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NOTE

ISSR markers for genetic relationships in Caricaceae and sex differentiation in papaya

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ABSTRACT – *ISSR markers are polymorphic and their results easily reproducible. They are therefore intensely used in phylogenetic studies and sex differentiation of some economically interesting plant species. The objectives of this study were to analyze the genetic diversity in Caricaceae using ISSR markers, to identify a specific ISSR band that could distinguish female from hermaphrodite papaya genotypes and to verify whether this marker could be used for early sex differentiation. The ISSR-PCR was performed with nine primers and they could distinguish all species. It was observed that Jacaratia spinosa was closer to Vasconcellea than to Carica. The species C. papaya was only distantly related to both genera. A 500 bp ISSR marker was found in 25 % of the papaya genotypes studied. Specifically in these cases this marker could be used for early sex differentiation in papaya.*

Key words: *genetic diversity, early molecular sexing.*

INTRODUCTION

The Caricaceae family consists of 35 species distributed in six genera (Badillo 2000). All described species have 2n = 2x = 18 chromosomes and are regarded as diploids. Although papaya (*Carica papaya* L.) is an important fruit crop in tropical countries, especially in Brazil, *Vasconcellea* species are also considered important, as a source of desirable traits in papaya improvement (Manshardt and Wenslaff 1989, Drew et al. 1998).

Several compatibility levels are observed within *Vasconcellea*. However, they are drastically reduced in crosses with *C. papaya* (Warmke et al. 1954, Jimenez and Horovitz 1958, Horovitz and Jimenez 1967, Mekako and Nakasone 1975, Manshardt and Wenslaff 1989). Some interspecific crosses using embryo rescue were successful (Magdalita et al. 1996, Drew et al. 1998, Manshardt and Drew 1998). For a better understanding of the genetic relationships in Caricaceae, several molecular studies have been performed, suggesting a considerable genetic distance between *Carica* and *Vasconcellea* (Jobin-Decor et al. 1997, Aradhya et al. 1999, Olson 2002, Van Droogenbroeck et al. 2004, Kyndt et al. 2006).

Sex determination in *C. papaya* is controlled by a single gene with three allelic forms: M_1, M_2 and m. Therefore, female, male and hermaphrodite plants are mm, M_1m, and M_2m, respectively, while M_1M_1, M_2M_2, and M_1M_2, are non-viable genotypes (Hofmeyr 1938, Storey 1938). Nevertheless, the sex can only be identified when plants are flowering. There are no reports of heteromorphic or unpaired chromosomes nor rDNA sites associated with a putative sexual chromosome pair (Costa et al. 2008, Damasceno

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Junior et al. 2009), as would be expected for advanced sex chromosomes, although a sex-determining region was defined elsewhere (Liu et al. 2004, Yu et al. 2008).

In papaya plantations in Brazil, hermaphrodite plants are raised, obtained from crosses between hermaphrodite types. Of these crosses, 33.3 % are female whose fruits are worthless; only hermaphrodite trees produce marketable fruit. Thus, three seedlings are usually planted to ensure the desired number of fruit-bearing hermaphrodite trees in the plantation.

Recently, sex-linked molecular markers have been successfully developed in some dioecious species such as *Asparagus officinalis* (Reamon-Buettner and Jung 2000), *Cannabis sativa* (Flachowsky et al. 2001), *Pistacia vera* (Yakubov et al. 2005) and *Humulus lupulus* (Danilova and Karlov 2006). A SSR marker associated to male seedlings was found in *C. papaya* (Parasnis 1999).

The inter simple sequence repeat (ISSR) markers are polymorphic and their results are easily reproducible. To use ISSR-PCR, no prior information about DNA sequences is required and many of the technical limitations of other molecular markers are overcome by its high reproducibility and simplicity. For these reasons, the applicability of this method was studied to estimate the genetic diversity in Caricaceae species and to find a specific sex marker that could distinguish female from hermaphrodite papaya seedlings and be used for early sex differentiation.

MATERIAL AND METHODS

Plant material

Four genotypes of *V. monoica* and of *J. spinosa*, collected in Brazil, two genotypes of *V. goudotiana* that were kindly supplied by USDA, and bulks of hermaphrodite and female *C. papaya* (variety *SS72/12*) were investigated in the divergence study. All accessions belong to the UENF germplasm collection.

A putative sex marker was found during this study, differentiating female from hermaphrodite *SS72/12* plants, leading to another investigation to confirm this assumption. In this study, four accessions of the Solo group (*SS783*, *Caliman AM*, *SS72/12* and *Kapoho Solo*), four of the Formosa group (*Tailândia*, *Sekati FL*, *FR 45* and *Sekati*) and four hybrids (*Tainung 01*, *UENF/CALIMAN 01*, *Sel. Caliman* and *Tainung H*) were analyzed to validate the sex identification. Five female and five hermaphrodite plants were analyzed for each genotype.

DNA isolation

Total DNA was isolated from papaya seedling leaves, as described by Costa et al. (2006). The DNA concentration was determined by electrophoresis and comparison with a known quantity of Lambda DNA digested with *HindIII* (Amersham Pharmacia, Biotech).

ISSR-PCR analyses

To detect molecular polymorphisms among and within accessions, 30 primers were tested as described below and nine primers were used for the amplification of polymorphic loci. The annealing temperatures (AT) were optimized for each primer (Table 1).

Amplification reactions were performed in 0.02 cm^3 reaction mixture that contained: 5 ng of genomic DNA, 0.4 µM of each primer, 2 mM of $MgCl_2$, 100 µM of each dNTP, 0.6 U Taq DNA polymerase, 5 % DMSO and 1x enzyme buffer. After 4min at 94 ^oC, 42 cycles of PCR were performed as follows: 94 ^oC for 1min, AT for 1min and 72 ^oC for 3min. A final extension at 72 ^oC for 7min ended the reaction. The amplification products (bands) were size-separated by standard horizontal electrophoresis on 2 % agarose gels and stained with ethidium bromide.

The ISSR markers were scored as band presence (1) or absence (0), providing a data matrix used to calculate a genetic dissimilarity matrix (Jaccard's arithmetic complement index). The dendrogram was constructed using UPGMA cluster algorithm. All statistical analyses were performed using software Genes (Cruz 2006).

Presence or absence of the putative sex-linked marker was verified for each papaya genotype, using primer $(AGC)_5Y$. Each gel was stained with ethidium bromide and images were captured for analysis.

RESULTS AND DISCUSSION

Several primers were used to check whether informative genomic fingerprints could be generated (Table 1). The primers were 15-18 bases long and the annealing temperatures optimized for each one. A total of 94 amplification products were generated by nine primers with an average frequency of 10.4 bands per primer; 87 bands were polymorphic. The band sizes ranged from 180 to 1900 bp and best results were obtained with primer $(AGC)_5GR$, whereas $(GACA)_4$, $(GATA)_4$, $(GTG)_5RG$, $(CT)_8TG$ and $(CT)_8RC$ produced either no amplification products or unscorable profiles (data not shown).

Table 1. ISSR Primers used for PCR amplification of Caricaceae species and total number of amplified fragments generated from the analyzed accessions

Primers*	Annealing temperature (°C)	Scorable bands	Polymorphic bands	Fragment sizes (bp)
$(AGC)_5GR$	65	19	18	280 - 1900
$(AGC)_5AY$	65	9	8	300 - 1500
$CA(GA)_8$	58.5	11	9	250 - 1200
$(GCT)_5Y$	58.5	14	14	300 - 1200
$GC(GA)_8$	58.5	10	9	270 - 800
$(AGC)_5Y$	56	8	7	320 - 1000
$GGGT(GGGGT)_2G$	56	8	7	300 - 1100
$(GA)_8YC$	53	8	8	180 - 700
$(CAGA)_4$	45	7	7	300 - 1100

* Y = C or T; R = A or G.

The results obtained by ISSR polymorphism agree with previous studies at the species level. The UPGMA dendrogram (Figure 1) shows four main groups, separating each genotype analyzed. Surprisingly, the *Jacaratia* genus was closer to *Vasconcellea* than to *Carica*. In agreement with previous phylogenetic investigations *C. papaya* was distantly related to both genera.

The close relationship between *V. goudotiana* and *V. monoica* was first reported by Warmke et al. (1954). Some F_1 hybrids were obtained in interspecific crosses between these species using *V. goudotiana* as female parent. Their hybrids were fertile with intermediate morphological characteristics, but a monoecious sex type as the male parent. The F_2 plants segregated widely for both characters.

The genetic distance between *Vasconcellea* and *Carica* has been observed and documented in numerous investigations. Previous studies based on ovary morphology (Badillo 1993) and interspecific hybridization barriers (Manshardt and Wenslaff 1989) indicate that papaya is only distantly related to *Vasconcellea*. Results of cpDNA analysis of Aradhya et al. (1999) suggest that *C. papaya* must have diverged from the South American *Vasconcellea* species early in the evolution and evolved in isolation, probably in Central America. These data supported a recent rehabilitation of *Vasconcellea* as a genus category (Badillo 2000). On the other hand, Van Droogenbroeck et al. (2004) analyzed the diversity of 18 *Vasconcellea* species and corroborated the monophyly of Caricaceae in the genera *Carica*, *Jacaratia* and *Cylicomorpha* by RFLP. According to these authors, Caricaceae species are divided into two lineage groups, one with some *Vasconcellea* spp., including *V. monoica* and *V. goudotiana* and the second with the other *Vasconcellea* species, *C. papaya*, *Jacaratia* and *Cylicomorpha*.

The ISSR marker detected only in hermaphrodite papaya samples of *SS72/12* and absent in female seedlings motivated the second part of this investigation. An additional experiment using different genotypes and a large number of plants was carried out to validate the previous results. Fragments generated by primer $(AGC)_5Y$ ranged from 300 to 2000 bp. Figures 2A and 2B showed a marker around 500 bp, cosegregating with sex, in the genotypes *Tailândia*, *SS72/12* and *Tainung H* (arrows). This marker was present only in hermaphrodite samples of these three genotypes, representing 25 % of the genotypes investigated. In the other cases, the band was present in female and hermaphrodite plants or absent in both sexes, as in *SS783* and *Sekati* (Figure 1C and 1D).

The search for molecular markers cosegregating with sex in papaya has been intensified in the last years. Production costs are high and include the maintenance of all trees in the plantation until flowering, when the commercially worthless plants (female) can be eliminated. Although Parasnis et al. (1999) found a microsatellite marker to differentiate sex in papaya, the marker could only distinguish female from male plants. In our case, this is not relevant, since no male trees grow in Brazilian plantations. Nevertheless, the cited study demonstrates the relevance of such an investigation in academic and commercial terms.

Results of our study demonstrated that the sex-cosegregation marker in papaya is limited to some genotypes, but not restricted to specific groups e.g., *Solo*, *Formosa* or *Papaya hybrids*. In other words, case-by-case validation will be required. The 500 bp marker can be considered a starting point, while the search for other molecular markers or alternatives to optimize the early sex

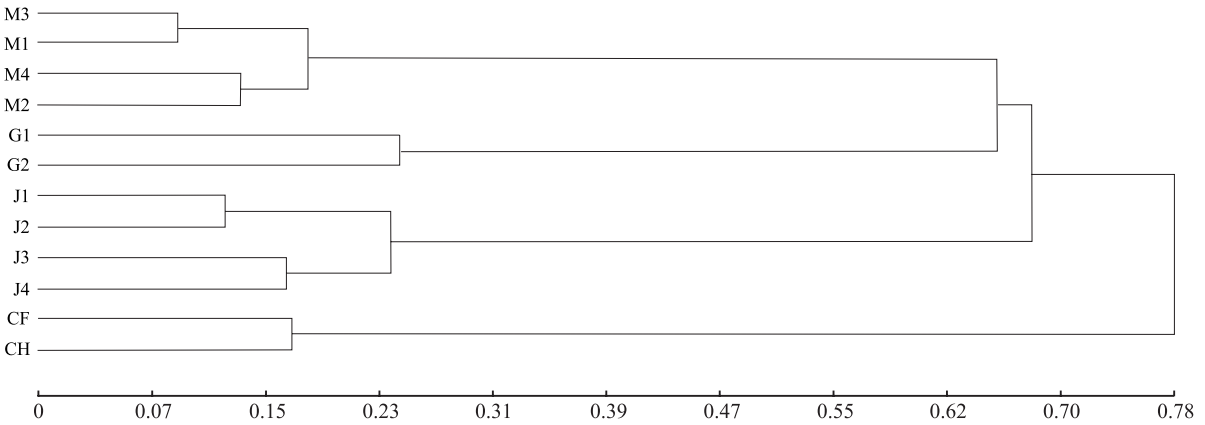

Figure 1. UPGMA dendrogram showing the relationships among four Caricaceae species. M: *V. monoica*, J: *J. spinosa*, G: *V. goudotiana*, CF: *C. papaya*, female bulk, CH: *C. papaya*, hermaphrodite bulk.

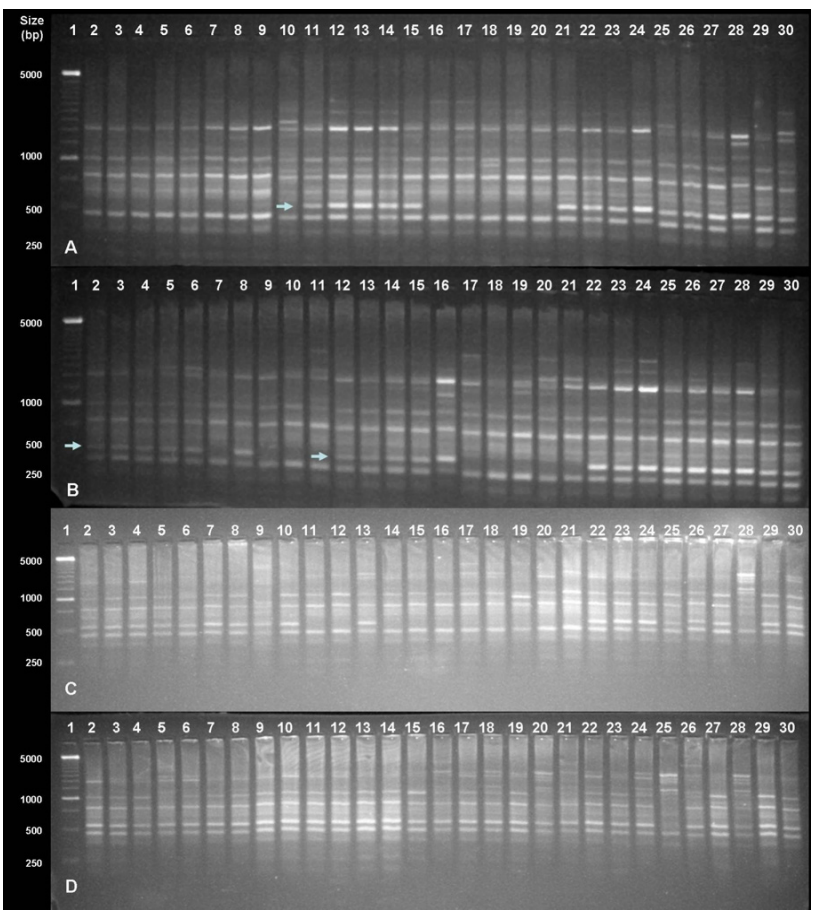

Figure 2. ISSR markers in 12 accessions of *C. papaya* using $(AGC)_5Y$. A) 1- *Ladder* 250 bp; 2-6, *SS783* hermaphrodite; 7-10, *SS783* female; 11-15, *Tailândia* hermaphrodite; 16-20, *Tailândia* female; 21-25, *Caliman AM* hermaphrodite; 26-30, *Caliman AM* female; B) 1- *Ladder* 250 bp; 2-6 and 8, *SS72/12* hermaphrodite; 7 and 9-11, *SS72/12* female; 12-16, *Tainung H* hermaphrodite; 17-21, *Tainung H* female; 22-26, *Kapoho Solo* hermaphrodite; 27-30, *Kapoho Solo* female; C) 1- *Ladder* 250 bp; 2-6, *Sekati FL* hermaphrodite; 7-10 and 13, *Sekati FL* female; 11-12 and 14-16, *Sekati* hermaphrodite; 17-21, *Sekati* female; 22-26, *UENF/CALIMAN 01* hermaphrodite; 27-30, *UENF/CALIMAN 01* female; D) 1- *Ladder* 250 bp; 2-6, *FR 45* hermaphrodite; 7-11, *FR 45* female; 12-16, *Sel. Calima hermaphrodite*; 17-21, *Sel. Caliman* female; 22-26, *Tainung 01* hermaphrodite; 27-30, *Tainung 01* female.

identification of a larger number of papaya genotypes should be intensified. Maybe in the future, a suitable system of early sex differentiation in papaya can be established.

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Marcadores ISSR nas relações genéticas em Cariaceae e na identificação sexual do mamoeiro

RESUMO – *Os marcadores ISSR apresentam amplo polimorfismo e alta reprodutibilidade de resultados, o que tem intensificado seu uso em estudos filogenéticos e na diferenciação sexual de algumas espécies de interesse econômico. Os objetivos deste trabalho foram analisar a divergência genética em Caricaceae utilizando-se marcadores ISSR, identificar uma marca capaz de diferenciar plantas hermafroditas e femininas em mamoeiro e verificar se este marcador pode ser utilizado na sexagem precoce de diferentes genótipos da espécie. O estudo foi conduzido com nove primers, os quais foram capazes de distinguir todas as espécies. Observou-se que Jacaratia spinosa ficou mais próxima de Vasconcellea do que de Carica. A espécie C. papaya mostrou-se geneticamente distante de ambos os gêneros. Verificou-se ainda a presença de um fragmento ISSR de 500 pb em 25 % dos genótipos de mamoeiro estudados, podendo ser usado para auxiliar a sexagem precoce do mamoeiro especificamente nesses casos.*

Palavras-chave: *diversidade genética, sexagem molecular precoce.*

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