0.3% pectolyase Y23, 0.3% cytohelicase in 30 mM citrate buffer, pH 4.5) (Zhong et al.1996) and incubate at 37^{o}C for 70-90 minutes (Fig. A13). The volume of enzyme mixture should be 10 µl times the number of root tips to be digested. A total of 50 µl of enzyme mixture should be enough for 5 root tips to be digested.

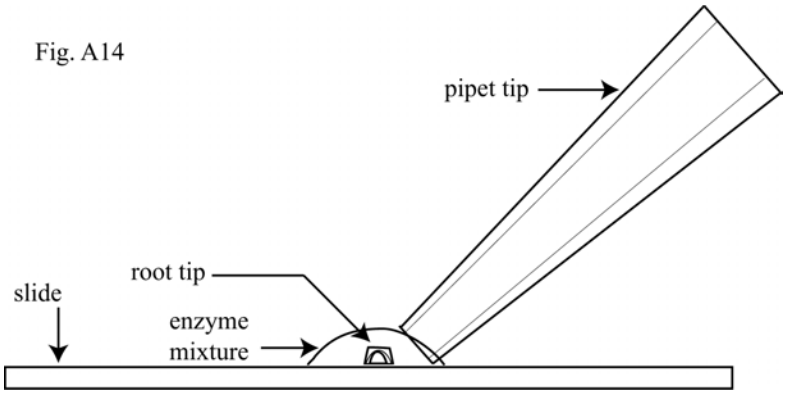


Tubes were placed on ice as soon as the digestion was finished to prevent over digestion. Digestion time was adjusted to make sure the meristem tissue was just digested while the epidermal tissue and the remaining root cap tissue were not completely digested. The thinner primary cell walls of the rapidly dividing meristem cells were more easily digested than elongated or matured cells that may have secondary wall structure. Before the root epidermis was completely digested, it was easy to handle the digested root tips with forceps.

A.2.6. Remove the undesired tissue of root epidermis and remaining root cap

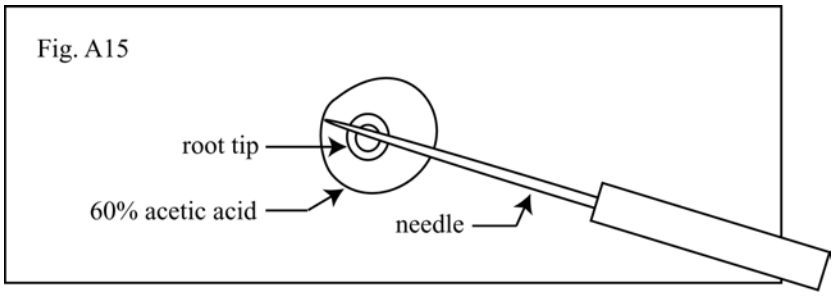
The digested root tips were transferred with a pipette tip from a microcentrifuge tube onto a slide. The pipette tips were cut to enlarge the opening sufficiently for the root sections (Fig. A13). Each root tip was transferred to a cleaned slide (Fig. A14).

Fig. A14



The digestion mixture was removed as much as possible by blotting with a piece of tissue paper. Attention should be paid here with the blotting. It is very easy for the

Fig. A15



digested root tip to be picked up by the tissue paper. Blotting should be done under the dissection microscope. Subsequently, add 3 µl of 60% acetic acid to the root tip on the slide. After adding acetic acid, gently and quickly press the root tip from the top several times with a needle (Fig. A15). By this time the root epidermis should look like an earring.

The debris can be removed with both a surgical blade and a needle under the dissection microscope. Move the knife till the knife-edge touches the acetic acid; the liquid and the cells will flow toward the knife blade and the debris will be left behind. Then move the blade further toward the debris and use a needle to move the debris to the upper side of the blade (Fig. A16-A18).

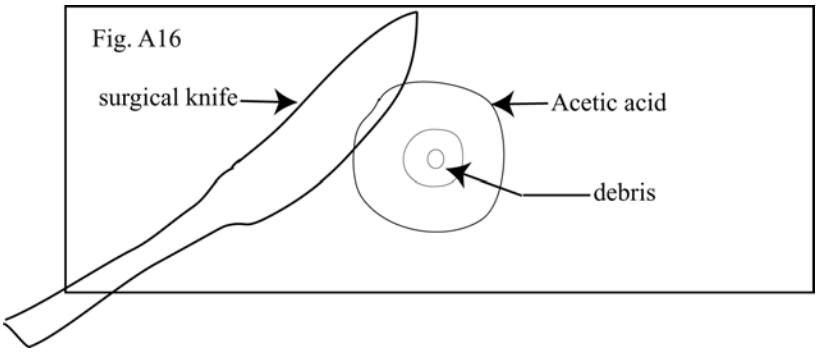


Fig. A16

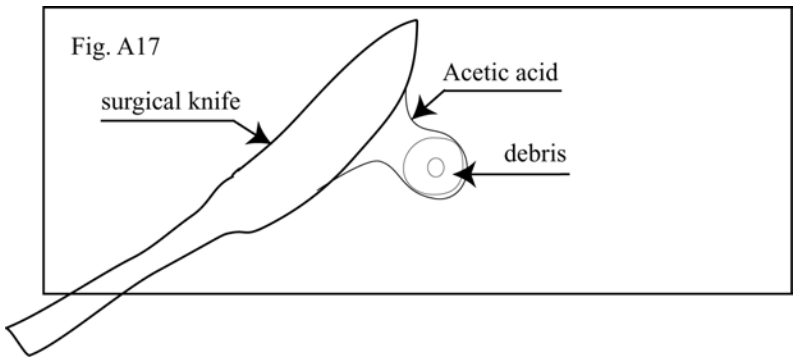
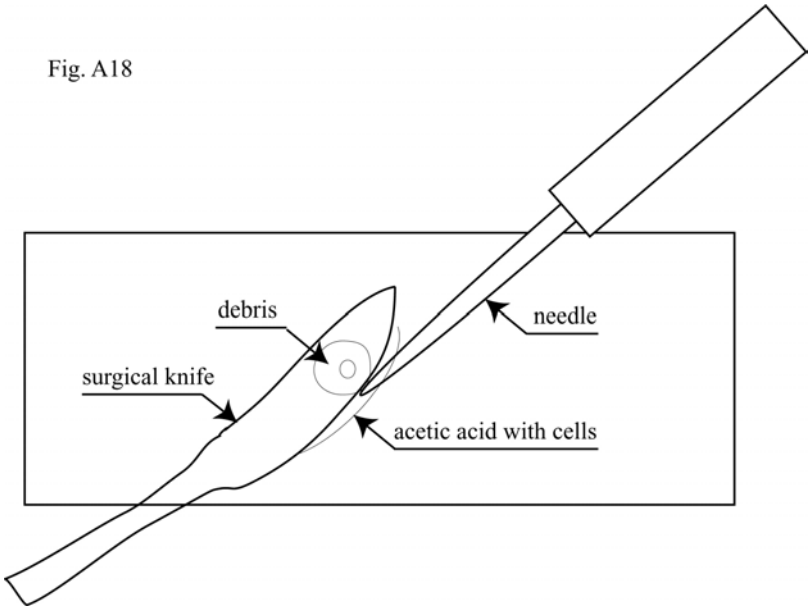


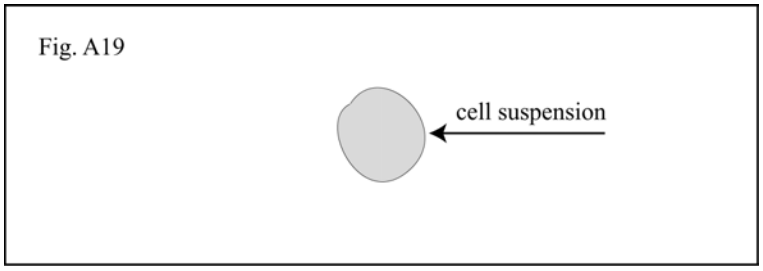
Fig. A17

Fig. A18



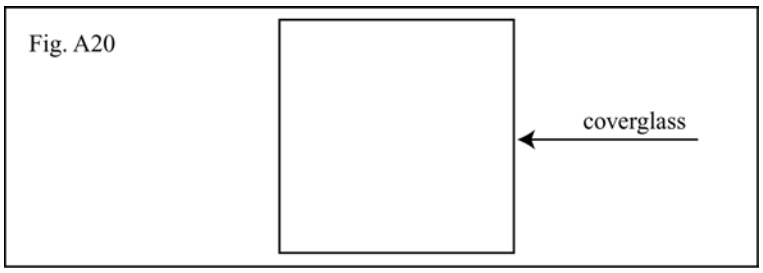
After the debris is removed, only acetic acid with evenly suspended meristem cells should remain. No obvious tissue should be seen under the dissection microscope (Fig. A19).

Fig. A19



Place a coverglass on top of the liquid (Fig. A20) and remove any bubbles as much as possible by tapping the top of the coverglass with the blunt end of the forceps. To avoid sideways movement of the cover glass, hold the coverglass by the edge when tapping.

If every step is carried out quickly, the liquid should be just enough to spread to fill the area under a 22 x 22 mm coverglass.



A.2.7. Removing the cover glass

Place the slide in liquid nitrogen for 30 seconds and remove the coverglass quickly with a razor blade. The slides are then kept in a slide jar with 60% acetic acid for 30 minutes. Dehydrate the slide in 100% ethanol for a couple of minutes and air-dry the slide in the laminar flow hood. The slides are now ready to check for further experimentation.

A.3.Recipes

A.3.1. 100 mM (10 x) citrate buffer:

For 50 mls of H_2O,	add	0.5169 g	citric acid
		0.6849 g	Na^+ citrate
		pH should be around 4.5.	

A.3.2. 2% acetocarmine:

Add 2 g of carmine in 100 ml of 45% acetic acid, heat slowly until the color turns, boil gently for 5 minutes, let it cool down and filter through whatman filter paper No.1 (Whatman Ltd. Maidstone, England) (Parrott 1999).

A.3.3. Cocktail for GISH/FISH

Reagent	Add amount (μl)	Final concentration
Formamide (100%)	4-6	50%
Dextran sulphate (50%)	4	10%
20X SSC	2	2X
Salmon sperm DNA (10μg/μl)	1	500 ng/μl
Block DNA (800 ng/μl)	3	120 ng/μl
Probe	3-4(GISH)	4-6 ng/μl
	3-4(FISH)	0.6-1.2 ng/μl
Total	20 μl	

References:

Parrott, W. (1999) Cytogenetics handout for 1999 fall cytogenetics class

Schwarzacher, T. and Heslop-Harrison, P. (2000) Practical in situ hybridization. Springer-Verlag New York Inc. pp. 60

Zhong, X. B., Fransz, P. F., Eden, J. W. V., Zabel, P., Kammen, A. and de Jong, J. H. (1996) High-resolution mapping on pachytene chromosomes and extended DNA fibers by Fluorescence in situ hybridization. Plant Molecular Biology. 14: 232-242