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Morphological, molecular and cytological analyses of *Carica papaya* × *C. cauliflora* interspecific hybrids

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Abstract Morphological, molecular and cytological analyses were performed to assess the hybridity of 120 putative interspecific hybrids of *Carica papaya* L. × *C. cauliflora* Jacq. In the putative interspecific hybrids the number of main leaf veins was intermediate between the two parents while the hermaphrodite flower sex form and the low vigour were distinctive features of these hybrids. Petiole length, stem diameter, leaf length, leaf width and flower colour were similar to *C. papaya*, whereas leaf shape, type, serration, venation, petiole hairiness and flower shape were similar to *C. cauliflora*. Markers generated by the polymerase chain reaction using 72 10-mer primers (random amplified polymorphic DNA) revealed a high level of polymorphism (64%) between *C. papaya* and *C. cauliflora*. Seventeen of these primers yielded reliable and easily scorable polymorphic banding patterns that were further screened to reveal hybrids. A range of 1–5 RAPD primers consistently confirmed that all 120 plants were genetic hybrids, with all of them containing at least one band from the male parent. Cytological analysis revealed that 7–48% of the cells in many of the interspecific hybrids were aneuploid suggesting that chromosome elimination was occurring. The frequency of aneuploid cells was negatively associated ($r = 0.88$) with the number of bands from the male parent integrated into the hybrid. Pollen fertility of the hybrids was from 0.5 to 14.0% while *C. papaya* and *C. cauliflora* had 88.0–99.0% and 90.0–97.0% fertile pollen, respectively.

Key words *Carica papaya* L. · *C. cauliflora* Jacq. · Random amplified polymorphic DNA (RAPD) · Interspecific hybrids · Aneuploid

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Introduction

The papaya (*Carica papaya* L.) is a widely grown tree crop in many countries which is used as a desert fruit and, when green, as a vegetable and a source of papain, an enzyme employed in medical and industrial preparations. Annual production of *C. papaya* worldwide is approximately 5.7 M tonnes (FAO 1993) and Australia produces 5000 tonnes worth approximately $A10–12 million dollars (NFF 1993). The greatest production constraint worldwide is papaya ringspot virus type-P (PRSV-P). This disease has been devastating the Australian industry since its first occurrence in 1991 (Thomas and Dodman 1993). The only reliable long-term solution to PRSV-P control is the development of genetically resistant cultivars. Resistance within the species is not available but some wild species (e.g. *C. cauliflora*) carry genetic resistance to PRSV-P (Alvizo et al. 1987; Magdalita et al. 1988). However, introgression of the resistance genes from *C. cauliflora* to *C. papaya* has been prevented due to genome incompatibility barriers which have lead to the abortion of hybrid embryos soon after their formation. The technique of embryo rescue has been used to overcome some incompatibility barriers and to aid in the formation of some hybrid plants (Manshardt and Wenslaff 1989; Chen et al. 1991).

Intermediate morphology (Khuspe et al. 1980) and isozyme patterns (Manshardt and Wenslaff 1989; Chen et al. 1991) have been used as ways of identifying

interspecific hybrids. However the expression of these hybridity markers is influenced by environmental conditions, genetic segregation and epistasis (Beckman and Soller 1986; Tanksley et al. 1989). Under optimal growth conditions, intermediate morphological markers would be a useful technique for the visual identification of hybrids. Isozymes that identify *C. papaya* × *C. cauliflora* hybrids can be affected by the developmental stage of the plant, protein content and other physiological factors for growth (Moore and Litz 1984). Because of these limitations, DNA-based techniques, which are more stable and reliable than isozymes, are now more commonly used for hybrid detection. Restriction fragment length polymorphisms (RFLPs; Williams et al. 1990), ribosomal DNA probes (Pental et al. 1988) and species-specific DNA sequences (Pehu et al. 1990) have all been used for the identification of interspecific hybrids. However, these approaches employ procedures which are costly, labour intensive and involve radioactivity. A simpler and quicker technique based on DNA amplification via the polymerase chain reaction (PCR) with arbitrary primers (random amplified polymorphic DNA, RAPD: Welsh and McClelland 1990; Williams et al. 1990) has been used as an alternative. Since this methodology is based on PCR, only small amounts of DNA are required. The present study utilized RAPD markers in combination with morphological and cytological data to confirm the hybridity of *C. papaya* × *C. cauliflora* putative interspecific hybrids.

Materials and methods

Plant materials and the production of interspecific hybrids

Field-grown trees of *C. papaya* and *C. cauliflora* in Redlands Research Station, Cleveland, Queensland, were used for interspecific hybridization. The *C. papaya* female parent (Clone 2001) is a high-yielding cultivar but susceptible to PRSV-P, while *C. cauliflora*, used as the pollen donor, is a wild relative with resistance to the Australian strain of PRSV-P (Persley and Thomas 1994). The interspecific hybrids were produced via embryo rescue (Magdalita et al. 1996). All materials used for morphological, cytological and molecular analyses of hybridity were from glasshouse-grown plants.

Morphological analysis

The morphological characters (height; petiole length; stem diameter number of main veins; leaf length, width, shape, serration, venation; petiole hairs; flower shape, colour, number per node, sex and plant vigour) of the interspecific hybrids (20 plants), and of *C. papaya* (five plants) and *C. cauliflora* (five plants) were examined. Plant height was measured from the base of the plant to the apex while stem diameter was measured 100 mm from the base of the plant using a vernier caliper. Petiole length was measured from the node up to the base of the leaf in five mature leaves. Leaf length was measured from the base of the middle leaflet midrib up to the tip, while leaf width was measured at maximum breadth using the same leaves. All characters employed were based on the descriptors for *C. papaya* provided by the International Board for Plant Genetic Resources (IBPGR 1988).

DNA extraction

A young leaf from near the apex was severed from each of the putative interspecific hybrids (120 plants), as well as from *C. papaya* and *C. cauliflora* (three plants each). Each leaf was individually washed, blotted dry and the midrib removed. One gram of leaf tissue was placed in a sterile polycarbonate tube, frozen in liquid nitrogen and stored at −70°C until ready for use. Extraction of the DNA was achieved using a modified hexadecyltrimethylammonium bromide (CTAB, Sigma Chem., Mo., USA) method (Graham et al. 1994). The DNA concentration obtained from each leaf extract was measured using a TKO 100 Mini-Fluorometer following the procedures provided by the manufacturer (Hoefer Scientific Inst., Calif., USA).

DNA amplification and analysis

Seventy two 10-mer primers (Operon Technologies Inc., Calif., USA) were tested for DNA amplification of an interspecific hybrid, and of *C. papaya* and *C. cauliflora*. The PCR was carried out in 25-µl reaction containing 20 ng genomic DNA, 2 µM primer, 0.1 mM deoxyribonucleotide triphosphate, 3 mM $MgCl_2$, reaction buffer (10 mM Tris-HCl, pH 8.3, 50 mM KCl) and 1.0 unit of *Taq* DNA polymerase (Boehringer Mannheim, Mannheim City, Germany). The mixture was overlayed with 30 µl of paraffin oil (light white mineral oil, Sigma Chem. Co., Mo., USA). The thermal cycling program was run on a Perkin-Elmer 480 DNA Thermal Cycler (Perkin Elmer Cetus Co., Conn., USA), with one initial cycle of denaturation at 94°C for 5 min followed by 40 cycles of denaturation at 94°C for 1 min, annealing at 35°C for 1 min and extension at 72°C for 2 min. The RAPD products were analyzed by electrophoresis in 1.2% (w/v) agarose in 1 × TBE buffer at 100 V for 2.5 h, stained with ethidium bromide for 20 min and photographed under UV light.

Primers giving high resolution of polymorphism were identified and further screened. Five RAPD primers were used in testing the hybridity of 120 putative interspecific hybrids. The percentage polymorphism between *C. papaya* and *C. cauliflora* was determined. The presence or absence of the parental bands in each of the 120 putative hybrids was examined.

Cytological analysis

The somatic chromosome number of a sample of interspecific hybrids (20 plants), and of *C. papaya* (three plants) and *C. cauliflora* (three plants) was ascertained. Root tips were pre-treated for 3 h in a saturated aqueous monobromonapthalene solution, fixed for 24 h in Farmer's solution (3 ethanol : 1 glacial acetic acid, v/v; Darlington and La Cour 1976) and then hydrolyzed in 1 M HCl for 15 min. They were stained in aceto-orcein and HCl solution (1 : 9 1% aceto-orcein : 1 M HCl) and then destained and squashed in 45% acetic acid (v/v in distilled water). Between 20 and 32 cells in each of the putative interspecific hybrids and 50 cells in each plant sample of the parents were examined for somatic chromosome number. The percentage of aneuploid cells of the hybrids was regressed (Gomez and Gomez 1984) with the number of male bands inherited from *C. cauliflora*. The percent pollen fertility of the interspecific hybrids (five plants) and their parents (five plants each) was checked using an aceto-carmine staining technique (Cohen et al. 1989). Pollen from flowers at full-balloon stage were dusted onto glass slides, stained with aceto-carmine (45%) and covered with a cover slip. Five-hundred pollen grains from each plant sample of the hybrid and the parents *C. papaya* and *C. cauliflora* were examined in a phase-contrast microscope (10 × magnification; Olympus BH-2,

Olympus optical Co. Ltd., Tokyo, Japan) and the number of fertile and unfertile pollen grains was counted.

Results

Morphology

The putative interspecific hybrids had morphological characteristics that were not seen before in either parent, were identical to one parent, or intermediate between those of the two parents (Table 1). Out of 16 characters examined, only the number of main leaf veins was exactly intermediate between *C. papaya* and *C. cauliflora*. Two characters, hermaphrodite flower sex form and low vigour, were distinctive to the interspecific hybrids. The other characters, such as petiole length, stem diameter, leaf length, leaf width and flower colour, were similar to those of *C. papaya*. Leaf shape, type, serration, venation, as well as hairs on petioles and flower shape, were similar to those of *C. cauliflora*. All the interspecific hybrids showed cupping of the leaves and three plants had a branched stem, which was not observed in either parent.

RAPD analysis

The 72 10-mer primers screened for DNA amplification of *C. papaya*, *C. cauliflora* and the putative interspecific hybrids generated a total of 207, 225 and 182 bands respectively. Overall, 64% of bands were polymorphic between *C. papaya* and *C. cauliflora*. Seventeen (24%) of these primers showed high resolution of the integrated parental bands into the putative interspecific hybrids. In repeated trials, five operon primers (OP) (OPA-07, 5′CAAACGGGTG3′; OPA-09, 5′GGGTAACGCC3′; OPA-19, 5′CAAACGTCGG3′; OPB-12, 5′CCTTGACGCA3′; OPC-06, 5′GAACGGACTC3′) consistently amplified the *C. papaya* and *C. cauliflora* bands in all 120 putative interspecific hybrids when results were pooled. Using these five primers a total of ten polymorphic bands (two bands from each primer, five bands each from each parent) were amplified in the putative interspecific hybrids. For example, OPA-09 and OPB-12 amplified an 800- and 940-bp fragment, respectively, in the hybrids which were from the male parent (Figs. 1, 2). Only 31 (26%) of the putative hybrids had all five RAPD bands from the male parent, and almost half of these were lacking one or more maternal bands (Fig. 3). All the putative hybrids tested had at least one paternal RAPD band. However, any one of the 10-mer primers alone cannot identify all of the putative interspecific hybrids as the primers failed to assay other male bands when used individually. Furthermore, while all 120 putative interspecific hybrids had *C. papaya* female RAPD bands but only 70 (58%) of the plants had the complete female bands. At least one *C. papaya* female RAPD band was absent in the rest of the putative interspecific hybrids.

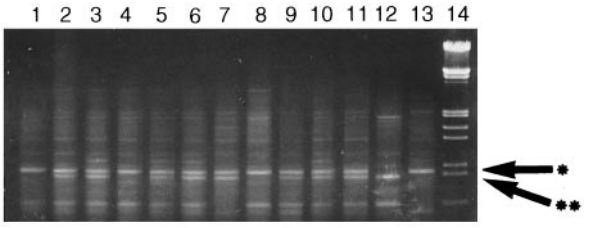

Fig. 1 RAPD profile generated by primer OPA-09. *Lanes 1–11* putative interspecific hybrids, *12 C. cauliflora*, *13 C. papaya*, *14* molecular-weight marker. Note that the 850-bp fragment of *C. papaya* (*) and the 800-bp fragment of *C. cauliflora* (**) are present in the putative interspecific hybrids (*lanes 2*, *3*, *5–7*, *9–11*) but the 800-bp fragment is absent in other putative interspecific hybrids (*lanes 1*, *4*, *8*). Resolution was on a 1.2% agarose gel

Table 1 The morphological characteristics of *C. papaya*, *C. cauliflora* and the putative interspecific hybrids. The data are the mean ± standard error determined from five plants each of *C. papaya* and *C. cauliflora* and from 20 interspecific hybrid plants. Descriptions of flower characteristics were determined from five putative interspecific hybrids

Characteristics	Genotypes		
	C. papaya	Hybrid	*C. cauliflora*
Height (cm)	195.0 ±2.0	109.0 ±2.0	283.0 ±6.0
Petiole length (cm)	9.8 ±0.3	6.2 ±0.2	18.1 ±0.8
Stem diameter (cm)	6.2 ±0.2	5.9 ±0.2	16.0 ±0.7
Main veins (no.)	9.0	7.0	5.0
Leaf length (cm)	11.2 ±0.1	12.9 ±0.4	22.1 ±0.8
Leaf width (cm)	10.4 ±0.2	11.4 ±0.3	21.0 ±0.6
Leaf shape	*C. papaya*-type	*C. cauliflora*-type	*C. cauliflora*-type
Leaf serration	Shallow	Deep	Deep
Leaf venation	Palmate	Palmitifid	Palmitifid
Petiole hairs	Absent	Present	Present
Plant vigour	High	Low	High
Flower shape	*C. papaya*-type	*C. cauliflora*-type	*C. cauliflora*-type
Flower colour	Cream	Cream	White
Flowers per node	Multiple	One	Multiple
Flower sex form	Female	Hermaphrodite	Male

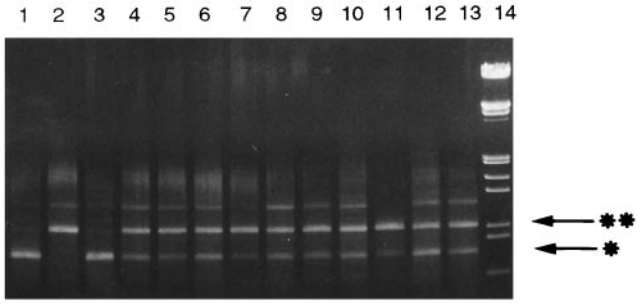

Fig. 2 RAPD profile generated by primer OPB-12. *Lane 1 C. papaya*, *2 C. cauliflora*, *3–13* putative interspecific hybrids, *14* molecular-weight marker. Note that the 830-bp fragment of *C. papaya* (*) and the 940-bp fragment of *C. cauliflora* (**) are present in the hybrids (*lanes 4–13*) but the 940-bp fragment is absent in one putative interspecific hybrid (*lane* 3). Resolution was on a 1.2% agarose gel

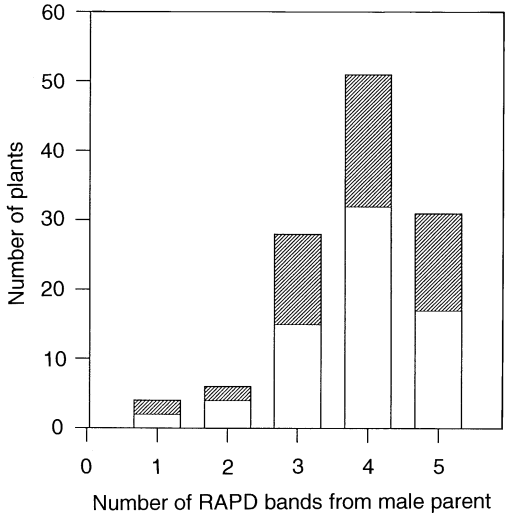

Fig. 3 Frequency of interspecific hybrid plants having different numbers of RAPD bands from the male *C. cauliflora* parent. The *bars* represent the number of plants having all female bands present (□) or at least one female band absent (▨)

Cytological analysis

C. papaya, *C. cauliflora* and the putative interspecific hybrids had a somatic chromosome number of 2n = 18 (Table 2). However, some of the putative interspecific hybrids had up to 48% aneuploid cells and the frequency was negatively associated ($r = 0.88$) with the number of RAPD bands from the male parent (Fig. 4). Pollen fertility of *C. papaya* and *C. cauliflora* was from 88 to 98% and from 90 to 97%, respectively (Table 2). However, the interspecific hybrids had only 0.5–14% fertile pollen.

Discussion

The number of main leaf veins was consistently seven in the interspecific hybrids, which was intermediate between that of the two parents. This morphological character could be used as a reliable visual marker for preliminary identification of the hybrid prior to genetic analysis. Intermediate morphological characters have been used previously for the identification of *C. papaya* × *C. cauliflora* interspecific hybrids (Khuspe et al. 1980; Chen et al. 1991). Also, the low vigour (slow-growing, weakness, oversensitive to slight changes in growing conditions) of the putative interspecific hybrids in contrast to the high vigour of *C. papaya* and *C. cauliflora* could be another way of identifying hybridity. Furthermore, the hermaphrodite sex form of all five putative hybrids make them distinctive as they have flowers that were clearly different from either parent. The crossing of dioecious *C. papaya* plants has been known to produce male and female progenies only in a 1:1 ratio (Storey 1976). However, in the present study, where female *C. papaya* plants were crossed with male *C. cauliflora*, neither single sex form was observed in all five interspecific hybrid progenies that flowered.

Table 2 Pollen fertility and chromosome number of *C. papaya*, *C. cauliflora* and the putative interspecific hybrids. The data for pollen fertility were determined from 500 pollen grains of five plants each of *C. papaya* and *C. cauliflora* and five putative interspecific hybrid plants. The data for chromosome number were determined from 50 cells of each of three plants of *C. papaya* and *C. cauliflora* and from 20 to 32 cells in each of the 20 putative interspecific hybrids

Genotypes	Pollen fertility (%)	Chromosome number (2n)
C. papaya	88.0–98.0	18
C. cauliflora	90.0–97.0	18
Interspecific hybrid	0.5–14.0	16–18

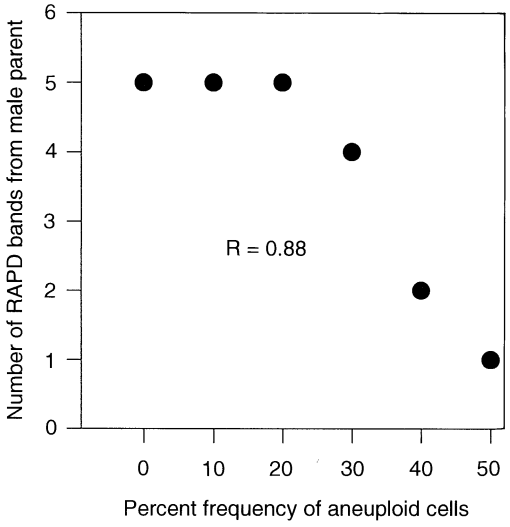

Fig. 4 Relationship between the frequency of aneuploid cells and the number of RAPD bands from the male *C. cauliflora* parent amplified in the interspecific hybrids

A high level of polymorphism (64%) which existed between *C. papaya* and *C. cauliflora*, as indicated by the numerous RAPD bands produced, confirms that these two species are genetically distant. Consistent amplification of the integrated parental RAPD bands into the putative interspecific hybrids using the five selected primers indicates hybridity of all the 120 putative interspecific hybrid plants. Since a band from each parent was consistently amplified in the hybrid, this implies the presence in the hybrid of two chromosome sets, consisting of one set from each parent. For example, using OPA-09, 850- and 800-bp fragments from *C. papaya* and *C. cauliflora*, respectively, were identified in the hybrids (Fig. 1). Variation in the number of hybrids having the male RAPD bands (Fig. 3) suggests the loss of paternal chromosomes. For instance, some hybrids (lanes 4 and 8) do not have the 800-bp fragment from the male *C. cauliflora* parent, as shown by OPA-09, but the same hybrids (lanes 6 and 10) had a 940-bp fragment of the male parent amplified by OPB-12 (Figs. 1 and 2). This result indicates that a single primer cannot detect hybrids in all cases. Although the hybrids are expected to have an additive banding profile of the two parents, an absence of a male band (Fig. 2, lane 3) where the plants had obvious morphological attributes of *C. cauliflora* signifies chromosome elimination, or else that this locus of the male *C. cauliflora* parent is possibly in a heterozygous condition. Likewise, the absence or presence of a female RAPD band in the hybrids also suggests heterozygosity of the locus in the *C. papaya* female parent. In interspecific hybrids of barley (*Hordeum vulgare* × *H. bulbosum*, Kasha and Kao 1970) and potato (*S. tuberosum* × *S. phureja*, Clulow et al. 1991) some of the paternal chromosomes were preferentially eliminated. The lower number of RAPD bands amplified in the putative interspecific hybrid compared with the parents may suggest competition for primer binding at sites of similar homology. A similar primer binding competition has been seen in interspecific somatic hybrids of potato (*Solanum tuberosum*) × *S. brevidens*, where all the detectable amplification products were from *S. brevidens* (Xu et al. 1993).

Further analysis of the somatic chromosome number indicated a high frequency (up to 48%) of aneuploid cells in the putative interspecific hybrids, suggesting that these plants were aneuploid mosaics. Similar results were observed previously in interspecific hybrids of *C. papaya* × *C. pubescens* and *C. quercifolia* where one of the genomes of the wild species was eliminated in the backcross progenies (Manshardt 1992). The significant negative association between the frequency of aneuploid cells and the number of male bands integrated into the hybrids (Fig. 4) again suggests that chromosome elimination explains some of the loss of RAPD bands. Moreover, the low pollen fertility (Table 2) of the five interspecific hybrids that flowered and the failure of most hybrids to flower indicate that this may be due to chromosome imbalance caused by incompatibility barriers. The same observation has been made on the tomato interspecific hybrids *Lycopersicon esculentum* Mill × *L. peruvianum* (L.) Mill (Kirkham and Halloran 1982).

The present results demonstrate that the combination of morphological and cytological data, together with RAPD markers, are all useful for determining the hybridity status of the *C. papaya* × *C. cauliflora* plants produced. Earlier RAPD markers have been used to positively confirm the hybridity of *S. tuberosum* × *S. brevidens* interspecific hybrids (Baird et al. 1992). In the current study, the RAPD markers generated for the interspecific hybrids may be used for tracking the flow of *C. cauliflora* genetic material among progenies that can be produced in future backcrosses to *C. papaya*. RAPD analysis, being a simple and quick technique, can facilitate the screening of a very large population of putative interspecific hybrids of *C. papaya* and other *Carica* species. These markers are also useful in identifying individuals with a high level of aneuploid mosaicism. RAPD markers show dominance and therefore have some limitations in their application; for example, this technique cannot detect both parental alleles. However, this can be overcome by using co-dominant markers such as RFLPs, although this requires more time and large amounts of DNA. Alternatively, RAPD markers can be converted into sequence-tagged sites to allow both alleles to be identified.

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