

SHORT COMMUNICATION

An apospory-specific genomic region is conserved between Buffelgrass (*Cenchrus ciliaris* L.) and *Pennisetum squamulatum* Fresen

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Summary

Twelve molecular markers linked to pseudogamous apospory, a form of gametophytic apomixis, were previously isolated from *Pennisetum squamulatum* Fresen. No recombination between these markers was found in a segregating population of 397 individuals (Ozias-Akins *et al.*, 1998, *Proc. Natl Acad. Sci. USA*, 95, 5127–5132). The objective of the present study was to test if these markers were also linked to the aposporous mode of reproduction in two small segregating populations of *Cenchrus ciliaris* (= *Pennisetum ciliare* (L.) Link), another apomictic grass species. Among 12 markers (sequence characterized amplified regions, SCARs), six were scored as dominant markers between aposporous and sexual *C. ciliaris* genotypes (presence/absence, respectively). Five were always linked to apospory and one showed a low level of recombination in 84 progenies. Restriction fragment length polymorphisms (RFLPs) were observed between sexual and apomictic phenotypes for three of the six remaining SCARs from *P. squamulatum* when used as probes. No recombination was observed in the F_1 progenies. Preliminary data from megabase DNA analysis and sequencing in both species indicate that an apospory-specific genomic region (ASGR) is highly conserved between the two species. Although *C. ciliaris* has a smaller genome size to *P. squamulatum*, a higher copy

number for markers linked to apospory found in the former may impair the progress of positional cloning of gene(s) for apomixis in this species.

Introduction

Apomixis is a unique mode of reproduction by which plants clonally propagate themselves through seeds (Asker and Jerling, 1992). In apomicts, meiosis is circumvented in the differentiation of the female gametophyte and the megaspore mother cell or its meiotic derivatives degenerate (Nogler, 1984). In apospory, a form of gametophytic apomixis, an embryo sac differentiates by mitotic division of a somatic cell within the ovule. In this study we tested the synteny of apospory-linked molecular markers (Ozias-Akins *et al.*, 1998) in two species which reproduce by apospory, *Pennisetum squamulatum* Fresen and *Cenchrus ciliaris* L. (= *Pennisetum ciliare* (L.) Link; buffelgrass).

Pennisetum squamulatum is native to eastern Africa (Jauhar, 1981) while buffelgrass is found in tropical and southern Africa, the Mediterranean region, India and Pakistan. *Pennisetum squamulatum* is a hexaploid ($2n=6x=54$) (Dujardin and Hanna, 1984), whereas most accessions of buffelgrass are tetraploid with $2n=4x=36$ (Hignight *et al.*, 1991). These two polyploid species differ greatly in their vegetative and floral morphology (Figure 1a,b). The genus *Cenchrus* is taxonomically separated from the genus *Pennisetum* by involucral bristles fused at their point of origin (Nath *et al.*, 1970); however, the validity of such a distinction is disputed (Hignight *et al.*, 1991). In both species, aposporous genotypes display the *Panicum*-type embryo sac phenotype (Nogler, 1984; Vielle *et al.*, 1995). In *P. squamulatum* no sexual genotype is known and genetic analysis of the aposporous trait has been conducted by inter-specific crosses with pearl millet (*P. glaucum* (L.) R. Br.) using *P. squamulatum* as the pollen donor (Ozias-Akins *et al.*, 1998). In buffelgrass, no sexual genotype was known until one was found inadvertently (Bashaw, 1962). Crossing this sexual genotype as the female parent to apomictic genotypes as male parents produced progenies of three phenotypic classes, obligate sexual, obligate apomict and facultative apomict. In a facultative apomict both four-nucleate *unreduced* embryo sacs and eight-nucleate sexual *reduced* embryo sacs can

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be observed in ovules within the same inflorescence (Sherwood *et al.*, 1980).

We previously isolated 12 molecular markers linked to the aposporous trait in progenies produced from a single cross between pearl millet used as female parent and *P. squamulatum* (Ozias-Akins *et al.*, 1998). Sequence-tagged sites (STS) and sequence-characterized amplified regions (SCARs) were designed from clones of restriction fragment length polymorphism (RFLP) probes and random amplified polymorphic DNA (RAPD) fragments, respectively. All 12 PCR-based, dominant markers were linked in *cis* to the trait with no recombination between them in a population of 397 individuals (Ozias-Akins *et al.*, 1998). In another study we showed that at least two of these molecular markers linked to the aposporous trait in *P. squamulatum* were also present in related apomictic species, including

Cenchrus ciliaris (Lubbers *et al.*, 1994), but linkage between these markers in these species was not determined. The present research was undertaken to determine if the molecular markers isolated from *P. squamulatum* could be mapped with higher resolution in a buffelgrass population segregating for sexual and apomictic genotypes.

Results and Discussion

Six SCARs totally linked to an apospory-specific genomic region in P. squamulatum are also linked to apomictic reproduction in C. ciliaris

Among the 12 SCARs previously mapped in *P. squamulatum* (Ozias-Akins *et al.*, 1998), six (UGT-197, P16R, Q8M, U12H, V4 and X18R) could be amplified from the two apomictic buffelgrass parents but not the sexual parent. The amplification products for SCAR P16R are shown in

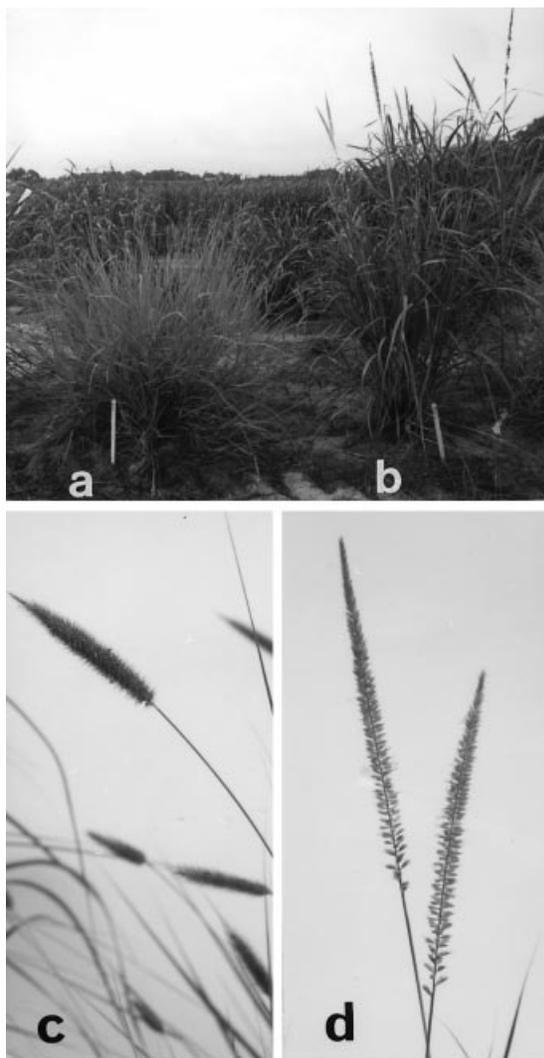


Figure 1. Two grasses reproducing by apospory, a form of apomixis. (a) *Cenchrus ciliaris* L. cv. B-12-9 and (b) *Pennisetum squamulatum* Fresen. Inflorescences of (c) *C. ciliaris* and (d) *P. squamulatum*.

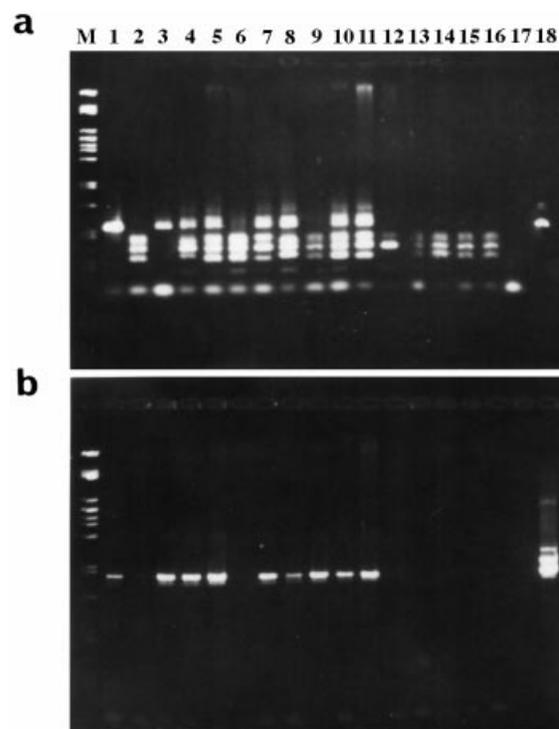


Figure 2. PCR analysis of apomictic and sexual genotypes. (a) SCAR pattern for *Pennisetum* sp. and *Cenchrus ciliaris* genotypes with X18R primers. *P. squamulatum* PS26 (lane 1), *P. glaucum* 23DE (lane 2), apomictic F_1 (*P. glaucum* X *P. squamulatum*) MS228-18 (lane 3), *C. ciliaris* apomictic lines cv Higgins (lane 4), B-12-9 (lane 5), sexual line B-2s (lane 6), apomictic *C. ciliaris* progenies from B and H population (lanes 7–11), sexual *C. ciliaris* progenies from B and H population (lanes 12–16). (b) SCAR pattern for the same genotypes with P16R primers. In both panels, two controls are included, no DNA (H_2O) and cloned RAPD fragment in pGEMT vector, on lanes 17 and 18, respectively. Molecular marker of *Pst*I-cut lambda is shown in lane M.

Figure 2(b), in which a conserved molecular size is observed among *P. squamulatum*, its apomictic progeny MS228-18, both apomictic buffelgrass parents, and obligate and facultative apomicts in the two segregating buffelgrass populations. No amplification products could be obtained from the sexual buffelgrass parent (B-2s) and all sexual progenies. The molecular sizes of five other PCR-based markers (Q8M, UGT-197, U12H, V4, X18R) were also conserved between *P. squamulatum* (and its derived progenies) and apomictic *C. ciliaris* (data not shown). The only exception to absolute linkage between apomictic reproduction and the presence of a dominant SCAR marker was with the marker X18R where no amplification in an apomictic F_1 individual, 182-8, was obtained (Figure 2a, lane 9). This result was confirmed by repeating the amplification several times using multiple independent DNA preparations from the same plant. This plant was classified as an apomictic plant by cytological analysis in both locations (Pennsylvania and Georgia), and showed the expected amplification products with the five other SCAR markers, as indicated here using one example (P16R, Figure 2b, lane 9). However, besides being a potential recombinant, it is possible that a mutation may have altered one of the priming sites. Further investigation by direct genome sequencing or RFLP analysis with X18R was not possible due to the highly repetitive nature of sequences located between the two X18R priming sites in both *P. squamulatum* and *C. ciliaris* (data not shown). In this study, we could not confirm a previous finding of recombination between the mode of reproduction and UGT-197 (Gustine *et al.*, 1997). In that study, 18-mer

primers were used to yield a 144 bp fragment. Further sequencing within the ASGR of a lambda genomic clone hybridizing to UGT-197 yielded two 24-mer primers amplifying an 800 bp fragment which proved to be more reliable for typing (Ozias-Akins *et al.*, 1998; Roche, unpublished results).

Four RFLP markers are linked in cis to the aposporous trait but again show no recombination in C. ciliaris as previously documented in P. squamulatum

Of the remaining six SCARs previously documented as tightly linked to apospory in *P. squamulatum*, three detected RFLPs when genomic blots of buffelgrass parental lines and segregating progenies were probed with the amplified DNA fragments. A thirteenth putative apospory-linked SCAR isolated from *P. squamulatum* (C16) could also be mapped as an RFLP in *C. ciliaris*. The RFLP fragments hybridizing with A10H, C4, C16 and 07M were mapped using restriction enzymes *Pst*I, *Dra*I, *Hind*III and *Dra*I, respectively. In 84 progenies, occurrence of these RFLPs was always associated with the aposporous mode of reproduction with no observation of any recombinant. As an example, several individuals from population B in which genomic DNA was digested with the enzyme *Pst*I and probed with the labelled A10H PCR-generated fragment are shown in Figure 3. Apomictic parental line, B-12-9 and all obligate- and facultative-apomictic progenies displayed a dominant RFLP fragment (indicated by an arrow) which was not observed in the sexual parental line (B-2s) and all sexual progenies. No allelic fragment was identified in the sexual genotypes with this probe-enzyme combination (A10H/*Pst*I) or in the other three digests tested (data not shown).

In *C. ciliaris*, nine markers are completely linked to the aposporous mode of reproduction and, as in *P. squamulatum*, all segregate as a single dominant locus (i.e. DNA region). In the latter species we named this DNA region as an apospory-specific genomic region or ASGR (Ozias-Akins *et al.*, 1998). We previously calculated within segregating gametes of *P. squamulatum* the likelihood for two unlinked molecular markers (or subsets of markers) to independently segregate. Assuming no major genetic differences between the two apomictic *C. ciliaris* parental lines in their determination of apomixis, we can combine populations B and H for linkage analysis and apply the same probabilistic approach to 84 *C. ciliaris* individuals. Therefore, for two markers (or two subsets of markers) residing on different linkage groups, the probability of independent segregation is $P=0.75$, and thus we should expect 63 out of 84 individuals to display independent segregation. No segregants were observed and the likelihood for more than two unlinked markers to independently

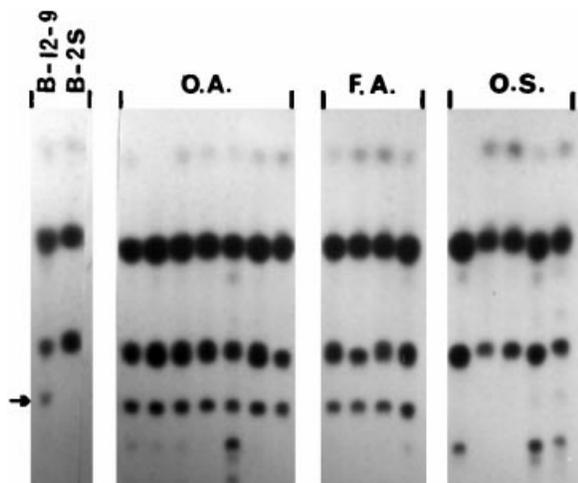


Figure 3. Restriction fragment length polymorphism (RFLP) pattern within the B population of *Cenchrus ciliaris* for A10H-SCAR used as a probe and *Pst*I-digested DNA.

Each lane was loaded with 12.5 µg of DNA. All obligate (O.A.) and facultative apomicts (F.A.) and obligate sexual (O.S.) progenies arose from a cross between the two parental lines indicated on the left (B12-9; B-2s). The arrow indicates the RFLP of interest (1.8 kb).

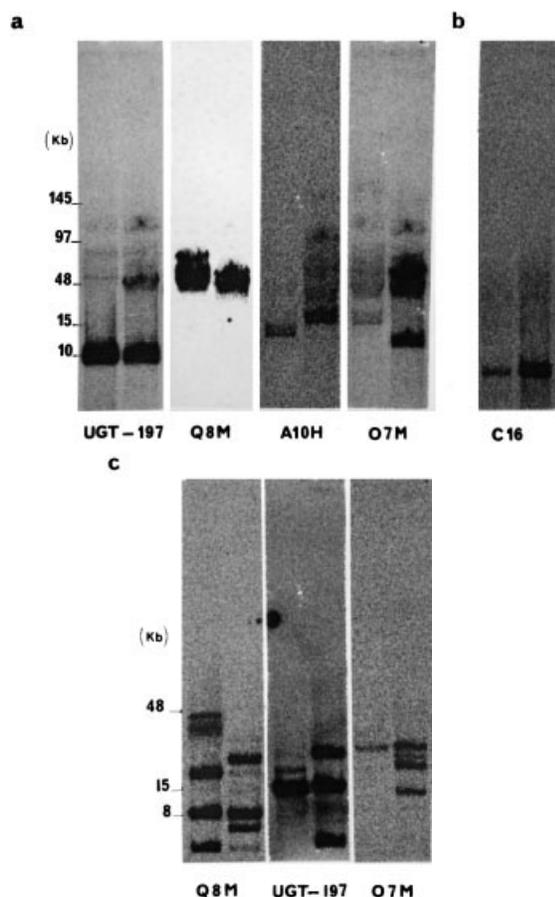


Figure 4. Southern analysis of pulsed-field separated megabase DNA from MS228-18 (apomictic F_1 *P. glaucum* \times *P. squamulatum*) (left lane in all double compositions presented) and buffelgrass apomictic line B-12-9 (right lane).

(a) Megabase DNA from these two lines were digested simultaneously with *Nru*I/*Bss*HI restriction enzymes. 32 P-radiolabelled probes from Q8M, UGT-197, O7M and A10H SCARs were used successively on the same blot.

(b) Megabase DNA was digested simultaneously with *Nru*I/*Sfi*I restriction enzymes and 32 P-radiolabelled probe from C16 SCAR was used.

(c) Megabase DNA was cut with *Afi*III restriction enzyme. Radiolabelled probes from Q8M, UGT-197 and O7M SCARs were successively used on the same blot.

segregate is even more remote. Therefore, we could safely assume that all 10 markers tested in buffelgrass are linked and belong to a similar ASGR as described previously in *P. squamulatum*.

Pulsed-field electrophoresis and sequencing data further confirm conservation of the ASGR between C. ciliaris and P. squamulatum

Megabase-DNA from one apomictic buffelgrass parent (B-12-9) and the apomictic MS228-18, derived from *P. squamulatum*, hybridized as low-copy DNA with seven of the 13 SCAR markers (A10H, C4, C16, O7M, P16R, Q8M, UGT-197). The remaining six SCARs either

did not hybridize with *C. ciliaris* DNA (A14M) or detected middle to highly repetitive sequences in both species and consequently could not be used in pulsed-field electrophoresis experiments. When megabase DNAs cut simultaneously with *Nru*I and *Bss*HI restriction enzymes were probed with Q8M and UGT-197 (Figure 4a), both genotypes displayed common RFLP fragments of estimated sizes of approximately 50 kb and 10 kb, respectively. However, when hybridized with SCAR fragments such as A10H and O7M, polymorphism was observed between the two genotypes (Figure 4a). With double digestion (*Nru*I/*Sfi*I) of genomic DNA from both species, hybridization with a C16 SCAR-derived probe indicated a common RFLP of approximately 10 kb (Figure 4b). In a second experiment, in which the enzyme used (*Afi*III) cuts DNA more frequently, one DNA fragment hybridizing to Q8M, UGT-197 and O7M SCARs was conserved between the two apomictic lines (Figure 4c). In summary, relative conservation of the aposporous trait and fragments detected by UGT197, Q8M, A10H, C16 and O7M SCAR-derived probes between both species was observed by pulsed-field gels. However, with most probes, a larger number of copies was observed in buffelgrass than in the *P. squamulatum*. The total size of the ASGR could not be estimated since all restriction enzymes yielded relatively small hybridizing fragments (<100 kb).

For both *P. squamulatum* and *C. ciliaris* (B12-9 parent) we sequenced approximately 1100 bp within two SCARs linked to ASGR (UGT-197 and Q8M) and a chloroplast sequence (*psbC-trnS* primers; 1690 bp-SCAR; Demesure *et al.*, 1995). We intended to compare the substitution rates for all these non-coding DNA regions between the two species in order to assess their respective rates of divergence. Chloroplast DNA sequences are maternally inherited. Likewise, the nuclear DNA sequences in an obligate apomict are inherited through an asexual lineage. An identical rate of substitution of 0.3% was found (data not shown), documenting a low rate of divergence.

Uniqueness of the ASGR in *C. ciliaris* is not maintained in terms of hemizygoty since there were always more DNA fragments which cross-hybridized to a given SCAR-fragment in *C. ciliaris* than in *P. squamulatum*. These observations conflict with the common consensus that one would expect more copies of a sequence in a larger genome than in a smaller one. The genome of tetraploid *C. ciliaris* is approximately one-third smaller than the genome of hexaploid *P. squamulatum* (Ozias-Akins, unpublished results). In spite of these copy-number differences between the two species, when we tested a *P. squamulatum* ASGR-linked marker on buffelgrass progenies we found a consistent dominant SCARs or RFLPs in the obligate and facultative apomictic

tic lines of buffelgrass. In our initial work with RAPDs in the bulked-segregant analysis of *P. squamulatum* progenies (Ozias-Akins *et al.*, 1998), unique alleles were always associated in coupling with apomixis and never with sexuality. The uniqueness of the ASGR was unexpected but were perhaps predicted in a visionary statement made by Harlan *et al.* (1964): 'the genes controlling normal reproduction are not allelic to those controlling apomixis in the conventional sense'. We are currently in the process of constructing a DNA-contig made from BAC clones of both species, an approach which will unambiguously yield minimal physical size(s) for both ASGRs and allow us to study the microcolli-nearity of these two DNA regions in both species.

Experimental procedures

Plant material

Plant MS228-18 is an apomictic polyhaploid F_1 progeny produced by the cross *P. glaucum* × *P. squamulatum* (Dujardin and Hanna, 1986). Two buffelgrass populations segregating for mode of reproduction were generated at the Pasture Systems and Watershed Management Research Laboratory, University Park, PA, USA (Gustine *et al.*, 1997). Two obligately apomictic genotypes (B-12-9 and cv. Higgins) were used as male parents in crosses with the same sexual line (B-2s) to produce two segregating populations referred to as B (46 individuals) and H (38 individuals), respectively. Plants were characterized for mode of reproduction in Pennsylvania (1993–95) and Georgia (1996–97) by the cleared-pistil technique of Young *et al.* (1979).

DNA extraction

Leaf and shoot tissues from field- or greenhouse-grown plants were ground in liquid nitrogen and DNA was isolated according to Ozias-Akins *et al.* (1993), a slight modification of Tai and Tanksley (1990).

Amplification of SCARs

All but one of the PCR-based markers linked to apospory used in this study have been described previously with respective annealing temperatures and thermocycling conditions (Ozias-Akins *et al.*, 1998). An additional SCAR marker designated C16, was included in this study (forward primer 5'CACTCCAGGCACATATTTT 3'; reverse primer 5'CTATGATATGCTTTTTTTTGC 3', annealing temperature at 55°C).

Restriction fragment length polymorphism analysis

Plant DNA (15 µg) was digested overnight with 50 units of each restriction enzyme (New England Biolab, Beverly, MA, USA) and separated by electrophoresis for 15 h at 3 V cm⁻¹ in 1% GTG agarose (FMC, Rockland, ME, USA) in 1× TBE buffer. DNA was transferred to Genescreen Plus nylon membranes (NEN, Boston, MA, USA) by the alkaline transfer method (Sambrook *et al.*, 1989). Hybridization was conducted with ³²P-labeled insert DNA generated by PCR amplification from RAPD clones using SCAR-specific

primers. Hybridized blots were washed at a final stringency of 0.1× SSPE (0.18 M NaCl/10 mM sodium phosphate, pH 7.4/1 mM EDTA) containing 0.1% SDS at 65°C for 30 min.

Preparations of megabase DNA

Nuclei from fresh shoots were prepared as described previously (Zhang *et al.*, 1995) and embedded in 100 µl plugs of InCert agarose (FMC) at a final concentration of 10⁷ nuclei per ml. Embedded nuclei were lysed at 50°C for 48 h in 0.5 M EDTA (pH 9.3), 1% (w/v) sodium lauryl sarcosine and 1 mg ml⁻¹ proteinase K (Boehringer Mannheim, Indianapolis, IN, USA) with one change of buffer. Plugs were then washed sequentially for 1 h each in 0.5 M EDTA (pH 9.3) at 50°C, in 0.05 M EDTA (pH 8.0) on ice and TE buffer (10 mM Tris.HCl pH 8.00:1 mM EDTA) on ice, followed by three 30 min washes in the latter buffer containing 0.1 mM phenylmethyl sulfonyl fluoride. Plugs were finally equilibrated in TE three times on ice for 30 min each.

Digestion of megabase DNA by restriction enzyme

Each plug was individually equilibrated on ice (twice for 45 min each) in 0.5 ml of 1× restriction enzyme buffer containing 2 mM spermidine-HCl and 100 µg ml⁻¹ of bovine serum albumin (if required for proper digestion). Restriction enzymes (New England Biolabs) were added at a concentration of 50 units per plug containing approximately 5–10 µg of megabase DNA. Reaction mixtures were equilibrated for 30 min on ice to allow adequate diffusion of enzymes into the agarose matrix, and reactions were incubated in a water bath overnight at 37°C for most enzymes or 50°C for *Sfi*I. The following morning, 25 U of enzyme was added and reactions were incubated for an additional 8 h.

Pulsed-field electrophoresis of megabase DNA

Digested DNA was separated by pulsed-field electrophoresis in 0.9% SeaKem Gold agarose gels (FMC) (gel volume of 110 ml) in 3.5 l of 0.5×TBE using a CHEF-DR11 apparatus (Biorad, Inc, Hercules, CA, USA). Run conditions were as follows, 5.5 V cm⁻¹, 7.5 h constant shift of 70 sec followed by 7.5 h at constant shift of 6 sec. Buffer temperature was maintained between 8 and 14°C with circulation through a cooling water bath. Gels were stained for 30 min in water containing 1 µg ml⁻¹ of ethidium bromide and photographed. DNA in gel was UV-nicked for 16 sec with a DNA transfer lamp (Fotodyne, Hartland, WI, USA). The gel was equilibrated in a 1.5 M NaCl/0.4 M NaOH solution for 20 min. The latter solution was used in the 48 h-long capillary membrane transfer. The membrane was then neutralized in 0.5 M Tris pH 7.0 for 5 min and briefly rinsed in 2×SSC. Blots were hybridized and washed according to the above protocol described for the RFLP analysis.

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