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# Molecular markers shared by diverse apomictic *Pennisetum* species

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**Abstract** Two molecular markers, a RAPD (randomly amplified polymorphic DNA) and a RFLP/STS (restriction fragment length polymorphism/sequence-tagged site), previously were found associated with apomictic reproductive behavior in a backcross population produced to transfer apomixis from *Pennisetum squamulatum* to pearl millet. The occurrence of these molecular markers in a range of 29 accessions of *Pennisetum* comprising 11 apomictic and 8 sexual species was investigated. Both markers were specific for apomictic species in *Pennisetum*. The RFLP/STS marker, UGT 197, was found to be associated with all taxa that displayed apomictic reproductive behavior except those in section *Brevivalvula*. Neither UGT197 nor the cloned RAPD fragment OPC-$04_{600}$ hybridized with any sexually reproducing representatives of the genus. The cloned $C04_{600}$ was associated with 3 of the 11 apomictic species, *P. ciliare*, *P. massaicum*, and *P. squamulatum*. UGT197 was more consistently associated with apomictic reproductive behavior than $OPC04_{600}$ or cloned $C04_{600}$, thus it could be inferred that UGT197 is more closely linked to the gene(s) for apomixis than the cloned $C04_{600}$. The successful use of these probes to survey other *Pennisetum* species indicates that apomixis is a trait that can be followed across species by using molecular means. This technique of surveying species within a genus will be useful in determining the relative importance of newly isolated markers and may facilitate the identification of the apomixis gene(s).

**Key words** Apomixis · Agamospermy · Pearl Millet · Interspecific hybrids · RFLP · RAPD

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## Introduction

The major cultivated species in the genus *Pennisetum* is pearl millet, *P. glaucum* (L.) R.Br. (Terrel et al. 1986), which reproduces sexually (Brunken et al. 1977). Apomixis, asexual reproduction through seed (Nogler 1984), occurs in many wild species of *Pennisetum* in the form of pseudogamous apospory (Dujardin and Hanna 1984; Jauhar 1981). If apomictic reproduction could be easily controlled in a cultivated crop, hybrids could be readily maintained and stable new cultivars could be developed more quickly. Regions of the world that rely on low-input agriculture would benefit from hybrid apomictic genotypes that should require fewer resources for maintenance than sexually reproducing hybrids.

Substantial efforts have been made to transfer apomixis from the tertiary gene pool of *Pennisetum* (*P. setaceum* (Forsk). Chiov., *P. orientale* L. C. Rich., and *P. squamulatum* Fresen) to pearl millet (Hanna and Dujardin 1985). The most successful attempt to date uses a backcross (BC) program initiated with a *P. squamulatum*/*P. glaucum*/*P. purpureum* complex cross as the male parent and pearl millet as the recurrent female parent (Dujardin and Hanna 1989). *P. purpureum* was used as a bridging species to maintain male fertility in the offspring of this complex trispecific cross. The backcross program produced a single obligately apomictic individual, designated $BC_3$, from which a $BC_4$ population was derived. Two molecular markers for apomixis were identified in the apomictic $BC_3$ line (Ozias-Akins et al. 1993). One marker was a RAPD (randomly amplified polymorphic DNA), $OPC04_{600}$, and the other was a RFLP (restriction fragement length polymorphism), UGT197. UGT197 was converted to a PCR (polymerase chain reaction)-amplifiable STS (sequence-tagged site). These molecular markers were unique to the *P. squamulatum* parent and were not found in the sexual parents. In general, these markers were not found in $BC_4$ sexual plants but were found in $BC_4$ obligately apomictic plants.

Cosegregation of $OPC04_{600}$, UGT197 STS, and apomixis in the $BC_4$ population plus infrequent transmission ($< 5\%$) of this linkage group from $BC_3$ to $BC_4$ suggested that the chromosome of interest did not have a homolog or homeolog in $BC_3$ with which pairing and recombination could occur. Only one other chromosome from *P. squamulatum* was detected in the apomictic $BC_3$ line. The two chromosomes of *P. squamulatum* assorted independently in the $BC_4$ generation (Ozias-Akins et al. 1993). With no apparent recombination between alien and pearl millet chromosomes, any markers on the apomixis-associated chromosome would appear strictly cosegregational with genes on that chromosome including the gene(s) for apomixis. With strict cosegregation, genetic distance between any molecular marker and the apomixis gene(s) in our backcross program could not be determined.

*Pennisetum* species used in the present study have basic chromosome numbers of 5, 7, 8, and 9, which suggests that chromosome repatterning is likely to have occurred during evolution within the genus. If a common genetic origin/mechanism for apomixis throughout the genus and the subfamily Panicoideae is assumed, as suggested by Brown and Emery (1958), markers more distant from the gene(s) for apomixis should have a greater probability of becoming rearranged during speciation. Conversely, markers that remain associated with apomixis across species should have a higher probability of being closely linked to apomixis. Simple recombination and segregation affect linkage of markers and traits. Multiple translocations have repatterned the chromosomes of pepper and tomato (Tanksley et al. 1988), sorghum and maize (Whitkus et al. 1992), and rye and wheat (Devos et al. 1993). As in recombinational events, translocation-produced breakpoints would be more likely to occur between distantly linked loci, thereby separating loci to different chromosomes. Species such as potato and tomato, in which inversions and not translocations are common (Tanksley et al. 1992), show a repatterning of chromosomes that could affect PCR priming sites and thus eliminate PCR-based markers. Markers that remain linked with a phenotypic trait would be valuable in a breeding program for marker-assisted selection or perhaps in the ultimate isolation of the gene(s) controlling the trait via map-based cloning. The genus *Pennisetum* contains many apomictic species.

**Table 1** Accession descriptions for the plants used in this experiment grown at the Coastal Plain Experiment Station (CPES)

| CPES designation | Species | Taxonomic section[a] | Reproductive behavior | Source of plant material |
|---|---|---|---|---|
| PS938 | *P. alopecuroides* (L.) Spreng. | Not assigned | Sexual[b] | Mary Meyer |
| PS2 | *P. basedowii* Summerhayes & Hubbard | Not assigned | Sexual[c] | PI257782 |
| Tift23BE | *P. glaucum* (L.) R. Br. | *Penicillaria* | Sexual[d] | CPES-UGA |
| PS156 | *P. hohenackeri* Hochst. ex Steud. | *Gymnothrix* | Sexual[e] | ICRISAT |
| PS38 | *P. nervosum* (Nees) Trin. | Not assigned | Sexual[b] | Mexico |
| PS187 | *P. nervosum* (Nees) Trin. | Not assigned | Sexual[b] | Argentina |
| N109 | *P. purpureum* Schumach | *Penicillaria* | Sexual[f] | Spain |
| N168 | *P. purpureum* Schumach | *Penicillaria* | Sexual[f] | Kenya (Ibrahim) |
| PS29 | *P. ramosum* (Hochst.) Schweinf. | *Gymnothrix* | Sexual[e] | P13311699 |
| PS63 | *P. ramosum* (Hochst.) Schweinf. | *Gymnothrix* | Sexual[e] | deWet & Harlan |
| PS243 | *P. schweinfurthii* Pilger | *Heterostachya* | Sexual[b] | ICRISAT (IP8627) |
| PS163 | *P. subangustum* (Schum.) Stapf & Hubb. | *Brevivalvula* | Apospory[b] | Nigeria |
| PS185 | *P. ciliare* (L.) Link | Not assigned | Apospory[g] | Llano |
| PS186 | *P. ciliare* (L.) Link | Not assigned | Apospory[g] | Nueces |
| PS32 | *P. flaccidum* Griseb. | Not assigned | Apospory[h] | PI271601 |
| PS95 | *P. flaccidum* Griseb. | Not assigned | Apospory[h] | D. Timothy |
| PS9 | *P. massaicum* Stapf | *Gymnothrix* | Apospory[i] | PI365021 |
| PS962 | *P. macrourum* Trin. | Not assigned | Apospory[c] | Zimbabwe |
| PS12 | *P. orientale* L. C. Rich. | Not assigned | Apospory[j] | PI315867 |
| PS13 | *P. orientale* L. C. Rich. | Not assigned | Apospory[j] | PI218097 |
| PS16 | *P. pedicellatum*Trin. | *Brevivalvula* | Apospory[k] | PI266185 |
| PS304 | *P. pedicellatum* Trin. | *Brevivalvula* | Apospory[k] | Senegal (Harlan) |
| PS19 | *P. polystachyon*(L.) Shult. | *Brevivalvula* | Apospory[c] | PI189347 |
| PS264 | *P. polystachyon* (L.) Shult. | *Brevivalvula* | Apospory[c] | PI284770 |
| PS22 | *P. setaceum* (Forsk.) Chiov. | *Eu-pennisetum* | Apospory[e] | PI300087 |
| PS25 | *P. setaceum* (Forsk.) Chiov. | *Eu-pennisetum* | Apospory[e] | PI364994 |
| PS24 | *P. squamulatum* Fresen | *Heterostachya* | Apospory[c] | PI248534 |
| PS158 | *P. squamulatum* Fresen | *Heterostachya* | Apospory[c] | ICRISAT |
| PS249 | *P. villosum* R. Br. ex Fresen | *Eu-pennisetum* | Apospory[e] | Israel |

[a] Stapf and Hubbard 1934, all species have not been put into a section

[b] unpublished data.

[c] Dujardin and Hanna 1984

[d] Brunken et al. 1977

[e] Narayan 1962

[f] Hanna 1981

[g] Synder et al. 1955

[h] Chatterji and Timothy 1969a

[i] D'Cruz and Reddy 1968

[j] Chatterji and Timothy 1969b

[k] Kalyane and Chatterji 1981

The present study was conducted to determine if other *Pennisetum* species carry the molecular markers previously shown to be linked to apomixis, thereby enhancing the utility of the markers and species for genetic studies and gene introgression.

## Materials and methods

### Plant material

Descriptions of the plant material are found in Table 1. Herbarium specimens of each accession were collected. One accession, PS9, originally labeled as *P. macrourum*, was reclassified as *P. massaicum* [syn. *P. mezianum* Leeke (Jauhar 1981)]. *Pennisetum ciliare* (L.) Link is synonymous with *Cenchrus ciliaris* L.

### DNA isolation

Plant DNA was isolated following the method of Tai and Tanksley (1990) modified for fresh-frozen tissue. Inner, whorled leaf tissue was ground to a fine powder in liquid nitrogen and then added to the extraction buffer (approximately 75 ml buffer per 10 g tissue). Tissue was incubated in extraction buffer for 1 – 3h and subsequently processed according to the published protocol. DNA was quantified on a TKO-100 fluorometer (Hoefer Scientific Instruments, San Francisco, Calif.).

### PCR DNA amplification

The molecular markers linked to apomixis that were used in this study have been described by Ozias-Akins et al. (1993). PCR reaction mixes (50 μl) contained 50 m*M* Tris-HCL (pH 9.0), 50 m*M* KCL, 1.5 m*M* MgCl$_2$, 0.1% Triton X-100, 100 *μM* each of dATP, dCTP, dGTP, and dTTP, 0.5 μ*M* of each primer, 25 ng genomic DNA, and 0.5U *Taq* DNA polymerase (Promega Corp, Madison, Wis.). Cycling was performed on a Perkin-Elmer/Cetus DNA Thermal Cycler (Norwalk, Conn.) programmed as follows for primer OPC-04 (Operon Technologies, Alameda, Calif., 5′ CCGCATCTAC 3′): 3 cycles of 1 min at 97 °C, 1 min at 42 °C, and 2 min at 72 °C; followed by 32 cycles of 1 mm at 94 °C, 1 min at 42 °C, and 2 min at 72 °C with a 3-s auto-segment extension of each cycle. The cycling parameters for STS marker UGT197 (synthesized by the Molecular Genetics Facility, University of Georgia; forward primer 5′ CTGCAGACCTCCAAACAG 3′; reverse primer 5′ CTGCAGCATGTGAACCAT 3′) were 3 cycles of 1 min at 97 °C, 1 min at 55 °C, and 2 min at 72 °C; followed by 32 cycles of 1 min at 94 °C, 30 at 55 °C, and 30 s at 72 °C with a 3-s auto-segment extension of each cycle.

### Electrophoresis, blotting, and hybridization

PCR-amplified DNA was electrophoresed in 2% NuSieve: SeaKem 1:1 agarose (FMC Corp, Rockland, Me.) in 1 × TBE. Genomic DNA was digested with *Dra*I (Promega Corp, Madison, Wis.) according to the maufacturer's instructions and was electrophoresed in 0.8% SeaKem agarose in 1 × TBE. DNA was transferred to nylon membrane (Genescreen Plus, NEN, DuPont, Boston, Mass.) according to the manufacturer's instructions. OPC04$_{600}$ was cloned from PS26, a *P. squamulatum* germ plasm introduction, using the pGEM-T vector system (Promega Corporation, Madison, Wis.) according to the manufacturer's instructions. Southern blots of *Dra*I-digested genomic DNA were hybridized with radiolabelled cloned C4$_{600}$ that was PCR amplified from plasmid using M13 and M13r primers. Gel-purified UGT197 insert was radiolabelled and hybridized to Southern blots of both *Dra*I-digested genomic DNA and DNA amplified with UGT197 STS primers. Probes were radiolabelled with [$^{32}$P] by the random hexamer method according to the maufacturer's instructions (BRL, Gaithersburg, Md. and Promega Corp, Madison, Wis.). Southern blots were prehybridized and hydridized at 65 °C in 6 × SSPE, 1% SDS, and 50 μg/ml sheared salmon sperm DNA (50 ml prehybridization solution/400 cm$^2$ membrane reduced to 20 ml fresh solution/400 cm$^2$ for hybridization). Hybridized blots were washed at a final stringency of 0.1 × SSPE with 1% SDS at 65 °C for 30 min.

## Results and discussion

PCR amplification of DNA using RAPD primer OPC04 produced numerous DNA fragments from the sexual and apomictic *Pennisetum* species as detected on an ethidium bromide-stained gel (Fig. 1). Many of the species displayed amplified fragments comparable in size to the OPC04$_{600}$ of *P. squamulatum*. Size comparison alone was misleading since hybridization of a genomic Southern blot showed that the species with DNA homologous to cloned C4$_{600}$ were fewer than those implied by the comparison of band sizes in the ethidium bromide-stained gel (Fig. 2). Three of the apomicitic species (*P. squamulatum*, *P. ciliare*, and *P. massaicum*) showed both strong amplification with the

**Fig. 1** Ethidium bromide-stained gel of PCR amplification of DNA from *Pennisetum* species using the OPC-04 RAPD primer. *Arrows* indicate the location of the informative 600-bp bands

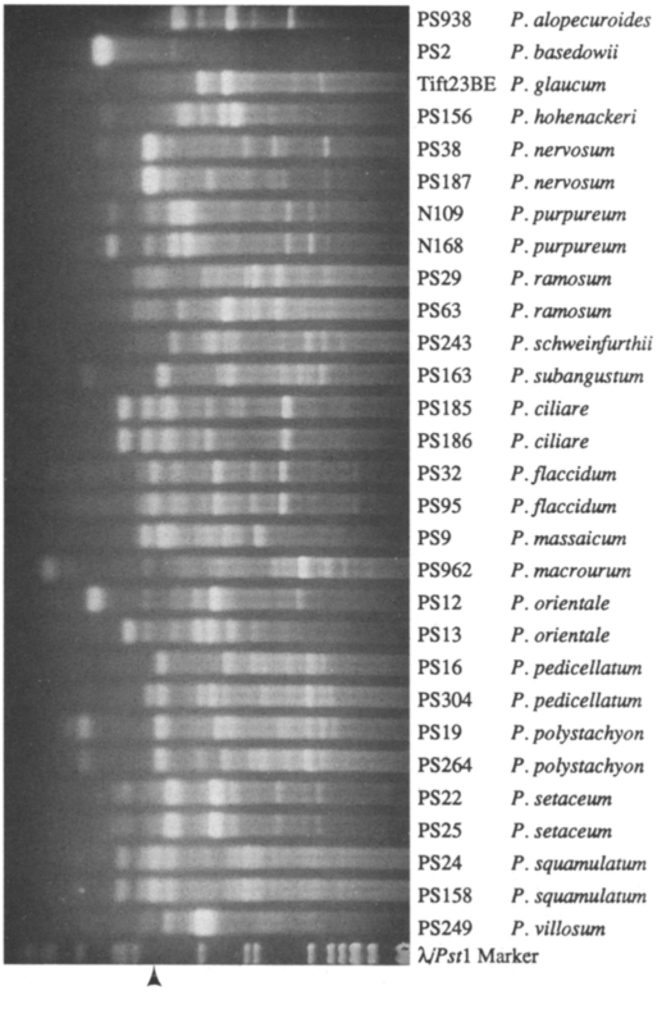
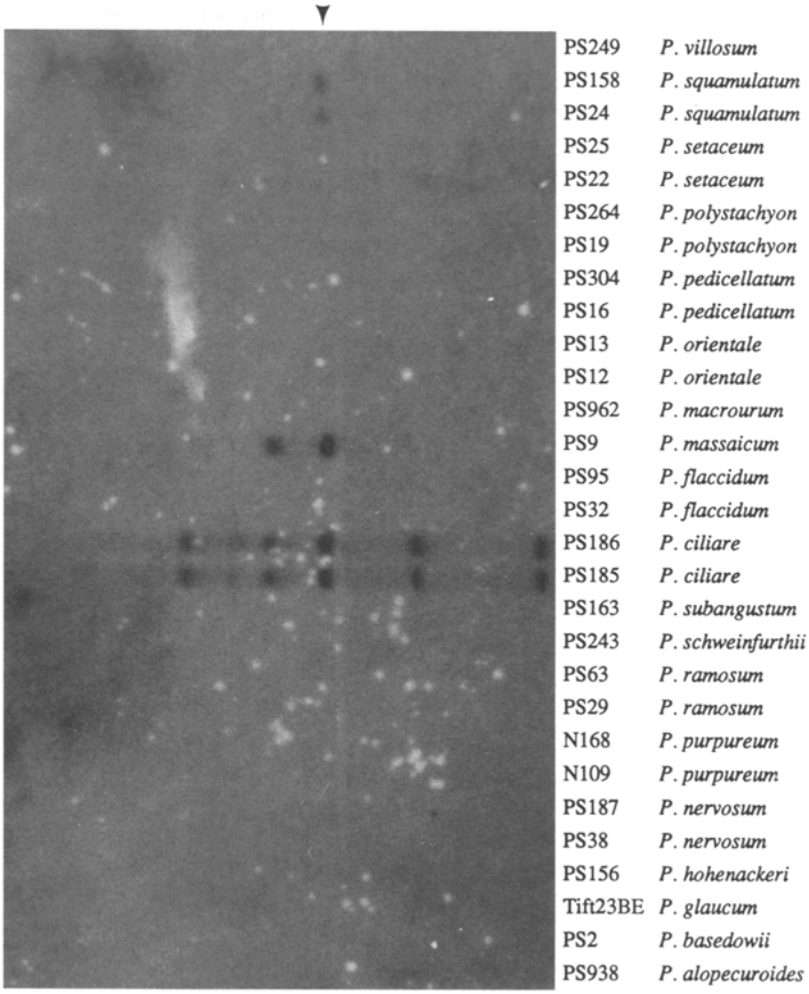


**Fig. 2** Autoradiogram of *Dra*I-digested genomic DNA of *Pennisetum* species hybridized with cloned $C04_{600}$. *Arrows* indicate the location of the common informative band



OPC04 primer and strong homology with cloned $C4_{600}$.

Single-to-low DNA copy number in the above apomictic subset was indicated when we used cloned $C4_{600}$ as a probe on a genomic Southern blot (Fig. 2), whereas there was an apparent dispersed repeat pattern in the subset as well as in *P. glaucum*, *P. purpureum*, and some other *Pennisetum* species when $OPC04_{600}$ amplified from *P. squamulatum* was excised out of a gel and used as a radiolabelled probe (data not shown). It appears that co-migrating DNA sequences were responsible for the dispersed repeat pattern. Williams et al. (1990) and Paran et al. (1991) have used excised RAPD bands as the source for their hybridization probes. Both noted hybridization patterns consistent with repetitive DNA, which prevented the use of some RAPD fragments as hybridization probes for RFLPs. Paran and Michelmore (1993) frequently found that DNA sequences other than the informative and predominant sequence were cloned from an excised band. It is apparent from our results that excised RAPD bands used as RFLP probes can produce misleading hybridization patterns.

PCR amplification of DNA from the sexual and apomictic *Pennisetum* species using UGT197 STS primers showed an intense band of the size predicted from a known DNA sequence (144 bp; Ozias-Akins et al. 1993) from all apomictic *Pennisetum* species except for those in the section *Brevivalvula* (*P. pedicellatum*, *P. polystachyon*, and *P. subangustum*) (Fig. 3, right panel). Hybridization of UGT197 to a Southern blot of the PCR-amplified products verified that the PCR products were homologous to the DNA clone (Fig. 3, left panel). In some sexual *Pennisetum* species, very faint amplification products of about 144 bp were detected after excessive overexposure of the autoradiogram. These faint autoradiographic bands have had a visible counterpart on a stained gel only once with *P. nervosum*. Ozias-Akins et al. (1993) showed that UGT197 hybridized to genomic DNA of *P. squamulatum* and $BC_3$ but not to that of *P. glaucum* or *P. purpureum*. The banding pattern and intensity of hybridization in that study suggested that UGT197 was single-copy DNA. Slight contamination of the DNA between samples could account for the appearance of faint bands; however, the presence of bands persisted with new reagents for PCR amplification, and the bands were not consistently amplified from one PCR amplification run to the next using the same DNA source. Contamination during isolation

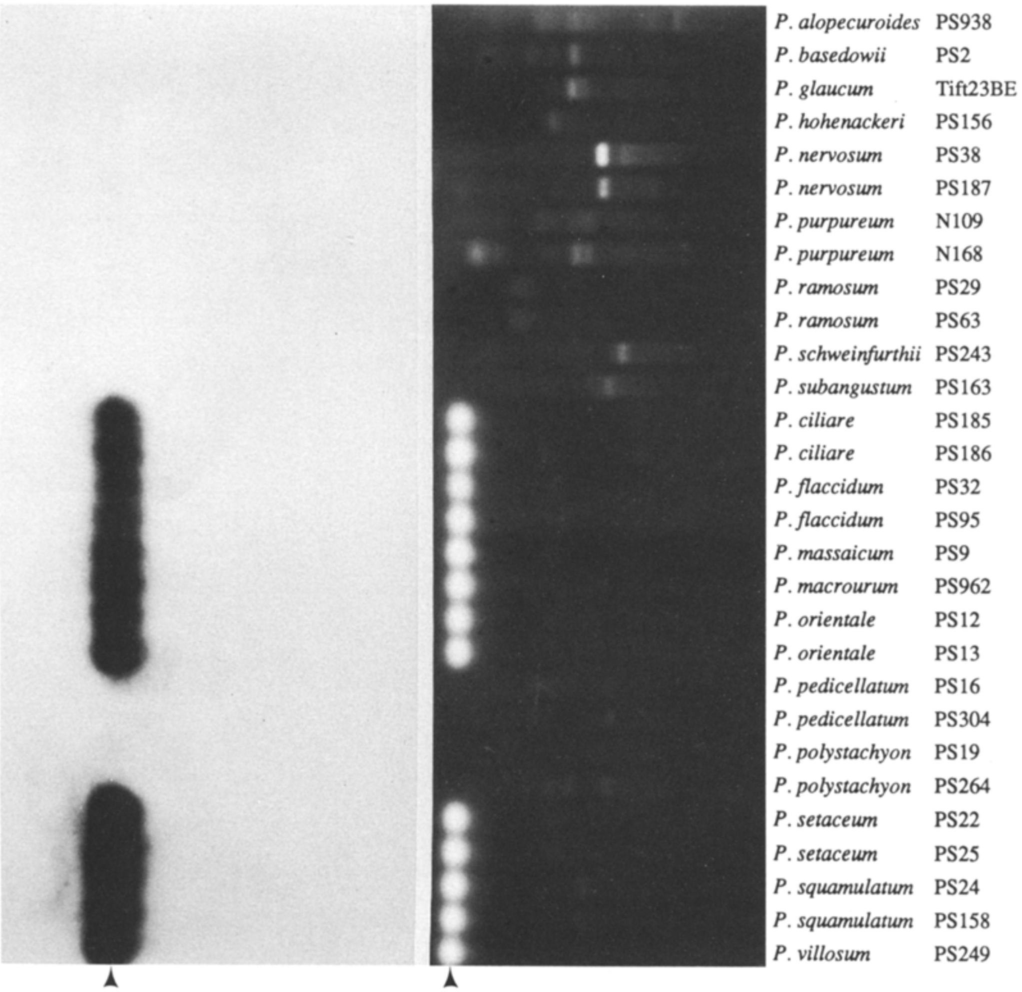


**Fig. 3** PCR amplification of DNA from *Pennisetum* species using UGT197 STS primers. *Right panel* is the ethidium bromide-stained gel. *Left panel* is an autoradiogram of the Southern blot of the gel in the right panel using UGT197 as the probe. Arrows indicate the location of the informative 144-bp bands

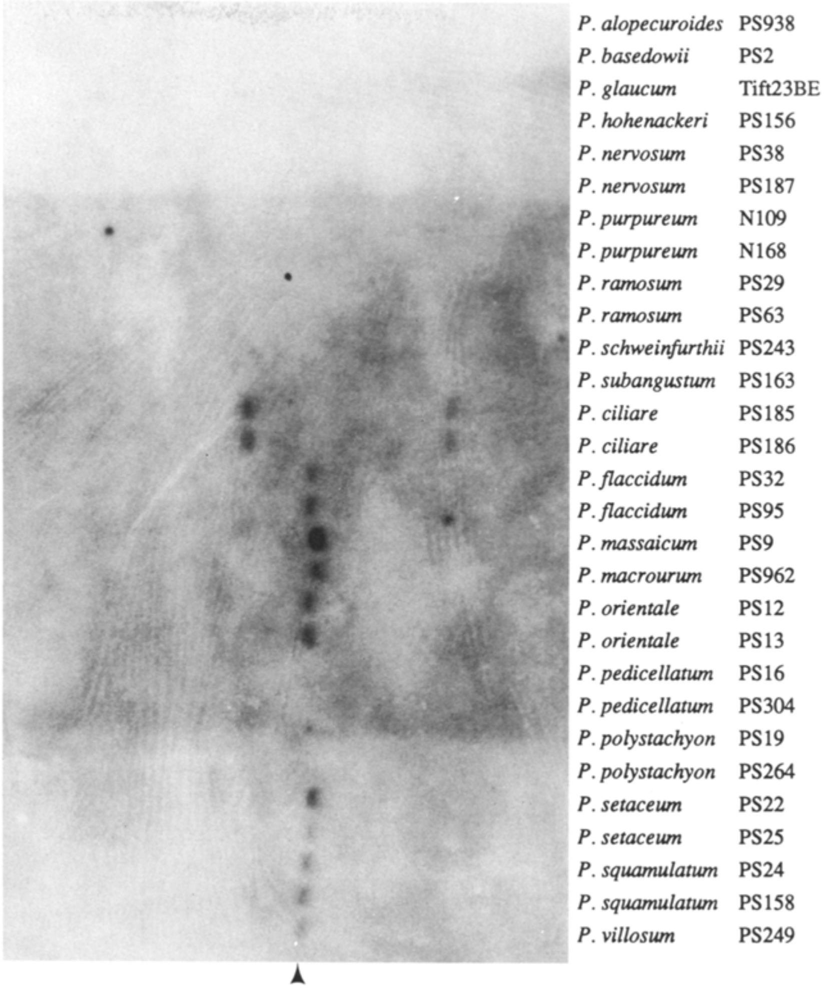
of the DNA should not have occurred because the items used for DNA extraction were either disposed of or autoclaved after each sample had been processed.

UGT197 did not hybridize to *Dra*I-digested genomic DNA from sexual *Pennisetum* species, whereas it did hybridize to genomic DNA from all apomictic species that showed the intense PCR-amplified UGT197 STS (Fig. 4). All of the other apomictic species except *P. ciliare* had a single band roughly comparable in size (2.8 kb) to the original source of the probe, *P. squamulatum*. *P. ciliare* had two *Dra*I fragments, one at 5.1 kb and one at 2.4 kb. The single band in *P. flaccidum* and *P. orientale* consistently appeared slightly smaller (2.7 kb) than the single band in the *P. squamulatum*-type species (*P. massaicum, P. macrourum, P. setaceum, P. squamulatum*, and *P. villosum*). UGT197 did not hybridize with genomic DNA from the apomictic species in the section *Brevivalvula*, which confirmed the absence of UGT197 STS in these species.

The apomictic species could be separated into four groups based on their banding pattern for UGT197: the section *Brevivalvula* (no hybridization), *P. flaccidum* and *P. orientale* (2.7-kb band), *P. ciliare* (two bands), and the *P. squamulatum*-type apomicts (2.8-kb band). Other evidence for the validity of two of these groupings exists. Species in the section *Brevivalvula* have been shown to be closely related to each other based on prolamin seed protein patterns (Lagudah and Hanna 1990) and mitochondrial DNA restriction fragment patterns (Chowdhury and Smith 1988). Clegg et al. (1984) noted that selected portions of the chloroplast genome were invariant between *P. flaccidum* and *P. orientale*. We observed 200-bp *Dra*I repeat ladders common to *P. flaccidum* and *P. orientale* on ethidium bromide-stained gels (data not shown). These data support a close relationship between the two species.

In conclusion, we have demonstrated that two markers isolated from the apomict *P. squamulatum* are specific for apomictic species in *Pennisetum*. We previously used these two markers in the transfer of apomixis to sexual pearl millet. These probes show no homology with any of the sexual species of *Pennisetum* that were tested. UGT197 hybridized exclu-

**Fig. 4** Autoradiogram of *Dra*I-digested genomic DNA of *Pennisetum* species hybridized with UGT197. *Arrows* indicate the location of the informative 2.8-kb bands



sively to apomictic species except those in the section *Brevivalvula*. The absence of this fragment of DNA from the section *Brevivalvula* could be explained by either recombination and segregation or deletion during speciation. Three apomictic species (*P. ciliare*, *P. massaicum*, and *P. squamulatum)* showed hybridization with the cloned $C4_{600}$ probe. UGT197 was more consistently associated with apomictic reproductive behavior than either the RAPD $OPC04_{600}$ or the cloned $C4_{600}$, thus it could be inferred that UGT197 is more closely linked to the gene(s) for apomixis than cloned $C4_{600}$. The successful use of these probes to survey other *Pennisetum* species indicates that apomixis is a trait that can be followed across species by using molecular means. This technique of surveying species within a genus will be useful in determining the relative importance of newly isolated markers. Therefore, the use of related species to facilitate the identification of the apomixis gene(s) appears feasible. The isolation of other probes linked to the apomixis gene(s) is proceeding so that a genetic and physical map of the single chromosome of interest can be produced from an interspecific population segregating for mode of reproduction.

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## Book review

**Singh, R. J. 1993. Plant Cytogenetics.** CRC Press, Boca Raton, Florida, USA. 416 pp., 99 figs., 122 tables, Hard Bound, DM US $. 96.00 ISBN 0-8493-8656-X.

Cytogenetics has played an important role in understanding the chromosomal and genetic architecture of plant species. Since the publication of chromosomal theory of inheritance (1902–1903), a great wealth of information has become available on chromosome pairing, crossing over, chromosome maps, and genomic relationships. Various cytogenetic stocks representing numerical and structural aberrations of chromosomes have been developed and employed in constructing genetic and molecular maps in several plant species. More recently, chromosome engineering techniques have become an integral part of genetic and breeding research.

Since the publication of an excellent text on plant cytogenetics, (Discussions in cytogenetics) by C. R. Burnham in 1962, plant cytogenetics has witnessed many advances. Thus there was a great need for an updated and comprehensive book on plant cytogenetics. The publication of this book is thus very timely.

The contents of the book are arranged into eight chapters: (1) introduction, (2) the handling of plant chromosomes, (3) cell division, (4) genetic control of meiosis, (5) karyotype analysis, (6) chromosomal aberrations: structural and numerical chromosomal changes, (7) genome analysis, and (8) chromosomal aberrations in cell and tissue culture derived callus and their regenerants.

The book provides an excellent review of various techniques in handling of chromosomes, karyotype analysis, genetics of meiosis, genomic relationships, and chromosome manipulations. In addition to his own studies, Dr. Singh has made extensive use of the information published in various journals.

In chapter1, Mendel's laws of inheritance have been discussed at length. However, inclusion of some of the major discoveries in chromosome research and discussion of parallelism between Mendel's laws of inheritance and chromosomal theory of inheritance would have been useful. Several simplified procedures for handling of meiotic and mitotic chromosomes have been presented in chapter 2 with photographs. Cell division has been well explained with simple and clear photographs describing various aspects of mitotic and meiotic divisions. However, brief introduction to meiotic divisions during megasporogenesis in apomictic species would have been appropriate. In subsequent chapters, the author has elegantly described genetic control of meiosis, analysis of chromosomal aberrations-both structural and numerical changes. Procedures for development and characterization of different types of aneuploids and chromosomal interchanges have been well illustrated. The methodology and usefulness of such cytogenetic stocks in genetic mapping with relevant examples have been nicely presented. Table 6.76 on alien chromosome substitutions should have been titled alien chromosome additions. Genomic relationships have been well explained with selected examples on wheat, cotton, soybean and tobacco. The last chapter describes chromosomal aberrations in cell and tissue culture and their regenerants in a simplified form .

A chapter on advanced techniques in cytogenetics particularly molecular cytogenetics such as fluorescence in situ hybridization (FISH) and chromosome image analyzing system would have added to the value of the book. Also, an additional chapter highlighting the application of various cytogenetic techniques in plant improvement would have added to the usefulness of the text.

The contents of the book are well arranged and easy to read and understand. It should serve as a useful text book for students taking courses in genetics and cytogenetics and as a reference book for scientists engaged in plant cytogenetic research.

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