First Cytogenetic Investigation in Populations of *Acacia heterophylla*, Endemic from La Réunion Island, with Reference to *A. melanoxylon*

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Five populations of tetraploid *Acacia heterophylla*, endemic from La Réunion island, were compared together and with their Australian diploid relative *A. melanoxylon* for cytogenetic and DNA characteristics. *A. melanoxylon* ($2n = 26$) had 1.59 pg nuclear DNA; *A. heterophylla* ($2n = 4x = 52$) had double this value (3.19 pg), and there was no difference between populations within species. Both species had 39% GC. Interchromosome connections were evident at metaphase and mitotic irregularities at anaphase were twice as frequent in *A. heterophylla* as in *A. melanoxylon*, again with no difference between populations within species. These results argue for a recent autotetraploid origin of *A. heterophylla* from *A. melanoxylon*. Yet, fluorochrome banding showed that in some *A. heterophylla* populations, GC-rich bands had slightly changed from the supposed ancestral pattern, probably by means of translocations involving parts of nuclear organizer areas. No clear relation was found between banding patterns and ecological factors.

Key words: *Acacia heterophylla* Willd., *Acacia melanoxylon* R. Br. (Blackwood), interpopulation variation, autotetraploidy, insular evolution, flow cytometry, DNA content, base ratio, cytogenetics, fluorochrome banding.

INTRODUCTION

Acacia heterophylla Willd., endemic from La Réunion island (21° S, 55° E), is the single indigenous *Acacia* species of Mascarenes (Polhill, 1990). It is a tree or a shrub growing from an altitude of 1200 to 2300 m within several original native formations covering a wide pedo-climatic range. These formations, sometimes dominated by *A. heterophylla*, contain a rich endemic flora (Cadet, 1980) whose conservation is necessary. *A. heterophylla* has a local economic value for timber and for reclamation of impoverished secondary areas.

The biogeography of *A. heterophylla* is particularly interesting because few species of the large *Acacia* genus have arisen outside of Australia (the main diversity centre with 729 species; Maslin and Pedley, 1982), Africa or tropical America (Moran, Muona and Bell, 1989). *A. heterophylla* is taxonomically close to another phyllodinous species, *A. koa* A. Gray, endemic to Hawaii, and heterophyllous species do not occur on continents except Australia where they arose (Pedley, 1975). According to Vassal (1969) and Pedley (1975), these two *Acacia* species are polyploids ($2n = 4x = 52$) from a common diploid ancestor, *A. melanoxylon* R. Br. ($2n = 2x = 26$) growing on the eastern coast of Australia, widely used for timber furniture and recently studied by Muona, Moran and Bell (1991). Biochemical studies (Bell and Evans, 1978) confirmed the link between *A. heterophylla* and Australian species, but not with *A. koa*.

Despite its high economic and ecological value, little is known about the biology, genetic structure of populations or even dynamics of ecosystems involving *A. heterophylla*. However, the species displays an obvious phenotypic variation (Cadet, 1980) and constitutes an interesting example of colonization and speciation on a recent island. We have initiated a biosystematic and ecological study and this paper shows the first results of cytogenetic investigations. Representative populations of *A. heterophylla* have been compared together and with *A. melanoxylon* populations for DNA amount and base composition, chromosome numbers, mitotic behaviour, and fluorochrome banding.

MATERIAL AND METHODS

Seeds were collected from 25 trees in each of five isolated *A. heterophylla* populations (Table 1). Populations Maïdo, Chicots and Bélouve are on the slopes of the former volcano (Piton des Neiges), Volcan and Vallée Heureuse on ancient lava flows of the active shield volcano (Piton de la Fournaise). For DNA measurement, five seedlings from each of five randomly chosen parents per population were firstly grown in a greenhouse for three months. For cytogenetic studies, one to five seeds of each individual from each population were germinated. Seeds from three Australian *A. melanoxylon* populations constituted by bulked progenies of ten trees were kindly provided by G. F. Moran, of CSIRO (Canberra). These populations were from

TABLE 1. Origin of *A. heterophylla* populations sampled from La Réunion, with some ecological details

Population*	Area	Altitude (m)	Soil†	Vegetation
Maïdo	W	1950	Poorly differentiated leached humic andosol	High altitude shrub forest disturbed by extensive grazing and invasion by alien species
Chicots (Plaine des)	NW	1400	Podzol with mascarenite	Native <i>A. heterophylla</i> forest including pastures and pathways
Bélouve	NE	1500	Podzol with mascarenite	Managed <i>A. heterophylla</i> forest (30 years) replacing a native forest
Volcan (Route du)	E	1600	Podzol and perhydrated differentiated andosol	Patches of relictual native forest replaced during the 19th century by intensive pastures
Vallée Heureuse	SE	1700	Podzol with mascarenite	Native shrub forest with little disturbance

* Populations Chicots and Vallée Heureuse harvested by D. Strasberg and C. Thebaud.

† From Raunet (1991).

Ravenshoe (Queensland; altitude 1000 m), Tallaganda (New South Wales; 1120 m) and Welshpool (Victoria; 250 m).

Flow cytometry

Total DNA amount in nuclei was assessed by flow cytometry with *Petunia hybrida* PxPc6 ($2C = 2.85$ pg; 41.0 % GC) as internal standard, according to the technique of Brown *et al.* (1991). Three 25 mm² patches were sampled on juvenile leaves of similar rank on each individual and chopped together with 25 mm² of *Petunia* leaf in 400 µl buffer modified from Galbraith *et al.* (1983) containing 5 µl ml⁻¹ β-mercaptoethanol. Histograms of fluorescence intensities were based on at least 5000 nuclei per sample. Total 2C DNA content of each *Acacia* sample was determined as the ratio of the modal position of its 2C nuclei relative to those of *Petunia*, using the intercalating dye ethidium bromide.

Base ratio was calculated by the same means after staining with AT-specific fluorochrome 4', 6-diamino-2-phenylindole (DAPI, Aldrich) and matching the ethidium bromide result, following Godelle *et al.* (1993). Two plants per diploid and one per tetraploid population were first assessed. As interpopulation variation within species was not larger than intra-individual variation, the plants were considered as representative of their populations.

Cytogenetics

For observation of mitosis, root tips from 4-d-old seedlings were fixed in 3:1 ethanol:acetic acid for at least 24 h at 4 °C. After 10 min hydrolysis with 1 N HCl at 60 °C, meristems were stained with Schiff reagent and squashed in acetocarmine following Östergren and Haneen's technique (1962). For chromosome morphology and banding, seedlings were pretreated before fixation with 0.002 M 8-hydroxyquinoline at 16 °C during 3 h for *A. heterophylla* and 2.5 h for *A. melanoxylon*. None of the other antimetabolites tested (colchicine, 1-chloronaphthalene, oryzalin, cold water, or their combinations) could improve chromosome spreads. Before banding, hydrolysis was performed with

45 % acetic acid at 60 °C for 13 min. Squashed preparations were made permanent following Conger and Fairchild (1953). Fluorochrome banding with the GC-specific anti-biotic Chromomycin A₃ (CMA₃, Serva) followed the technique of Ambros and Schweizer (1987), using distamycin A (Serva; 0.1 mg ml⁻¹, 10 min) as enhancer before incubation in 0.2 mg ml⁻¹ CMA₃ for 7 min in the dark. AT specific banding used 2 min incubation in bisbenzimidazole (Hoechst 33258) according to Martin and Heisemann (1988). Interphase nuclei were observed in differentiated cells near the meristem. Numerical comparisons between populations for mitotic irregularities (on 615 metaphase plates, 497 anaphases and 1272 telophases) and interphase fluorescent prints of 1287 nuclei used χ^2 tests of Pearson at $P = 0.05$.

RESULTS

Flow cytometry

Nuclear DNA histograms showed nearly all the nuclei at the 2C stage. Coefficients of variation for 2C population fluorescence ratios were low (on average 3.00 % for ethidium bromide and 2.60 % for DAPI). There was no interpopulation variation of nuclear total DNA amount within species. Total DNA amount in *A. heterophylla* ($2C = 3.19$ pg; s.d. = 0.004) was exactly twice DNA amount of *A. melanoxylon* ($2C = 1.59$ pg; s.d. = 0.004). GC percentage was the same in all the populations of both species (39.0 %).

Chromosome observations

Chromosome counts (Coulaud, Godelle and Siljak-Yakovlev, 1992) showed that all the *A. melanoxylon* populations were diploid with $2n = 2x = 26$ chromosomes (Figs 1–2) and all the *A. heterophylla* populations tetraploid with $2n = 4x = 52$ chromosomes (Fig. 3). In accordance with an early report in Leguminosae (Atchinson, 1948), endomitoses were rather frequent in meristems of both species (3/100 metaphase plates in *A. melanoxylon* and 4/97 in *A. heterophylla*).

In both species, a few big chromocentres were present in interphase nuclei (one to two in *A. melanoxylon* and one to

FIGS 1, 2 and 4. *Acacia melanoxylon* Welshpool cells ($2n = 26$). Others, *A. heterophylla* ($2n = 52$). All figures $\times 1500$.

FIGS 1–3. Pretreated metaphases (Figs 1 and 3) and prometaphase (Fig. 2) showing secondary constrictions (arrows), other lately spiralized chromosome arms (Fig. 2), and some interchromosome connections (Figs 2 and 3).

four in *A. heterophylla*). In meristematic cells, a number of small netted chromomeres (one per chromosome) appeared during early prophase in pericentromeric areas and were visible with any stain (reticulated type nuclei). Spiralization of chromosomes began in these areas and was not achieved before metaphase (Fig. 2). Nucleoli did not disappear before metaphase, as often observed in Mimosoideae (Tjio, 1948). Because of the persistence of telomeric and centromeric interchromosome connections (evident with any stain), metaphase chromosomes were particularly difficult to spread, but at least one pair in *A. melanoxylon* populations and two pairs in *A. heterophylla* bearing a secondary constriction (SC) could be noticed.

For mitotic irregularities, X^2 tests did not show significant differences between populations within species at any stage. The frequencies of metaphase irregularities (one to four slightly marginal chromosomes) were not significantly different between species (26%). Anaphase irregularities were significantly twice more frequent in *A. heterophylla* (54%) than in *A. melanoxylon* (22%). They appeared as laggard chromosomes (one to three in both species) or bridges (one to four in *A. melanoxylon* and one to eight in *A. heterophylla*), with most often two to four bridges in all the populations. Most of these irregularities had no consequence on chromosome distribution for telophase was generally normal (8% with bridges in *A. heterophylla* and 0.3% in *A. melanoxylon*).

Fluorochrome banding

CMA₃ fluorescent GC-rich bands appeared on metaphase plates and were present in interphase as several spots, generally nucleolus-associated. Spots were observed merging and dividing. X^2 tests showed interpopulation differences within both species for spot number in interphase nuclei.

FIGS 4 and 5. CMA₃-banding on metaphase plates. Diploid Welshpool population showing two bright subtelomeric bands (Fig. 4); Maïdo population showing four broad subtelomeric bands (Fig. 5).

FIGS 6–8. Interphase nuclei from the same meristem of Maïdo population, showing various numbers of CMA₃-fluorescent spots.

FIG. 9. CMA₃-banding on a prometaphase plate of Volcan population showing pericentromeric narrow bands (some arrowed).

FIG. 10. Interphase nucleus with four CMA₃-fluorescent spots associated with the nucleolus.

On metaphase plates of *A. melanoxylon*, one rather small chromosome pair bore a subtelomeric band at the level of a SC (Fig. 4) and one or two spots appeared in interphase. Fluorescent parts of chromosomes (i.e. SC) tended to separate from the chromosomes or to cling to each other, or to the other end of the chromosome, showing the fragility of the constriction. In the Ravenshoe population, metaphase bands were broad and interphase spots large and irregular in shape, with a clear tendency to merge and divide (as many one-spot as two-spot nuclei and 8% of cells with more than two spots). Both other *A. melanoxylon* populations had twice as many interphase cells with two spots than with one spot and thinner brighter bands, precisely located on both sides of the constrictions. In addition, one pair of submetacentric bands, weak and narrow, appeared on prometaphases of all three populations.

In *A. heterophylla*, Maïdo and Chicots clearly displayed four broad bands at metaphase (Fig. 5) on the SCs of rather large chromosome pairs, but although Maïdo had largely four-spot interphase nuclei (Fig. 6), Chicots had a majority of two-spot nuclei (Table 2). Both had (like Volcan) about 20% of three-spots (Fig. 7) and few one-spot (Fig. 8) interphase nuclei. All three other populations displayed more than four bands on most metaphase plates (Table 2). In Volcan, two to six bright bands most often corresponded to two interphase spots and, in addition, four to ten narrow pericentromeric bands appeared. Vallée Heureuse had a majority of two-spot nuclei though metaphase plates displayed four subtelomeric bands and four to eight narrow pericentromeric bands. Bélouve had a majority of one-spot interphase nuclei and the most metaphase bands (two bright

TABLE 2. *CMA₃-staining on interphase nuclei and metaphases of five A. heterophylla populations*

Population (individuals)	Spots in interphase (% of scored nuclei)					Nuclei scored	Bands in metaphase plates (plates scored)				
	1	2	3	4	> 4		1	2	3	4	> 4
Maïdo (7)	13	22	21	37	7	68	1	2	2	16	0
Chicots (6)	17	39	19	23	2	179	0	2	13	15	3
Bélouve (3)	50	26	8	13	3	175	0	1	0	1	22
Volcan (3)	9	44	21	17	9	145	0	0	1	4	10
Vallée Heureuse (6)	32	43	8	12	5	227	0	1	19	11	42

telomeric and nine to 20 narrow pericentromeric). Narrow pericentromeric bands were evident only at prometaphase stage (Fig. 9) but were clearly not due to spiralization artefacts. The position of interphase spots argued for nucleolus organizer activity (Fig. 10).

With Hoechst 33258 (Ho), interphase nuclei showed no spots and chromosome banding was neither important nor certain enough to allow sure comparisons. In both species, CMA₃ fluorescent SCs were Ho⁺, proving that they were exclusively GC-rich regions. *A. heterophylla* populations Maïdo and Vallée Heureuse displayed small Ho⁺ satellites on the long arm of four middle-sized chromosomes, but these were not found in other populations, though more than ten plates were observed in each case. *A. melanoxylon* population Tallaganda only displayed one pair of subtelomeric narrow bands and Welshpool no band, though both of these populations had CMA₃ small satellites on one chromosome pair.

DISCUSSION

Our present results confirm previous reports about chromosome number in *A. melanoxylon*: $2n = 26$ (Tjio, 1948) and tetraploidy of *A. heterophylla*: $2n = 4x = 52$ (Hamant, Lescanne and Vassal, 1975). They provide original estimates of 2C genome size (respectively 1.59 and 3.19 pg) corresponding to about 1.5×10^9 base pairs per basic 1C genome. The single previous valuation in *Acacia* was $2C = 1.16$ pg for *A. catechu* Willd. (Ohri and Kumar, 1986). These genome sizes are a common range in plants: out of 107 values cited by Arumuganathan and Earle (1991) for crops, 39 were higher, 45 lower and 23 between them, including several Leguminosae. They are also within the narrow range of the 44 tropical hardwoods studied by Ohri and Kumar (1986), who noticed that their variation was much smaller than that of angiosperms in general (Bennett, Smith and Heslop-Harrison, 1982). It is notable that though Grime and Mowforth (1982) proposed ecological justification for large genomes, none of the properties of smaller genomes appears as a universal feature (Arumuganathan and Earle, 1991).

Total DNA content, base composition and mitotic behaviour argue for a recent autotetraploid origin of *A. heterophylla* from *A. melanoxylon*. This hypothesis fits geological data showing the recent volcanic origin of La Réunion (3 million years; Fischer, Johnson and Heezen, 1967) and constant isolation from continents (Graham *et*

al., 1990). It is coherent with previous results showing that, though total chromatin lengths in tetraploid *Acacia* species are in general much less than twice diploids (Kumar and Kumar, 1958), for heterophyllous species chromatin length in tetraploids is approximately twice that of diploids (Vassal and Lescanne, 1976).

Suprachromosomal organization (according to Du Praw, 1970) has been reported for many plant species and attributed to heterochromatin (Stoian *et al.*, 1983). In both species, morphological studies of chromosomes were constrained by irregular spiralization and by these suprachromosomal connections, containing both GC- and AT-rich mixed repeated sequences. Their particular strength probably increases the importance of anaphase bridges in *A. heterophylla* that, at first view, might be attributed to recent disturbance of polyploidy.

Though nuclear DNA of both species is AT-rich (61%), there is no important grouping of AT sequences visible as bands on chromosomes. By contrast, GC-rich sequences appear in both species and reveal slight differences between populations. In *A. melanoxylon*, the GC-rich principal pair of bands clearly behaves like GC-repeated sequences of nucleolar organisers (NORs) containing most of the genomic satellite DNA, as described by Deumling and Greilhuber (1982). This hypothesis will be confirmed by NOR-specific staining. Interpopulation differentiation in *A. melanoxylon* fits the genetic and morphologic diversity corresponding to the natural geographic extension of this species (Muona *et al.*, 1991). The Ravenshoe population (belonging to the Northern group) appears the most distinctive, as for previously reported characters. Yet, the differentiation is not important enough to affect total DNA content or base composition within the limits of our present assay.

In *A. heterophylla*, Maïdo and Chicots GC-rich subtelomeric bands appear as the direct descendants of the NOR bands of *A. melanoxylon*. However, in Chicots, their greater tendency to merge may be the sign of a recent translocation between two SC regions involving parts of NORs. Genome reorganisation fitting with the observation of structural fragility at the level of SCs, seems more important in the three other populations. In Bélouve, only two main GC-rich bands, clearly tending to merge, remain, and the numerous narrow pericentromeric bands might come from translocations involving parts of the other NORs. Moreover, all the individuals may not be homozygous for translocations and intra-individual variations between cells are likely. Such variations of GC-banding pattern have been reported in

other species by Friebe, Cermeno and Zeller (1987), who could not propose any explanation for their origin. Deumling and Greilhuber (1982) stated that the size of heterochromatin bands could vary within a species, and even between homologues in one individual and invoked selection of some satellite sequences without offering any putative function. Parker and Wilby (1989) reported extreme rates of chromosomal variation within an herbaceous island species, and proposed several mechanisms, including centric shifts. Such mechanisms may exist in *A. heterophylla*, but with lower impact on population differentiation because of its long generation time.

Anyway, it is clear that a slight karyologic differentiation, involving mainly GC-rich regions, has occurred between populations of *A. heterophylla*. Western populations from the ancient volcano (Maïdo and Chicots) are the nearest to the autotetraploid pattern produced from *A. melanoxylon*, and the central population Bélouve is the furthest. There is no clear relation between banding pattern and ecological situation, especially altitude, soil, or vegetation type, however karyotypic differentiation may be due to isolation and chance only. The population ages cannot easily be deduced from datations of lava flows on which they have established. According to Raunet (1991), Maïdo and Chicots are on lavas dated 0.35–0.07 million years (my) and Bélouve on more recent (0.05–0.015 my) lavas that MacDougall (1971) previously dated 0.073 my. Populations from the active volcano slopes Volcan and Vallée Heureuse grow on ancient flows (respectively 0.11–0.015 and 0.36–0.32 my; Graham *et al.*, 1990). Yet, Raunet (1991) reports that the final pyroclastites of Piton des Neiges (0.040–0.015 my) thoroughly covered its previous lava flows, and even parts of the active volcano shield including Volcan area. Anyway, the first colonizing seeds did not necessarily arrive before the active volcano rose (at least 0.38 my). So it is hazardous to suppose that the ancient volcano populations are the oldest, even though seeds might have found lava substrata as early as 2 my ago, some of which probably have never been covered by younger flows (MacDougall, 1971).

NORs seem to be the main key to karyologic differentiation between populations and need further detailed investigations. We plan to complement the observed karyological variations by the study of genetic and morphological traits which may help to identify their expression, and so to understand the processes of past and present colonization of this recent oceanic island by a plant originating 8000 km to the east.

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