Theor Appl Genet (2000) 100:576–583

P.A. Butcher · G.F. Moran · R. Bell

Genetic linkage mapping in *Acacia mangium* 1. Evaluation of restriction endonucleases, inheritance of RFLP loci and their conservation across species

Received: 5. June 1999 / Accepted: 30 July 1999

Abstract Random genomic probes were used to assess levels of restriction fragment length polymorphism (RFLP) in two 2-generation outbred pedigrees of *Acacia mangium* Willd. Probes were evaluated for their ability to detect polymorphic loci in each pedigree and to determine the relative efficiency of different restriction enzymes in revealing polymorphisms. Sixty two percent of the probes which detected single- or low-copy number sequences revealed polymorphisms with at least one restriction enyzme. *Hpa*II was the most efficient in detecting polymorphism among first-generation individuals. The recognition sequence of *Hpa*II contains a CpG dimer, suggesting that cytosines in the CpG sequence may be hotspots for mutation in plant genomes, as previously reported in bacterial and mammalian genomes. Mendelian inheritance of 230 loci was demonstrated based on single-locus segregation in second-generation individuals. Less than 5% of loci showed evidence of segregation distortion. The proportion of fully informative loci (15%) was lower than previously reported in eucalypts reflecting the lower level of genetic diversity in *A. mangium*. The RFLP probes are suitable for the construction of a high-density genetic linkage map in *A. mangium*. Cross-hybridisation of the *A.mangium* RFLPs to DNA from species representing the three subgenera of the genus *Acacia* indicates that these markers could be used in breeding programs of other diploid acacias, for comparative studies of genome organisation, and for phylogenetic studies.

Key words *Acacia* · RFLP · Restriction enzymes · Genome mapping

Communicated by P.M.A. Tigerstedt

P.A. Butcher (✉) · G.F. Moran · R. Bell
CSIRO Forestry and Forest Products, PO Box E4008,
Kingston ACT 2604, Australia
e-mail: Penny.Butcher @ffp.csiro.au

Introduction

Acacia mangium Willd. is a diploid (2n=26), predominantly outcrossing, tree species which is in the early stages of domestication. Its fast growth rate, high pulp yields and tolerance of a range of soil types have led to rapid expansion of its plantation areas in the humid and subhumid tropics (approximately 600000 ha) (Turnbull et al. 1998). Breeding programs for the species rely on recurrent selection and the vegetative propagation of superior clones. To take advantage of the increased efficiencies offered by marker-assisted selection, a high-density genetic linkage map is being developed which will be used to locate markers linked to genes controlling wood quality and disease resistance traits.

Genetic linkage mapping relies on the ability to detect allelic differences in full-sib pedigrees – a task made more difficult in species with low genetic diversity. Isozyme variation in *A. mangium* is amongst the lowest detected in forest trees (Moran et al. 1989) and the species has an unusual pattern of diversity across a disjunct geographic range (Moran et al. 1989; Butcher et al. 1998). The level of RFLP variation is significantly higher in populations from New Guinea and Cape York than populations from other parts of the species' geographic range (Butcher et al. 1998). To maximise the number of segregating loci in the *A. mangium* mapping population, two crosses were made between parents from different populations in New Guinea.

The probability of detection of restriction site polymorphism is affected by the size and composition of the recognition sequence of restriction enzymes. Restriction enzymes with 6-bp in their recognition sequence have been reported to reveal higher levels of polymorphism than those with 4-bp (Landry et al. 1987; McCouch et al. 1988; Liou et al. 1996) and to have a higher probability of detecting insertion/deletion mutations (Devey et al. 1991). Differences have been reported in the enzymes which produced the highest proportion of polymorphic loci in different plant species (Landry et al. 1987; McCouch et al. 1988; Graner et al 1990; Miller and Tanksley 1990; Byrne et al. 1994; Liou et al. 1996). In humans, a signifi-

cantly higher proportion of polymorphisms were detected with enzymes containing the CpG dinucleotide in their recognition sequence (Barker et al. 1984), although similar findings have not been reported in any plant species. Evidence has also been presented suggesting that the level of polymorphism may be related to the size of fragments generated and whether polymorphisms are due to base substitutions or rearrangements (Landry et al. 1987; McCouch et al. 1988; Liou et al. 1996; Paterson 1996).

The utility of RFLP markers depends, in part, on whether polymorphism can be detected in other mapping populations and in other species. *Acacia* is a large pantropical genus which includes over 1200 species. Aspects of the taxonomy of the genus remain unresolved (Maslin and Stirton 1997) and markers that could be used across the genus would provide a useful tool for comparative studies of genome organisation and phylogenetic studies. To develop a generic map that can be used in other commercial acacia species, it is also important that loci can be assayed and are orthologous across as wide a group of species as possible. RFLPs have proved superior for this purpose to most other markers (Ahuja et al. 1994; Cheung and Landry 1996; Nelson et al. 1996; Devey et al. 1999).

In the present study RFLPs from a genomic library were used to evaluate the efficiency of different restriction enzymes in revealing polymorphism in *A. mangium*. The Mendelian inheritance of loci was assessed and their homology to species representing the three subgenera of *Acacia* was investigated.

Materials and methods

Genetic material

Two 2-generation pedigrees were used to determine the segregation ratios of RFLP loci in outbred F_1 progeny. Interprovenance crosses were made using parents from four populations in New Guinea, cross A from Bura (CSIRO seedlot 16587) × Bensbach (CSIRO 16548) and cross B from Boite (CSIRO 16586) × Bimadebun (CSIRO 16990). Crosses were made in a seed orchard at Cardwell, northern Queensland, in April–May 1995 following procedures outlined in Sedgley et al. (1992) and pods were collected in November 1995. Seeds were germinated and grown in the glasshouse in Canberra and leaves collected from 5–7 month-old plants for DNA extraction.

Library construction

A random genomic library was constructed from *A. mangium* total DNA (cross B mother) using standard methods (Sambrook et al. 1989). *Pst*I-restricted DNA fragments ranging from 0.5 to 2.3 kb were ligated into de-phosphorylated pUC19 and transformed into *Escherichia coli*. Transformant colonies were selected for the presence of recombinant plasmids using IPTG and Xgal and hybridised with labelled genomic DNA to detect plasmids containing highly repetitive DNA. Dilutions of overnight cultures of individual bacterial colonies (1:50 in d $H_2$0) were denatured and stored at –20°C. Probes were prepared by PCR-amplification of inserts, purified using spin columns (Qiagen Qiaquick PCR Purification Kit) and insert sizes were determined relative to *Hind*III-digested λ DNA on 0.8% agarose gels. Random genomic probes were given consecutive numbers (g1–920) and letter suffixes were added when more than one locus was detected by the same probe.

DNA procedures

Total genomic DNA was extracted from 5 to 10 g of leaves from parents and progeny using a modified CTAB procedure as described in Butcher et al. (1998). Four µg of DNA from each parent were digested with each of 5–12 restriction enzymes and fragments separated by electrophoresis on 0.8% agarose gels for 6-bp enzymes and 1.2% agarose for 4-bp enzymes. DNA was transferred to nylon membranes (Amersham Hybond-N+) by capillary blotting. Probes, varying from 200 to 2300 bp in size, were labelled with ^{32}P-dCTP using the random priming method (Feinberg and Vogelstein 1983). Filters were pre-hybridized for several hours at 65°C in 2 × SSPE, 1% SDS, 100 µg/ml of herring sperm DNA, and 5 × Denhardt's. Pre-hybridization solution was replaced with hybridization solution with the same ingredients plus 10% dextran sulphate and denatured labelled probe. Filters were hybridized overnight at 65°C, rinsed and washed twice at 65°C for 15 min in 2 × SSC, 0.1% SDS, exposed to Cronex film for 3–6 days at –80°C with intensifying screens. Filters were stripped for re-use in 1% SDS.

Survey of restriction enzymes

To determine the relative efficiency of different restriction enzymes in detecting polymorphism, 30 random genomic probes were hybridised to filters containing DNA from the two sets of parents digested with 12 restriction enzymes. These included six enzymes recognising 4-bp sequences (*Hpa*II, *Alu*I, *Hae*III, *Rsa*I, *Sau*3AI, *Mse*I) and six enzymes recognising 6-bp sequences (*Dra*I, *Eco*RI, *Hin*dIII, *Bgl*II, *Eco*RV, *Bcl*I). The sizes of the restriction fragments revealed by the probes were estimated by comparison with λ DNA cut with *Hind*III and the relationship between average fragment size and polymorphism was investigated. A further 250 probes were screened using the 6-base cutters and *Hpa*II, and 300 probes using *Eco*RI, *Eco*RV, *Hind*III, *Bcl*I and *Hpa*II.

Segregation of RFLP loci

Probe/enzyme combinations for mapping were selected to maximise the number of potentially segregating alleles based on RFLP patterns in the four parents. Segregation ratios were calculated for 265 RFLP loci, using 108 progeny in cross A and 123 progeny in cross B, and compared with expected Mendelian ratios by χ^2 analysis.

Homology of loci across species

To determine the potential for using *A. mangium* RFLP probes in studies of related species, 20 randomly selected genomic probes were hybridised to *Hin*dIII- and *Bcl*I-digested DNA samples from each of the species listed in Table 6. These included 38 acacia species representing three subgenera and eight sections of the genus. Individuals from the genera *Faidherbia*, *Archidendron* and *Pararchidendron* were included to determine whether the RFLP markers hybridise to other Mimosaceae. The hybridisation procedures and stringency conditions were the same as those listed above for *A. mangium*.

Results

Efficiency of restriction enzymes

Screening the first 30 probes revealed that, as expected, the average size of restriction fragments hybridising to clones was significantly greater for enzymes recognising 6-bp sequences (6.8 kb) than those recognising 4-bp se-

Table 1 Average fragment size and number of polymorphisms detected between four unrelated mapping parents using 30 probes and restriction enzymes recognising 4-bp and 6-bp sequences

Enzyme	*Dra*I	*Eco*RI	*Hin*dIII	*Bgl*II	*Eco*RV	*Bcl*I	*Hpa*II	*Alu*I	*Hae*III	*Rsa*I	*Sau*3A1	*Mse*I
Average fragment size (kbp)	3.2	8.8	4.2	8.6	10.4	6.1	5.5	0.5	1.8	2.0	0.9	0.4
Variable loci (%)	5 (17)	7 (23)	4 (13)	4 (13)	5 (17)	7 (23)	13 (43)	1 (3)	4 (13)	5 (17)	4 (13)	3 (10)

Table 2 Number of clones screened with each of 12 restriction enzymes and the proportion showing variation between mapping parents from two unrelated pedigrees

Probe	*Dra*I	*Eco*RI	*Hin*dIII	*Bgl*II	*Eco*RV	*Bcl*I	*Hpa*II	*Alu*I	*Hae*III	*Rsa*I	*Sau*3A1	*Mse*I
Total screened	290	583	583	290	583	583	531	32	32	32	32	32
% Loci variable in A	9	18	17	15	19	14	37	0	7	4	15	7
% Loci variable in B	13	18	17	13	20	18	40	4	15	19	7	7
% Loci variable in A or B cross	17	27	25	21	26	23	51	4	22	19	15	11
% Unique[a] polymorphisms	15	13	7	10	13	7	51	0	0	0	0	0

[a] Polymorphisms that were only detected with one enzyme

Table 3 Distribution of the number of alleles at 344 loci calculated from four first-generation individuals

Number of loci (%)					
2 alleles	3 alleles	4 alleles	5 alleles	6 alleles	7 alleles
248 (72%)	63 (18%)	17 (5%)	11 (3%)	3 (1%)	2 (0.5%)

quences (1.8 kb) ($P < 0.01$ determined by a one-way ANOVA). However, the average fragment length for DNA digested with *Hpa*II was five-times larger than the average of other 4-bp cutters (Table 1).

Differences among the 6-bp enzymes in the size of fragments detected by the 30 probes were also significant ($P < 0.01$). There was no evidence that enzymes which tended to produce larger restriction fragments detected higher levels of polymorphism. Regression of the number of polymorphic loci and the average length of restriction fragments detected using 6-bp enzymes was not significant. A positive correlation would be expected if the majority of polymorphisms were caused by insertion deletions rather than base substitutions (McCouch et al. 1988). For enzymes recognising 4-bp sequences the regression was significant ($P < 0.01$), but this reflected the larger fragment size and higher level of polymorphism detected using *Hpa*II. When *Hpa*II was omitted from the analysis the regression was not significant.

It is noteworthy that approximately half of the probes that were polymorphic with *Hpa*II were not polymorphic with other enzymes. In contrast, no probes were uniquely polymorphic with any of the other 4-bp enzymes whereas from 7 to 15% of probes were uniquely polymorphic with a 6-bp enzyme (Table 2).

Comparison of restriction enzymes, based on the 30 probes, revealed that a higher proportion of RFLPs were detected using enzymes which recognise 4-bp sequences (52%) than enzymes which recognised 6-bp sequences (30%). This difference was also attributable to *Hpa*II. Forty three percent of probes detected polymorphism between DNA from the mapping parents cut with *Hpa*II compared with 30% for all the remaining enzymes recognising 4-bp sequences. While 4-bp cutters may be expected to reveal a larger number of base-pair change variants because of the larger number of restriction sites detected per probe, the fragments were often too small (< 200 bp) to be detected on Southerns.

Library characterisation

No high-copy number clones were identified in the preliminary screening with labelled genomic DNA. Of the 580 genomic probes surveyed for copy number and polymorphism 86% were classified as single copy, 10% as multiple copy (3–10 bands per lane) and 4% repetitive (> 10 bands or a smear of undifferentiated DNA). Fifty two percent of probes showed allelic differences between the first-generation individuals of the mapping pedigrees with at least one enzyme, 28% were invariant and 6% were redundant, producing the same banding patterns as other probes.

The number of alleles was recorded for each locus (Table 3) and enzymes were selected for each probe

Table 4 The number of loci with expected segregation ratios and distorted segregation ratios ($P < 0.05$) from a total of 265 RFLP loci (percentages in parentheses)

Segregation ratio	1:1		1:2:1		1:1:1:1		Loci with distorted segregation ratios (%)	
Cross	A	B	A	B	A	B	A	B
*Hpa*II	37	38	5	4	19	24	2 (3.3)	5 (7.6)
*Bcl*I	18	24	3	1	4	3	1 (4)	1 (3.6)
*Eco*RI	18	21	1	1	2	2	0	0
*Eco*RV	20	22	4	6	0	2	1 (4.2)	1 (3.3)
*Hind*III	18	18	1	2	2	3	2 (9.5)	2 (8.7)

Table 5 Segregation (S) of RFLP loci in second-generation *A. mangium* individuals from two pedigrees (crosses A and B), showing all loci with distorted segregation ratios. Probabilities (*P*) determined from $\chi 2$ analysis

Locus	Enzyme	Observed S cross A				S-ratio	*P*	Observed S cross B				S ratio	*P*
g19a	*Hpa*II	44	66			1:1	0.036	32	27	39	24	1:1:1:1	0.238
g50a	*Hpa*II	30	56	25		1:2:1	0.928	50	75			1:1	0.025
g251	*Bcl*I	58	52			1:1	0.567	73	51			1:1	0.048
g285	*Hpa*II	44	67			1:1	0.029	35	35	28	26	1:1:1:1	0.546
g350	*Hin*dIII							46	79			1:1	0.003
g371	*Eco*RV	66	45			1:1	0.046						
g374	*Hpa*II	34	26	21	30	1:1:1:1	0.342	43	33	21	27	1:1:1:1	0.036
g454a	*Hin*dIII	45	66			1:1	0.046	50	75			1:1	0.025
g551	*Eco*RV							49	75			1:1	0.020
g630	*Hpa*II	26	31	34	20	1:1:1:1	0.255	43	17	19	35	1:1:1:1	0.001
g732	*Hpa*II							49	76			1:1	0.016
g733a	*Bcl*I	20	38	35	17	1:1:1:1	0.007	31	24	42	28	1:1:1:1	0.126
g870	*Hin*dIII	44	67			1:1	0.029	68	57			1:1	0.325
g877	*Hpa*II	57	54			1:1	0.776	78	47			1:1	0.006

which maximised the number of alleles in the first generation individuals. Fully informative loci are more useful in framework mapping and are also more likely to be variable in other families. In *A. mangium* the proportion of fully informative RFLP loci was relatively low with only 12 and 15% of the variable loci in crosses A and B respectively having 3 or 4 segregating alleles. Three quarters of these were detected using *Hpa*II.

Segregation of RFLP loci in the second generation

Segregation of RFLPs was assessed at 230 loci for the two pedigrees using 108 second-generation individuals from cross A and 123 second-generation individuals from cross B. The highest proportion of loci with distorted segregation ratios were detected using *Hin*dIII (Table 4). Of the 230 loci (62 loci variable in cross A only, 78 loci variable in cross B only, and 91 loci variable in both crosses) nine loci in cross B (5.3%) and six loci in cross A (3.9%) had distorted segregation ratios (see Table 5); within the 5% probability expected due to chance alone. Nine loci fitted expected segregation ratios in one cross but not the other, four were only variable in one cross, and only one locus showed segregation that was significantly different from expected in both pedigrees.

Approximately the same number of loci were segregating on the male and the female side. In both pedigrees 49% of loci segregrated on the female side and 51% on the male side.

Homology of probes across species

Twenty RFLP probes were hybdridised to DNA from 42 species representing the three subgenera of *Acacia*, the closely related genus *Faidherbia* (formerly included in the subgenus *Acacia*, see Maslin and Stirton 1997) and the genera *Archidendron* and *Pararchidendron*. RFLP loci were highly conserved across the subgenus *Phyllodineae* and in *Faidherbia* and *Archidendron* (Table 6). All *A. mangium* probes cross-hybridised under low-stringency conditions to species tested in the section *Juliflorae* with the exception of *A. aneura*. At least 85% of probes hybridised to species in other sections of the subgenus *Phyllodineae*. Eighty percent of probes hybridised to the two sub-species tested in the subgenus *Aculeiferum*. Hybridisation in the subgenus *Acacia* was more variable with from 45 to 85% of probes hybridising to DNA from different species. This group contains the highest proportion of polypoid species. The polyploid species display more complex RFLP banding patterns which are more difficult to interpret genetically.

Discussion

The proportion of high-copy clones in the *Pst*I library developed from *A. mangium* (14%) was lower than recorded in other trees including *Eucalyptus nitens* (11% after screening to remove 21% of plasmids with high-copy sequences) (Byrne et al. 1994), *Pinus taeda* (22%)

Table 6 Number of loci which hybridised to species representing three subgenera and seven sections of *Acacia* using 20 random genomic probes from *A. mangium* (ploidy level[a] in parentheses; $x = 13$)

Genus *Acacia* subgenus Phyllodineae	Number of loci hybridised	Genus *Acacia* subgenus Aculeiferum	Number of loci hybridised
Section Juliflorae		Section Aculeiferum	
Acacia mangium (2*x*)	20	*Acacia senegal* ssp. *senegal* (2*x*)	17
Acacia neurocarpa (2*x*)	20	*Acacia senegal* ssp. *leiorhachus* (2*x*)	16
Acacia holosericea (4*x*)	20		
Acacia cowleana (6*x*)	20		
Acacia elachantha (4*x*)	20	Genus *Acacia* subgenus Acacia	
Acacia thomsonii (4*x*)	20		
Acacia colei (6*x*)	20	*Acacia erioloba* (2*x*)	17
Acacia '*peregrina*' *ms* (2*x*)	20	*Acacia tortilis* ssp. *raddiana* (8*x*)	16
Acacia crassicarpa (2*x*)	20	*Acacia tortilis* ssp. *spirocarpa* (8*x*)	15
Acacia auriculiformis (2*x*)	20	*Acacia tortilis* ssp. *heteracantha* (4*x*)	15
Acacia spirorbis (2*x*)	20	*Acacia karroo* (4*x*)	15
Acacia aneura (4*x*)	18	*Acacia nilotica* (4*x*) Australian landrace	15
Section Plurinerves		*Acacia nilotica* ssp. *indica* (4*x*)	15
Acacia simsii (2*x*)	18	*Acacia nilotica* ssp. *adstringens* (8*x*)	12
Acacia flavescens (2*x*)	19	*Acacia nilotica* ssp. *tomentosa* (4*x*)	11
Acacia melanoxylon (2*x*)	20	*Acacia nilotica* ssp. *kraussiana* (4*x*)	10
Acacia simplex (2*x*)	20	*Acacia nilotica* ssp. *subalata* (4*x*)	9
Acacia brassii (2*x*)	19	*Acacia bidwillii* (2*x*)	14
Section Botrycephalae		*Acacia boliviana* (2*x*) introduced	14
Acacia dealbata (2*x*)	20		
Acacia mearnsii (2*x*)	19		
Section Phyllodineae		Other genera	
Acacia spinescens (2*x*)	18	*Faidherbia*	
Acacia falciformis (2*x*)	18	*Faidherbia albida* (2*x*)	17
Acacia victoriae (2*x*)	17	*Archidendron*	
Section Alatae		*Archidendron lucji* (2*x*)	18
Acacia alata (2*x*)	19	*Archidendron ramiflorum* (2*x*)	18
Section Lycopodiifoliae		*Pararchidendron pruinosum* (2*x*)	18
Acacia lycopodiifoliae (2*x*)	18		

[a] Ploidy levels from Hamant et al. (1975); Pedley (1987); Maslin and McDonald (1996); and Bukhari (1997)

(Devey et al. 1991), *Pseudotsuga menziesii* (> 50%) (Jermstad et al. 1994) and citrus (27%) (Liou et al. 1996). This suggests either a lower amount of repetitive DNA in the *A. mangium* genome or else that a higher proportion of high-copy sequences are methylated and therefore excluded from the *Pst*I library.

Efficiency of restriction enzymes

Comparison of restriction enzymes revealed that *Hpa*II was the most efficient in detecting RFLPs in *A. mangium*. To-date, 4-bp enzymes have not been used in the mapping of forest trees based on findings in crop species that they displayed RFLPs less frequently than 6-bp enzymes (Landry et al. 1987; McCouch et al. 1988). It has also been reported that the majority of polymorphisms in plant genomes result from localized rearrangements in DNA (Paterson 1996) which are more likely to be detected in the larger fragments generated by 6-bp enzymes. Rearrangements will usually be detected by more than one restriction enzyme, while a base substitution detected by one restriction enzyme will not be detected by other enzymes unless their recognition sites overlap. Findings of significant positive correlations between the average size of restriction fragments and the amount of polymorphism detected in rice (McCouch et al. 1988), lettuce (Landry et al. 1987) and tomato (Miller and Tanksley 1990) suggest that the majority of polymorphisms in these species were caused by rearrangements. These findings were supported in rice and citrus by positive correlations between the probability of detecting polymorphism with one enzyme and the number of other enzymes with which polymorphisms were detected (McCouch et al. 1988; Liou et al. 1996).

In *A. mangium* there was no correlation between the fragment size detected using 6-bp enzymes and the level of polymorphism. Similar results were reported for *E. nitens* (Byrne et al. 1994), suggesting that rearrangements are not the main cause of polymorphism in these species. In *A. mangium*, 43% of polymorphisms in cross A and 46% of polymorphisms in cross B appear to be due to single-base changes or small deletions as they were detected using only one enzyme.

The proportion of polymorphic probes in *A. mangium* was similar to that in *E. nitens* (48%), a species with high levels of genetic diversity (Byrne et al. 1994). However the proportion of fully informative markers (3 or 4 alleles) was low (13–15%) compared with *E. nitens* (40%). *A. mangium* is generally regarded as a species with low levels of genetic diversity (Moran et al.1989) and even the more-diverse populations from New Guin-

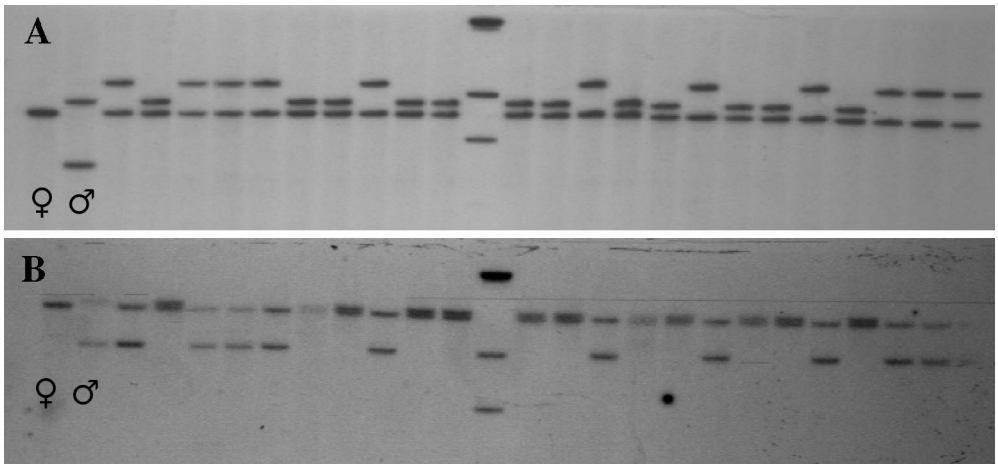

Fig. 1 DNA of parents (*lanes 1 and 2*) and progeny of cross B digested with *Hpa*II (**A**) and *Eco*RV (**B**) and hybridised with probe g492 (λ *Hin*dIII size marker in middle lane)

ea have approximately half the level of RFLP variation (H_E = 0.21) (Butcher et al. 1998) recorded in *E. nitens* (mean H_E = 0.37) (Byrne et al. 1998).

The relatively high proportion of polymorphic probes in *A. mangium* can be attributed to the number of polymorphisms detected using *Hpa*II. Of the polymorphic probes, approximately 70% detected polymorphism using *Hpa*II and three quarters of the fully informative loci were detected using this enzyme. The high proportion of probes that were polymorphic only with *Hpa*II (51%) compared to other enzymes (0–15%) suggests a high level of base substitution within the recognition sequence of this enzyme. *Hpa*II was the only enzyme surveyed with a CG dinucleotide in its recognition sequence (CCGG). In humans, restriction enzymes containing CpG dimers revealed more polymorphism, possibly because methylated cytosine at this position has a high mutation rate (Barker et al. 1984). Cytosine in DNA may spontaneously deaminate to form uracil, which, if not repaired, can result in a cytosine to thymine transition mutation at the next round of replication (Coulondre et al. 1978; Duncan and Miller 1980). Higher levels of polymorphism in sequences with CpG dimers have also been reported in procaryotes (Duncan and Miller 1980) but have not previously been reported in plants (Helentjaris et al. 1985; Landry et al. 1987; McCouch et al. 1988).

The average size of restriction fragments produced using *Hpa*II was larger than for other 4-bp enzymes. If all base pairs occur randomly and in equal frequency throughout the genome, all enzymes recognising 4-bp sequences would cleave more often and generate smaller fragments than 6-bp enzymes. However the CG dinucleotide has been shown to be under-represented in the genome of dicot plants (but not monocots) (Karlin and Burge 1995). It is also relatively rare in the human genome (Barker et al. 1984), and occurs at about one-fifth of the expected frequency in vertebrates (Swartz et al. 1962). Enzymes with CG in their recognition sequence, including *Hpa*II, would therefore cleave less often and produce larger fragments.

The larger than expected fragment sizes produced with *Hpa*II may also be associated with the relatively high content of methylated cytosine in plant DNA (Gruenbaum et al. 1981). If either of the cytosines in the recognition sequence of *Hpa*II (CCGG) are methylated, it will not cleave. With *Hpa*II there is a risk that cosegregation analysis may be confounded by polymorphism among the progeny or parents due to methylation. Such artefacts can appear as non-parental bands in the progeny or may result in segregation distortion. However, in this study, apparent artefacts due to methylation were only observed at one locus. In locus g492 in cross B an allele detected in the male parent was replaced by a different allele in the progeny (Fig. 1). It appears that a *Hpa*II site is methylated in this allele whenever it occurs in the progeny, resulting in the observed larger restriction fragment. The juvenile state of the second-generation plants suggests that the genomic region detected by the probe may be involved in maturation-related phase change. When the same probe was used on DNA cut with *Eco*RV, the same segregation pattern was observed in the progeny but there was no difference between alleles in the male parent and the progeny (Fig. 1). There was no evidence of other polymorphisms being caused by methylation – non-parental bands were not observed at other loci. In addition, segregation distortion was not observed more frequently using *Hpa*II than with other enzymes.

Homology of probes across species

The ability to use *A. mangium* RFLP probes across the genus provides opportunities for genetic studies involving other *Acacia* species. Studies of comparative genome organisation, phylogenetics, population genetics and synteny mapping are all possible. The genus includes more than 1200 species which are currently classified under three subgenera (Maslin and Stirton 1997). *A. mangium* is in the subgenus Phyllodineae which has more than 900 species and is largely confined to Australia. This group includes the main timber and pulp-producing species which are most likely to be of interest for comparative mapping. The subgenera Acacia and

Aculeiferum are pantropical and include 120–130 species and 180–190 species respectively with major centres of distribution of Acacia in Asia, Central and Southern America and for Aculeiferum, North and South America and Africa. The higher proportion of probes hybridising in the subgenera Phyllodineae and Aculeiferum supports current taxonomic views that the subgenus Acacia could be treated as a distinct genus and that Aculeiferum and Phyllodineae are closely related to one another (Pedley 1986; Chappill and Maslin 1995; Brain and Maslin 1996).

The higher proportion of RFLPs hybridising to *Faidherbia* and *Archidendron* compared with species in the subgenus Acacia suggests that ploidy level may also be an important factor affecting the homology of RFLP loci. In the section Juliflorae, all RFLPs hybridised to polyploids which were closely related to *A. mangium* while two loci failed to hybridise to the more-distantly related *A. aneura*. In the subgenus Acacia, fewer RFLPs hybridised to the polyploid species. This may reflect rapid genomic change which has been reported to accompany polyploidisation (Song et al. 1995). Lagercrantz (1998) suggested that allopolyploidisation is likely to result in an increase in aberrant meiotic pairing and translocations among homoeologous chromosomes.

The level of RFLP variation in *A. mangium* is sufficient to develop a framework map, despite the species' relatively low genetic diversity. The ability to cross-hybridise *A. mangium* RFLP probes to other species in the genus *Acacia* opens opportunities for comparative studies of genome organisation and for the development of a generic map. The construction of a map will enable the characterisation of genes controlling important traits and provide opportunities to improve the efficiency of breeding programs using marker-assisted selection.

Acknowledgements The assistance of Guiseppina Morosin and Juliet Ward with DNA extractions, Maurie Mills with the maintenance of plants, Jane Harbard and Peter Pomroy (Queensland Forest Research Institute) with controlled pollinations, is gratefully acknowledged. Les Pedley (Brisbane Herbarium), Greg Leach (Darwin Botanical Gardens), Australian National Botanic Gardens, Crispen Marunda (Zimbabwe Forestry Commission), Yves Ehrhart (CIRAD-Foret) and CSIRO's Australian Tree Seed Centre (ATSC) provided leaf material of acacia species. Jeff Glaubitz and Mike Devey reviewed the manuscript and special thanks are offered to Jeff for valuable discussions. Financial support was provided by ATSC and AusAID.

References

Ahuja MR, Devey ME, Groover AT, Jermstad KD, Neale DB (1994) Mapped DNA probes from loblolly pine can be used for restriction fragment length polymorphism mapping in other conifers. Theor Appl Genet 88:279–282

Barker D, Schafer M, White R (1984) Restriction sites containing CpG show a higher frequency of polymorphism in human DNA. Cell 36:131–138

Brain P, Maslin BR (1996) A serological investigation of the classification of *Acacia* subgenus Phyllodineae (Leguminosae: Mimosoideae). Biochem Syst Ecol 24:379–392

Bukhari YM (1997) Cytoevolution of taxa in *Acacia* and *Prosopis* (Mimosaceae). Aust J Bot 45:879–891

Butcher PA, Moran GF, Perkins HD (1998) RFLP diversity in the nuclear genome of *Acacia mangium*. Heredity 81:205–213

Byrne M, Moran GF, Murrell JC, Tibbits WN (1994) Detection and inheritance of RFLPs in *Eucalyptus nitens*. Theor Appl Genet 89:397–402

Byrne M, Parish TL, Moran GF (1998) Nuclear RFLP diversity in *Eucalyptus nitens*. Heredity 81:225–233

Chappill JA, Maslin BR (1995) A phylogenetic assessment of the tribe Acacieae. In: Crisp M, Doyle JJ (eds) Advances in legume systematics 7: Phylogeny. Royal Botanic Gardens, Kew, pp 77–99

Cheung WY, Landry BS (1996) Current status of genome mapping in the Cruciferae. In: Paterson AH (ed) Genome mapping in plants. Academic Press, California, pp 193–210

Coulondre C, Miller JH, Farabaugh PJ, Gilbert W (1978) Molecular basis of base-substitution hotspots in *Escherichia coli*. Nature 274:775–780

Devey ME, Jermstad KD, Tauer CG, Neale DB (1991) Inheritance of RFLP loci in a loblolly pine three-generation pedigree. Theor Appl Genet 83:238–242

Devey ME, Sewell MM, Uren TL, Neale DB (1999) Comparative mapping in loblolly and radiata pine using RFLP and microsatellite markers. Theor Appl Genet 99:656–662

Duncan BK, Miller JH (1980) Mutagenic deamination of cytosine residues in DNA. Nature 287:560–561

Feinberg AP, Vogelstein B (1983) A technique for radiolabelling DNA restriction endonuclease fragments to high specific activity. Anal Bioch 132:6–13

Graner A, Siedler H, Jahoor A, Herrmann RG, Wenzel G (1990) Assessment of the degree and the type of restriction fragment length polymorphism in barley (*Hordeum vulgare*). Theor Appl Genet 80:826–832

GruenbaumY, Naveh-Many T, Cedar H, Razin A (1981) Sequence specificity of methylation in higher-plant DNA. Nature 292:860–862

Hamant CN, Lescanne N, Vassal J (1975) Sur quelques nombres chromosomiques nouveaux dans le genre Acacia. Taxon 24:667–670

Helentjaris T, King G, Slocum M, Siedenstrang C, Wegman S (1985) Restriction fragment polymorphisms as probes for plant diversity and their development as tools for plant breeding. Plant Mol Biol 5:109–118

Jermstad KD, Reem AM, Henifin JR, Wheeler NC, Neale DB (1994) Inheritance of restriction fragment length polymorphisms and random amplified polymorphic DNAs in coastal Douglas fir. Theor Appl Genet 89:758–766

Karlin S, Burge C (1995) Dinucleotide relative abundance extremes: a genomic signature. Trends Genet 11:283–290

Lagercrantz U (1998) Comparative mapping between *Arabidopsis thaliana* and *Brassica nigra* indicates that *Brassica* genomes have evolved through extensive genome replication accompanied by chromosome fusions and frequent rearrangements. Genetics 150:1217–1228

Landry BS, Kesseli R, Leung H, Michelmore RW (1987) Comparison of restriction endonucleases and sources of probes for their efficiency in detecting restriction fragment length polymorphisms in lettuce (*Lactuca sativa* L.) Theor App Genet 74: 646–653

Liou P-C, Gmitter FG, Moore GA (1996) Characterisation of the *Citrus* genome through analysis of restriction fragment length polymorphisms. Theor Appl Genet 92:425–435

Maslin BR, McDonald MW (1996) A key to useful Australian acacias for the seasonally dry tropics. CSIRO, Melbourne

Maslin BR, Stirton CH (1997) Generic and infrageneric classification in *Acacia* (Leguminosae: Mimosoideae): a list of critical species on which to build a comparative data set. Bulletin of the International Group for the Study of Mimosoideae 20:22–44

McCouch SR, Kochert G, Yu ZH, Wang ZY, Khush GS, Coffman WR, Tanksley SD (1988) Molecular mapping of rice chromosomes. Theor Appl Genet 76:815–829

Miller JC, Tanksley SD (1990) Effect of different restriction enzymes, probe source and probe length on detecting restriction fragment length polymorphism in tomato. Theor Appl Genet 80:385–389

Moran GF, Muona O, Bell JC (1989) *Acacia mangium*: a tropical forest tree of the coastal lowlands with low genetic diversity. Evolution 43:231–235

Nelson JC, Dubcovsky J, McCouch SR, Sorrells ME (1986) Genome mapping in temperate grains and grasses in the family Gramineae (Poaceae). In: Paterson AH (ed) Genome mapping in plants. Academic Press, California, pp 243–266

Paterson AH (1996) Making genetic maps. In: Paterson AH (ed) Genome mapping in plants. Academic Press, California, pp 23–39

Pedley L (1986) Derivation and dispersal of *Acacia* (Leguminosae), with particular reference to Australia, and the recognition of Senegalia and Racosperma. Bot J Linn Soc 92:219–254

Pedley L (1987) Acacias in Queensland. Qld Dept Primary Industries, Brisbane

Sambrook J, Fritsch EF, Maniatis T (1989) Molecular cloning: a laboratory manual, 2nd edn. Cold Spring Harbor Laboratory Cold Spring Harbor, New York

Sedgley M, Harbard J, Smith R-MM, Wickneswari R, Griffin AR (1992) Reproductive biology and interspecific hybridisation of *Acacia mangium* and *Acacia auriculiformis* A. Cunn. ex Benth. (Leguminosae: Mimosoideae). Aust J Bot 40:37–48

Song K, Lu P, Tang K, Osborn T (1995) Rapid genome change in synthetic polyploids of *Brassica* and its implications for polyploid evolution. Proc Natl Acad Sci USA 92:7719–7723

Swartz MN, Trautner TA, Kornberg A (1962) Enzymatic synthesis of deoxyribonucleic acid. XI. Further studies on nearest-neighbor base sequences in deoxyribonucleic acids. J Biol Chem 237:1961–1967

Turnbull JW, Midgley SJ, Cossalter C (1998) Tropical acacias planted in Asia: an overview. In: Turnbull JW, Crompton HR, Pinyopusarerk K (eds) Recent developments in *Acacia* planting. ACIAR, Canberra, pp.14–28