

ISABELLA SANTIAGO DE ABREU CARVALHO

**EARLY SEX DISCRIMINATION IN *Carica papaya* L. BY  
MOLECULAR CYTOGENETICS**

Thesis presented to the  
Universidade Federal de Viçosa, as part  
of the requirements of the Genetics and  
Breeding Graduate Program, for the  
attainment of the title *Doctor Scientiae*.

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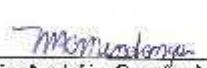
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To my dear parents, Messias and Cleonice, to my brother and friend  
Guilherme, and to my beloved husband Daniel, for their unconditional love  
and for supporting me all the time.

*I dedicate.*

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## **BIOGRAPHY**

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In March 2010, she joined the Graduate Program, in Doctor degree, in Genetics and Breeding at UFV, subsidized by CNPq, submitting to the defense of thesis in June 2014. In that time, she learned and developed researches in molecular biology and cytogenetics.

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## ABBREVIATION LIST

AFLP – amplified fragment length polymorphism  
BAC – bacterial artificial chromosome  
bp – base pairs  
DAPI – 4',6-diamidino-2-phenylindole  
FISH – fluorescence *in situ* hybridization  
FISHIS – fluorescence *in situ* hybridization in suspension  
HSY – hermaphrodite-specific region of the Y chromosome  
kb – kilobases  
LG1 – linkage group 1  
Mb – megabases  
miRNA – micro RNA  
MSY – male-specific region of the Y chromosome  
MYA – million years ago  
PCR – polymerase chain reaction  
PRSV – papaya ringspot virus  
RAPD – randomly amplified polymorphic DNA  
SCAR – sequence characterized amplified region  
sRNA – small RNA  
SSC – saline-sodium citrate  
SSR – simple sequence repeat

## ABSTRACT

CARVALHO, Isabella Santiago de Abreu, D. Sc., Universidade Federal de Viçosa, June, 2014. **Early sex discrimination in *Carica papaya* L. by molecular cytogenetics**. Advisor: Carlos Roberto de Carvalho.

The papaya, *Carica papaya* L., is the most economically important species of the family Caricaceae. Native of Central and South America, this herbaceous and fruitful crop is cultivated mainly in tropical and subtropical regions worldwide, and it is widely consumed for its edible fruit. *C. papaya* is characterized as a polygamous species with three sex types: male, female and hermaphrodite. Considering its preferred seminiferous propagation, inherent problems of papaya crop refer to the segregation of sex types and late sex detection. The sex identification is only possible after flowering, by inspection of the flowers, since there is no recognized chromosomal dimorphism or morphological difference between the three papaya sex types in seedling stage. In order to save time, labor and financial resources, it is desirable for growers that the sex type of this crop is known before transplanting. Researchers in molecular biology have developed a number of genetic markers in an attempt to distinguish the sex of papaya before reaching reproductive maturity. In this study, it was investigated seven sequence characterized amplified region (SCAR) markers previously described in the literature, by the polymerase chain reaction (PCR) technique, in two commercially important Brazilian varieties of *C. papaya* ('Golden' and 'Rubi'), and then, it was developed a fluorescence *in situ* hybridization (FISH) protocol by using a chosen marker as probe. Thus, we aimed to provide a molecular diagnosis of early sexing for the female and hermaphrodite plants. Firstly, genomic DNA was isolated from female and hermaphrodite young leaves of the both papaya varieties. After optimizing PCR conditions, amplifications of the seven SCAR primers were carried out in seven samples of each sex type from each variety. The fragments obtained were analyzed by agarose-gel electrophoresis. Among the genetic markers tested, three of them (NAPF-2, SDSP and SCARpm) generated prominent sex-specific fragments in all hermaphrodite samples of the both

papaya cultivars. Another SCAR marker (T12) produced a single sex-specific fragment for only hermaphrodite plants from 'Rubi' cultivar. NAPF-2 marker was selected to be used as FISH probe based on its consistently polymorphic banding pattern. FISH analyses showed a single strong fluorescent signal in 'Golden' hermaphrodite nuclei and many strong fluorescent signals in 'Rubi' hermaphrodite nuclei, while no detectable or very low intensity fluorescence signal was observed in female nuclei from both cultivars. In conclusion, the present study investigated successfully, for the first time, the discriminating potential of SCAR markers in two Brazilian commercial papaya cultivars, and proposed a new, reliable, efficient and relatively fast diagnostic method, based on FISH technique, for early identification of hermaphrodite plants from cultivars 'Golden' and 'Rubi'. In future studies, this method could be improved for a large-scale screening in commercial cultivation by flow cytometric approach after FISH in nuclei suspension.

## RESUMO

CARVALHO, Isabella Santiago de Abreu, D. Sc., Universidade Federal de Viçosa, junho, 2014. **Early sex discrimination in *Carica papaya* L. by molecular cytogenetics**. Orientador: Carlos Roberto de Carvalho.

O mamoeiro, *Carica papaya* L., é a espécie mais importante economicamente da família Caricaceae. Nativa da América Central e do Sul, esta espécie frutífera e herbácea é cultivada principalmente nas regiões tropicais e subtropicais, e é amplamente consumida pelo seu fruto comestível. *C. papaya* é caracterizada como uma espécie polígama com três tipos sexuais: macho, fêmea e hermafrodita. Considerando que o mamão é propagado preferencialmente via sementes, problemas inerentes desta cultura se referem à segregação dos tipos sexuais e à detecção sexual tardia. A identificação sexual só é possível após o florescimento, pela inspeção das flores, uma vez que não há dimorfismo cromossômico reconhecido nem diferenças morfológicas entre os três tipos sexuais do mamoeiro no estágio de plântula. Com o objetivo de economizar tempo, manejo e recursos financeiros, é desejável para os produtores que o tipo sexual desta cultura seja conhecido antes do transplante. Pesquisadores da área de biologia molecular têm desenvolvido um grande número de marcadores genéticos na tentativa de distinguir o sexo do mamão antes de atingir a maturidade reprodutiva. Neste estudo, foram investigados sete marcadores do tipo *sequence characterized amplified region* (SCAR) previamente descritos na literatura, pela técnica de *polymerase chain reaction* (PCR), em duas variedades brasileiras comercialmente importantes de *C. papaya* ('Golden' e 'Rubi'), e então, foi desenvolvido um protocolo de *fluorescence in situ hybridization* (FISH), usando um marcador selecionado como sonda. Dessa forma, objetivou-se prover um diagnóstico molecular de sexagem precoce para plantas fêmeas e hermafroditas. Primeiramente, DNA genômico foi isolado de folhas jovens coletadas desses dois tipos sexuais de ambas as variedades de mamão. Após a otimização das condições de PCR, amplificações dos sete primers SCAR foram realizadas em sete amostras de cada tipo sexual de cada variedade. Os fragmentos obtidos foram analisados

por eletroforese em gel de agarose. Dentre os marcadores genéticos testados, três deles (NAPF-2, SDSP e SCARpm) geraram fragmentos sexo-específicos significativos em todas as amostras hermafroditas de ambos os cultivares de mamão. Outro marcador SCAR (T12) produziu um único fragmento sexo-específico para somente plantas hermafroditas do cultivar 'Rubi'. O marcador NAPF-2 foi escolhido para ser usado como sonda FISH, baseado em seu padrão de bandas consistentemente polimórfico. Análises FISH mostraram um único e forte sinal fluorescente em núcleos de hermafrodita do cv. 'Golden', e muitos e fortes sinais fluorescentes em núcleos de hermafrodita do cv. 'Rubi', enquanto nenhum sinal fluorescente detectável ou de baixíssima intensidade foi observado em núcleos de fêmea de ambos os cultivares. Em conclusão, o presente trabalho investigou com sucesso, pela primeira vez, o potencial discriminativo de marcadores SCAR em dois cultivares comerciais brasileiros de mamão, e propôs um novo método diagnóstico confiável, eficiente e relativamente rápido, baseado na técnica de FISH, para a identificação precoce de plantas hermafroditas dos cultivares 'Golden' e 'Rubi'. Em estudos futuros, este método poderá ser melhorado para um escaneamento em larga-escala na produção comercial por meio de uma abordagem citométrica de fluxo após FISH em suspensão nuclear.

## 1. INTRODUCTION

*Carica papaya* L. species is the most economically important fruit crop of the family Caricaceae (BAJPAI & SINGH 2006, ZHANG et al. 2010, SUDHA et al. 2013). It is cultivated worldwide mainly in tropical regions, for its edible fruit (MING et al. 2007), and subtropical ones, for valuable papain proteolytic enzyme production (PARASNIS et al. 2000, YU et al. 2009). Papaya is referred to as the third major tropical fruit produced globally. Brazil is the second largest papaya producer of the world, with a production of about 1.5 million tonnes of fruits in 2012, according to FAOSTAT data. The largest plantations of the main Brazilian cultivars, 'Solo' and 'Formosa', are located in the states of Bahia, Espírito Santo and in other northeastern regions of the country (IBGE 2012).

Papaya is a polygamous species with three sex forms, female, male and hermaphrodite (YU et al. 2008, ZHANG et al. 2010), which are differentiated by their inflorescence and fruit shape (MING et al. 2007). Due to high productivity and pear-shaped fruits with lower ovarian cavity, hermaphrodite plants are the major type for commercial cultivation in tropical regions, including Brazil (MAGDALITA & MERCADO 2003, OLIVEIRA et al. 2007, YU et al. 2008, PINTO et al. 2013). Female plants are cultivated mainly for papain production (PARASNIS et al. 1999, 2000, REDDY et al. 2012), while males are useless for economic purposes (CHAVES-BEDOYA & NUÑEZ 2007, URASAKI et al. 2002a, b).

Over the years, the intriguing sex determination system of *C. papaya* has been subject of studies in evolutionary biology and molecular genetics (LIU et al. 2004, MA et al. 2004, MING et al. 2007). Understanding the molecular factors behind sex expression has great importance in both basic and applied researches (GANGOPADHYAY et al. 2007). Since the 1930's, many hypotheses about the sex determination system in papaya have been proposed: a single gene with three alleles (HOFMEYR 1938, STOREY 1938), a group of genes confined to a small region on the sex chromosome (STOREY 1953), genic balance between sex chromosomes and autosomes (HOFMEYR 1967), XX-XY sex chromosome system (HOROVITZ &

JIMÉNEZ 1967), a trans-regulatory element controlling flower organ development (SONDUR et al. 1996). All these hypotheses are in agreement on one aspect: there is a genetic factor that controls sex expression for male and hermaphrodite plants, existing as heterozygous, and for females, as homozygous.

More recent application of molecular techniques and biotechnology has revolutionized the field of sex determination research in *C. papaya*. Sex-linked DNA markers were developed by several research groups and linkage maps of the papaya genome were constructed. Apart from these studies play an important role in evolutionary biology researches, molecular markers and methods have been widely used for identifying the papaya sex type at an early developing stage to improve fruit production (MING et al. 2007). The sex type identification is a limiting factor on cultivation of papaya, since there are no distinguishing morphological features at the seedling stage or heteromorphic sex chromosomes between the sex forms (PARASNIS et al. 1999, 2000, CHAVES-BEDOYA & NUÑEZ 2007, GANGOPADHYAY et al. 2007), and such identification is only possible after flowering (DEPUTY et al. 2002). The selection of the appropriate papaya sex type prior to planting in the field is of great interest by growers, for reducing the production costs (PARASNIS et al. 1999, SANTOS et al. 2003, COSTA et al. 2011, REDDY et al. 2012).

A variety of molecular markers for sex types in papaya have been developed: randomly amplified polymorphic DNA (RAPD) markers (PARASNIS et al. 2000, LEMOS et al. 2002, URASAKI et al. 2002a, CHAVES-BEDOYA & NUÑEZ 2007, OLIVEIRA et al. 2007, REDDY et al. 2012); sequence characterized amplified region (SCAR) markers, converted from RAPD (PARASNIS et al. 2000, DEPUTY et al. 2002, URASAKI et al. 2002a, b, CHAVES-BEDOYA & NUÑEZ 2007); simple sequence repeat (SSR) markers (PARASNIS et al. 1999, COSTA et al. 2011). These markers were tested for the discrimination of sex type in different papaya cultivars worldwide. However, the use of molecular markers in large-scale cultivation is considered unfeasible, since this technology is expensive and intensive (DEPUTY et al. 2002, SANTOS et al. 2003).

Considering the economic importance in early sex identification of papaya crop, the present study aimed to develop an alternative diagnostic assay based on FISH technique, proposed to be reliable, efficient, less costly and relatively faster than PCR approach, in order to be applied in large scale. For this, we (i) investigated the discriminative potential of seven SCAR markers previously described in the literature by PCR methodology, in two commercially important Brazilian varieties of *C. papaya*, (ii) applied the PCR methodology for construction of labeled probe from a selected marker, (iii) developed a cytological protocol for obtaining nuclei suspension, used for slide preparation, and (iv) applied FISH technique in these slides, aiming to suggest a new diagnostic method based on molecular cytogenetics.

## 2. LITERATURE REVIEW

### 2.1. General aspects of *Carica papaya*

The common papaya, *Carica papaya* L., belongs to the small dicotyledonous family Caricaceae, which comprises 35 species (including 32 dioecious, two trioecious and one monoecious species) spread over six recently reclassified genera (VAN DROOGENBROECK et al. 2002, DREW 2003, MING et al. 2007, YU et al. 2008). *Vasconcellea* is the largest genus of the family, comprising 21 species, followed by *Jacaratia*, *Jarilla*, *Cylicomorpha*, *Horovitzia*, with seven, three, two and one species, respectively. *C. papaya* is the only species of the genus *Carica*. These herbaceous, shrubby or arborescent plants are native to Tropical America, except the genus *Cylicomorpha*, which is originated from equatorial Africa (BADILLO 1993).

*C. papaya* is by far the most economically important fruit crop of the family Caricaceae. It is cultivated mainly in tropical and subtropical regions worldwide (BAJPAI & SINGH 2006, ZHANG et al. 2010, SUDHA et al. 2013), for its edible and nutritious fruit rich in vitamin A and C (CHANDRIKA et al. 2003, YU et al. 2008), as well as for its milky latex. This latter is the source of papain, a commercially valuable proteolytic enzyme widely used in food processing, medical applications, and textile, dairy, pharmaceutical and cosmetic industries (PARASNIS et al. 1999, VAN DROOGENBROECK et al. 2002, MING et al. 2007, 2008, YU et al. 2009). According to FAOSTAT (2014), papaya is referred to as the third major tropical fruit produced globally. Global papaya production has grown significantly over the years, mainly as a result of increased production in India, corresponding to 41.6% of the world production during 2012. Brazil has been the second largest papaya producer (12.2%), followed by Indonesia, Dominican Republic, Nigeria and Mexico, and the second exporter in the world. In 2012, Brazil produced about 1.5 million tonnes of papaya fruits. The largest plantations of the main Brazilian cultivars, 'Solo' and 'Formosa', are located in the states of Bahia

(45.03%), Espírito Santo (31.93%) and in other northeastern regions of the country (IBGE 2012).

Papaya is among the limited number of plant species that are trioecious with three sex forms – female, male and hermaphrodite (YU et al. 2008, ZHANG et al. 2010). Male trees are characterized by long, pendulous, many-flowered inflorescences bearing slender male flowers lacking a pistil, except for occasional pistil-bearing flowers at the distal terminus. Female trees have short inflorescences with few flowers bearing large functional pistils without stamens. Hermaphroditic trees have short inflorescences bearing bisexual flowers that can be sexually variable (MING et al. 2007). Mainly in tropical regions as in Brazil, hermaphrodites are the major type for fruit commercial cultivation because of their higher productivity (MAGDALITA & MERCADO 2003, MING et al. 2007) and pyriform-shaped fruits (MA et al. 2004, YU et al. 2008, BLAS et al. 2012) with lower ovarian cavity (LEMOS et al. 2002, PINTO et al. 2013). Conversely, female plants are preferred in subtropical areas for their greater production under cool winter temperatures (YU et al. 2009). Female flowers are stable at low temperature while hermaphrodite flowers tend to fuse anthers to the carpels and produce deformed carpelodic fruit (MING et al. 2007). Besides, females are chosen for papain extraction, whose proteolytic activity and quantity obtained from these dioecious fruits are superior to the hermaphrodite ones (PARASNIS et al. 1999, 2000, REDDY et al. 2012). Male plants are useless for economic purposes (CHAVES-BEDOYA & NUÑEZ 2007, URASAKI et al. 2002a, b).

## **2.2. Sex determination in papaya**

Since sex represents a huge problem in evolutionary biology, understanding the molecular factor(s) behind sex expression has immense importance in both basic and applied researches (GANGOPADHYAY et al. 2007). The polygamous angiosperm species *C. papaya* ( $2n = 18$ ), which has male, female and hermaphroditic forms, offers several advantages for genetic and evolutionary studies (LIU et al. 2004, MING et al. 2008, WEI & WING 2008, ZHANG et al. 2010, ARYAL & MING 2014). These include a

relatively small genome of 372 megabases (Mb) (ARUMUGANATHAN & EARLE 1991) and  $2C = 0.65$  pg (ARAÚJO et al. 2010), diploid inheritance with nine pairs of chromosomes, a short generation time of 9 – 15 months, continuous flowering of various flower types throughout the year, a unique evolutionary process in female flowers, an intriguing primitive system of sex determination and a well-established transformation system (LIU et al. 2004, MING et al. 2008). Based on the knowledge and information available at the time, sex determination in papaya has been a frequent subject of genetic analyses (HOFMEYR 1938, 1967, STOREY 1938, HOROVITZ & JIMÉNEZ 1967, SONDUR et al. 1996, PARASNIS et al. 1999, LIU et al. 2004, MA et al. 2004, MING et al. 2007), because it is directly related to efficient commercial fruit production (SILVA et al. 2007). Thus, various hypotheses about the sex determination system in this species have been postulated over the years.

On the basis of segregation ratios from crosses among the three sex types, STOREY (1938) and HOFMEYR (1938) each proposed originally that sex determination in papaya is controlled by a single gene with three alleles:  $M$  (or  $M_1$ ),  $M^h$  (or  $M_2$ ) and  $m$ . Male ( $Mm$ ) and hermaphrodite ( $M^hm$ ) individuals are heterozygous, whereas females ( $mm$ ) are homozygous recessive. The dominant combinations of  $MM$ ,  $M^hM^h$  and  $MM^h$  are embryonic lethal, resulting in a segregation ratio of 2:1 hermaphrodite to female from self-pollinated hermaphrodite trees, and a 1:1 segregation of male to female, or hermaphrodite to female, from cross-pollinated female trees by male's pollen or hermaphrodite's pollen, respectively. When occasionally male trees are selfed, it occurs a segregation ratio of 2:1 male to female, or if male's pollen fertilizes hermaphrodite's pistil, a progeny of 1 male:1 hermaphrodite:1 female is generated (Table 1).

Later, STOREY (1953) revised the hypothesis to suggest that papaya sex is regulated not by a gene, but rather by a complex of genes confined to a small region on the sex chromosome within which recombination is precluded. The different segments in this region are so closely linked together that they behave as inherited unit factors. In addition to genes differing in functionality of the androecium and gynoecium, this sex locus also contains genes controlling several co-segregating secondary sexual characteristics (ovary shape, degree of corolla fusion, flower number and

Table 1. Segregation ratio from crosses between the three sex forms in *C. papaya*

Crosses	Sex ratio			
	Female ( <i>mm</i> )	Male ( <i>Mm</i> )	Hermaphrodite ( <i>M<sup>h</sup>m</i> )	Non-viable ( <i>M<sup>h</sup>M<sup>h</sup></i> )
<i>Mm</i> x <i>Mm</i>	1	2	0	1
<i>M<sup>h</sup>m</i> x <i>M<sup>h</sup>m</i>	1	0	2	1
<i>Mm</i> x <i>mm</i>	1	1	0	0
<i>M<sup>h</sup>m</i> x <i>mm</i>	1	0	1	0
<i>M<sup>h</sup>m</i> x <i>Mm</i>	1	1	1	1

Font: Storey (1938) and Hofmeyr (1938). Obs.: The Storey's designation for genotypes was adopted considering its convenience to separate the hermaphrodite allele *M<sup>h</sup>* from male allele *M*.

peduncle length), cross-over suppression (*C*) and lethality (*L*). Based on Storey's model, the genotypes of male, hermaphrodite and female were given as in Table 2.

Table 2. Genotypes of the three sexes of papaya according to the Storey's model

Sex type	Genotype
Male	<i>Mp l C + sg / + + + sa +</i>
Hermaphrodite	<i>+ l C + sg / + + + sa +</i>
Female	<i>+ + + sa + / + + + sa +</i>

*Mp* – long peduncles of male flowers, *l* – zygotic lethal factor, *C* – hypothetical factor for suppression of recombination at the sex determination region, *sa* – suppressor of the androecium, *sg* – suppressor of the gynoecium. Font: Storey (1953).

The gene *sa* controls the suppression of stamen development when it is recessive homozygous as in female trees. The gene *sg* controls the suppression of carpel development when it is recessive homozygous. The author suggested that most male and hermaphrodite trees were heterozygous for this second gene, because of sex reversal between hermaphrodite and male flowers, influenced by environmental changes, often observed in the field.

Alternatively, HOFMEYR (1967) published the genic balance theory for papaya sex determination, aiming to explain the zygotic lethality in the dominant homozygous condition and the differential sexual stability among the three sexes. It was assumed that *M<sub>1</sub>* (for males) and *M<sub>2</sub>* (for hermaphrodites) represent genetically inactive regions of "sex chromosomes"

of slightly different lengths from which vital genes are missing. Therefore, any combinations of  $M_1M_1$ ,  $M_1M_2$  or  $M_2M_2$  would be lethal, while  $M_1m$  and  $M_2m$  would be viable because an  $m$  “sex chromosome” is present in each genotype. Because the regions of masculinity ( $M_1$  and  $M_2$ ) have slightly different lengths, the sex types of papaya are the results of genic balance between the sex chromosomes and autosomes.

Based on intergeneric hybridizations between *Carica* and *Vasconcellea* species, HOROVITZ & JIMÉNEZ (1967) proposed a sex determination system in *C. papaya* of XX – XY chromosomal type, even though heteromorphic chromosomes have not been found in this species (PARASNIS et al. 1999, URASAKI et al. 2002a, LIU et al. 2004, CHAVES-BEDOYA & NUÑEZ 2007, YU et al. 2008, JUNIOR et al. 2010). Their hypothesis is similar to that suggested by STOREY (1938) and HOFMEYR (1938), by employing another terminology, namely: XX, XY and XY<sub>2</sub> for female, male and hermaphrodite genotypes, respectively, where Y<sub>2</sub> is the modified Y chromosome. As the combinations of Y<sub>(2)</sub> chromosomes are lethal, these researchers suggested that both chromosomes share the same lethal factor.

An additional hypothesis to explain the genetic basis of sex inheritance in *C. papaya* includes regulatory elements of the flower development pathway (SONDUR et al. 1996). In this model, the dominant male allele, designated *SEX1-M*, encodes a *trans*-acting regulatory factor that induces male floral parts while suppressing carpel development. The dominant hermaphrodite allele, *SEX1-H*, is considered intermediate with the ability to give rise to stamens, but only reduces carpel size rather than inhibiting it. The recessive female allele, *sex1-f*, was hypothesized as incapable of promoting male structures. Functional carpels would develop in heterozygous *SEX1-H/sex1-f* plants. The lethality of *SEX1-M* or *SEX1-H* homozygotes could result from an additional required function present only in the *sex1-f* allele. The variability observed in the secondary sexual characteristics could result from environmental or allelic interaction effects on the expression / function of *SEX1*. For instance, it is possible that the interaction between *SEX1-H* and its target, either a promoter sequence or another protein factor, could be less stable than the interaction between

*SEX1-M* and the target. This could account for the difference in carpel size between males and hermaphrodite flowers, and for the sex reversal sometimes seen in hermaphrodites but which is rare in males.

All the hypotheses mentioned above share the same foundation: a genetic factor controlling sex expression for male and hermaphrodite that exists as heterozygous, while the female's is homozygous. Over the following years, advances in genomic technology made it possible a lot of studies could be performed for characterization of the genomic region involved in papaya sex determination at the molecular level. Sex-linked DNA markers were developed by several research groups and linkage maps of the papaya genome were constructed (MING et al. 2007).

The first genetic linkage map of papaya using DNA molecular markers was constructed by SONDUR et al. (1996). This map consisted of 61 RAPD markers, of which the *SEX1* flower sex determinant locus was mapped on linkage group 1 (LG1), where it was flanked by two markers at 7 cM on each side. Nevertheless, this low density map provided no clue whether there was suppression of recombination at that locus (MING et al. 2007). Since then, different DNA markers flanking the *SEX1* locus have been developed, increasing the understanding of the genetic structure of this region: microsatellite (PARASNIS et al. 1999) markers, RAPD (LEMOS et al. 2002) markers, and SCAR markers, converted from RAPD (DEPUTY et al. 2002, PARASNIS et al. 2000, URASAKI et al. 2002a). However, these authors did not report detailed data on linkage.

High-density genetic linkage mapping of the papaya genome has revealed severe suppression of recombination around the sex determination locus (MA et al. 2004), thus validating the STOREY's (1953) hypothesis that the region containing the gene complex of sex determination behaves as a unique factor so that there is no crossing over inside. MA et al. (2004) analyzed 1501 amplified fragment length polymorphism (AFLP) and morphological markers, of which 225 co-segregated with sex types and were included on linkage group 1 (corresponding to the LG1 of the RAPD map by Sondur and co-workers). These data showed an extremely high level of DNA polymorphism in the genomic region immediately surrounding the sex locus.

Concurrently, a physical mapping and sample sequencing of bacterial artificial chromosomes (BACs) in the sex determination region also showed severe suppression of recombination and extensive divergence between homologues, in addition to DNA sequence degeneration, nucleotide insertions, deletions and substitutions. These findings provided evidence that sex determination in papaya would be controlled by a pair of incipient sex chromosomes, with a small male-specific region (MSY) that accounts for only about 10% of the Y chromosome. It was also found that hermaphrodite and male plants share nearly identical DNA sequences in the MSY region, suggesting that the Y and Y<sup>h</sup> chromosomes might have originated from the same ancestral chromosome. The incipient sex chromosomes of papaya may yield insights about earlier stages of sex chromosome evolution in plants (LIU et al. 2004).

More recently, other research groups also constructed physical maps for the hermaphrodite-specific Y<sup>h</sup> chromosome region (HSY) and its X counterpart (NA et al. 2012), and for MSY region as well (GSCHWEND et al. 2011), using BAC libraries. These mappings provide the foundation for sequencing the sex specific regions of papaya. The quality of genome information is enhanced, however, when complementary resources of each map are integrated. In this sense, YU et al. (2009) integrated a BAC-based physical map of papaya with high-density genetic map and genome sequence. The integrated map allowed identifying recombination hotspots, regions suppressed for recombination and estimating physical distances between genetic markers.

Cytological features of papaya chromosomes have been also linked with the existing genome sequence or with the genetic and physical maps, improving the knowledge of papaya genome organization and evolution at the chromosomal level (WAI et al. 2010). By physically allocating MSY-specific BACs on hermaphrodite chromosome Y (Y<sup>h</sup>) and, thereafter, sequencing some of them, YU et al. (2007) placed the MSY near the centromere of the chromosome Y<sup>h</sup> and observed extreme gene paucity, high density of retroelements and local sequence duplications in that region. WAI et al. (2010, 2012) and ZHANG et al. (2010) integrated chromosomal traits with genetic or physical maps by generating cytogenetic maps for localizing

molecular markers-tagged BACs directly on papaya chromosomes by fluorescence *in situ* hybridization (FISH). Representative BAC clones of all 12 linkage groups derived from genetic maps were reassigned to the nine chromosomes of papaya, corresponding to its haploid number (WAI et al. 2010, ZHANG et al. 2010). WAI et al. (2012), in turn, constructed a molecular cytogenetic map of the papaya sex chromosome (chromosome 1).

WANG et al. (2012) sequenced the HSY region, and its X counterpart, yielding an 8.1 Mb HSY pseudo molecule, and a 3.5 Mb sequence for the corresponding X region. In contrast to the Y chromosome in other organisms, Y chromosomes in Caricaceae have higher genetic diversity than the X chromosomes (WEINGARTNER & MOORE 2012). Sequence divergence between HSY and X regions and the expansion of the HSY are mostly due to retrotransposon insertions, inversions, recombination suppression and numerous additional chromosomal rearrangements (WANG et al. 2012). These authors inferred the oldest rearrangement event in papaya sex chromosomes occurred about 7 million years ago (MYA) and, the most recent, only 1.9 MYA. These data support theoretical models of early sex chromosome evolution in papaya.

An alternative approach involving analyses of small RNA (sRNA) libraries was explored by ARYAL et al. (2014), once it is known that sRNA plays an important role in DNA methylation and gene silencing, suggesting its participation in sex differentiation in plants. A total of 14 micro RNAs (miRNA) were differentially expressed among male, female, and hermaphrodite flowers, such as miR169 that regulates the genes in auxin signaling pathway and, hence, in carpel development. The results indicate potential function of these sRNAs in papaya sex determination.

All the studies mentioned above clarify the understanding of possible evolutionary mechanisms that drive the divergence of the papaya sex chromosomes, which will be better accessed by further researches (WEINGARTNER & MOORE 2012, ARYAL & MING 2014). Concerning commercial applications, knowing more about the sex determination gene may eventually lead to engineering a true breeding hermaphrodite cultivar to improve papaya fruit production (GSCHWEND et al. 2011, ARYAL & MING 2014).

### 2.3. Problems concerning papaya cultivation

The commercial cultivation of *C. papaya* is conducted conventionally via seeds derived from open pollination (DREW 1987, PARASNIS et al. 1999, BHATTACHARYA & KHUSPE 2001, CHAVES-BEDOYA & NUÑEZ 2007). Although vegetative propagation methods, such as cuttings, grafting and micropropagation in tissue culture, are available (MING et al. 2007), they are laborious and expensive (MAGDALITA & MERCADO 2003, SAKER et al. 1999, REDDY et al. 2012). Being an alogamous species, the inherent heterozygosity of papaya crop results in a mixture of genotypes with considerable variation regarding yield, fruit quality and susceptibility to various diseases (SAKER et al. 1999). Its most important pathogen is papaya ringspot virus (PRSV), a devastating disease that has a detrimental impact on both commercial papaya production and Caricaceae germplasm conservation (MATSUMOTO et al. 2010). The introduction of the commercial virus-resistant transgenic variety 'SunUp', in 1998, saved the Hawaii papaya industry from collapse in the mid-1990s (MING et al. 2007, WEI & WING 2008, REDDY et al. 2012). Because PRSV is widespread in nearly all papaya-growing regions, 'SunUp' could serve as a transgenic germplasm source to be used in breeding of suitable cultivars resistant to the virus in various parts of the world (MING et al. 2008).

Considering the trioecious nature of *C. papaya*, with male, hermaphrodite and female forms, the sex type identification is another limiting factor on cultivation of this crop. The lack of clearly distinct morphological features at the seedling stage and heteromorphic sex chromosomes have hampered the early sexing based on morphology or cytology (MAGDALITA & MERCADO 2003, PARASNIS et al. 1999, 2000, CHAVES-BEDOYA & NUÑEZ 2007, GANGOPADHYAY et al. 2007). Therefore, the sex of papaya plants is uncovered only after flowering (DEPUTY et al. 2002) which may take 5 – 8 months (PARASNIS et al. 1999, 2000, MA et al. 2004). Once hermaphrodite fruits are commercially preferred over that of females, and hermaphrodite plants segregate usually into hermaphrodites and females at the ratio of 2:1 (see Table 1), 3 – 5 seedlings

are commonly planted in one hill, followed by uprooting of undesired female trees, in order to guarantee a number of hermaphrodites evenly distributed in the field. Such practice raises the production costs, by wastage of cultivation area, labor, time and other resources (PARASNIS et al. 1999, LEMOS et al. 2002, URASAKI et al. 2002a, SANTOS et al. 2003, MA et al. 2004, COSTA et al. 2011).

The selection of the appropriate papaya sex type at the juvenile forms, prior to transplanting, would be beneficial especially for producers (MAGDALITA & MERCADO 2003, PARASNIS et al. 1999, CHAVES-BEDOYA & NUÑEZ 2007, REDDY et al. 2012). In order to overcome these constraints in cultivation practice and to make it more profitable, there has been a long-standing interest in developing strategies for identifying the papaya sexes at the vegetative stage (PARASNIS et al. 2000). Molecular markers for sex types in papaya that could be generated through DNA analysis using polymerase chain reaction (PCR) technology have been seen as a reliable strategy (MAGDALITA & MERCADO 2003).

#### **2.4. Molecular markers for sex identification in papaya**

Researches in molecular biology have led to the development of a variety of DNA markers linked to the sex determination locus in papaya (MAGDALITA & MERCADO 2003, PARASNIS et al. 1999, 2000, DEPUTY et al. 2002, LEMOS et al. 2002, URASAKI et al. 2002a, b, CHAVES-BEDOYA & NUÑEZ 2007, OLIVEIRA et al. 2007, COSTA et al. 2011, REDDY et al. 2012) (Table 3), as a means to sex the plants prior to flowering. The advantages of DNA markers include their abundance and stability, a relatively high rate of polymorphism in many populations, usually routine technologies for scoring, frequent comparability across species, clear dominance or codominance in most situations, presence at all stages and tissue types of plant growth, lack of epistasis and no detectable phenotypic effect (MA 2003, KANCHANA-UDOMKAN et al. 2014).

Table 3. DNA molecular markers used for predicting the sex type in *C. papaya*

Marker name	Primer sequence (5' → 3')	Product size	Type of marker	Sex detection <sup>a</sup>	Cultivars analyzed	Reference
(GATA) <sub>4</sub>	-	5 kb	SSR	H and M	Various	Parasnis et al. (1999)
OPF2	OPF2: GAGGATCCCT	831 bp	RAPD	M	From India and USA	Parasnis et al. (2000)
NAPF-2	F-Napf-76: GAGGATCCCTATTAGTGTAAG R-Napf-77: GAGGATCCCTTTTGCCTCTG	0.83 kb	SCAR	M	From India and USA	Parasnis et al. (2000)
T12	T12-F: GGGTGTGTAGGCACTCTCCTT T12-R: GGGTGTGTAGCATGCATGATA	0.8 kb	SCAR	H and M	'Sunrise', 'Kapoho', 'Rainbow', 'SunUp'	Deputy et al. (2002)
W11	W11-F: ATCTCGGTGTGTGCGTAGTC W11-R: TCATCTACTAGTGCGTAGTC	0.8 kb	SCAR	H and M	'Sunrise', 'Kapoho', 'Rainbow', 'SunUp'	Deputy et al. (2002)
PSDM	IBRC-RP07: TTGGCACGGG	450 bp	RAPD	H and M	Various	Urasaki et al. (2002a)
SCAR <sub>ps</sub>	SDP-1: GCACGATTTAGATTAGATGT SDP-2: GGATAGCTTGCCCAGGTCAC	225 bp	SCAR	H and M	Various	Urasaki et al. (2002a)
14 SCAR <sub>pm</sub>	SDP-2: GGATAGCTTGCCCAGGTCAC SDP-3: GGTAAGAGTTTTTCCCAAGC	347 bp	SCAR	H and M	'Sunrise'	Urasaki et al. (2002b)
T1	T1-F: GTCTCTCGTATAGTTCTCGT T1-R: ACGTCTCCACTCGCTTCCAT	1.3 kb	nd	H and F	'Cariflora', 'Cavite', 'Sinta'	Magdalita & Mercado (2003)
W11	W11-F: ATCTCGGTGTGTGCGTAGTC W11-R: TCATCTACTAGTGCGTAGTC	0.8 kb	nd	H	'Cariflora', 'Cavite', 'Sinta'	Magdalita & Mercado (2003)
OP-Y7 <sub>900</sub>	OP-Y7: AGAGCCGTCA	900 bp	RAPD	M	'Catira', 'ILS 647', 'ILS 649'	Chavez-Bedoya & Nuñez (2007)
SDSP	CFw: AAACCTACCGTGCCATTATCA CRv: AGAGATGGGTTGTGTCCTG	369 bp	SCAR	H and M	'Catira', 'ILS 647', 'ILS 649'	Chavez-Bedoya & Nuñez (2007)
BC210 <sub>438</sub>	BC210: GCACCGAGAG	438 bp	RAPD	H	'Sunrise', 'Calimosa', 'JTA', Tainung n <sup>o</sup> 1'	Oliveira et al. (2007)
(AGC) <sub>5</sub> Y <sup>b</sup>	-	500 bp	SSR	H	'Tailândia', 'SS72/12', 'Tainung H'	Costa et al. (2011)
OPL-13	-	-	RAPD	M and F	Various	Reddy et al. (2012)

<sup>a</sup> H – hermaphrodite, F – female, M – male; <sup>b</sup> Y = C or T; nd – non-described.

In predicting the sex type of papaya, MAGDALITA & MERCADO (2003) used two 20mer primer pairs, whose sequences were provided by University of Hawaii, in the PCR amplification. Hermaphrodites were distinguished by having two distinct bands (1.3 and 0.8 kilobases – Kb), females had a single band (0.8 Kb), while males had no band. The noted frequency of females, hermaphrodites and males, identified as such both by PCR and field observation, in three varieties ('Cavite', 'Cariflora' and 'Sinta' hybrid) showed 100% accuracy in the prediction.

LEMOS et al. (2002) carried out RAPD assays to differentiate between the sexual forms of three commercial *C. papaya* cultivars belonging to the 'Solo' group, grown in Brazil. From 152 RAPD primers tested, one (BC210) showed polymorphic banding pattern between hermaphrodite and female samples of all three cultivars. The efficiency of the BC210 molecular marker in detecting sex differentiation early in the development of papaya seedlings (one-month old) was evaluated. The amplification of sex-specific fragment was accurately detected in only hermaphrodite samples. The same BC210 marker was successfully tested in other Brazilian commercial genotypes, two varieties of the 'Solo' group and two hybrids of the 'Formosa' group (OLIVEIRA et al. 2007). In a different approach, REDDY et al. (2012) employed a RAPD marker to confirm the early identification of male and female sex types based on their leaf morphology and rate of growth.

PARASNIS et al. (2000), DEPUTY et al. (2002), URASAKI et al. (2002a, b) and CHAVES-BEDOYA & NUÑEZ (2007) selected sex-specific RAPD markers and converted them into more reliable SCAR markers, by cloning and sequencing the fragments and, then, designing longer primers. A PCR-based sex diagnostic assay using the SCAR marker (NAPF-2) developed by PARASNIS et al. (2000) revealed reliability of >90% on sex identification of juvenile papaya plants. These researchers inferred that the errors detected were mainly due to variations in concentration of the extracted DNA. A greater accuracy (99.2%) was achieved when using two other SCAR markers to discriminate hermaphrodite and male from female plants at seedling stage (DEPUTY et al. 2002). URASAKI et al. (2002a) developed a SCAR marker (SCARps) from the RAPD marker PSDM, and both were effective also in separating hermaphrodite and male from female

plants. PSDM marker was further tested on early sexual detection and the results were confirmed with 100% accuracy. Another SCAR marker, designed SCARpm, was used in a multiplex-PCR assay by the same researchers' group (URASAKI et al. 2002b).

CHAVES-BEDOYA & NUÑEZ (2007) reported an interesting finding. After screening 32 RAPD primers, one was amplified in male but not in female and hermaphrodite samples. This marker was converted into SCAR, which, in turn, amplified a 369-base pairs (bp) fragment from both male and hermaphrodite but not from female plants. This fact could be explained as an expected result of the higher specificity of SCAR marker since its primers are twice longer than the RAPD primer.

In contrast to most, PARASNIS et al. (1999) employed highly informative microsatellite and minisatellite probes to identify sex-specific differences in papaya. Among these, only the microsatellite probe (GATA)<sub>4</sub> demonstrated to be sex-specific in all the cultivars analyzed. Then, the diagnostic potential of this specific marker was exploited to sex papaya plants at the seedling stage (2-months old), generating unequivocal results. COSTA et al. (2011) have also demonstrated the feasibility of SSR markers on early molecular sexing in a variety of accessions belong to a Brazilian germplasm collection. Only one marker in three papaya genotypes proved to be discriminative.

Although molecular markers could be valuable tool for researchers to employ in sex determining of experimental material, some authors contest its practicability in routine sex testing by farmers, since that is an expensive and intensive technology (DEPUTY et al. 2002, SANTOS et al. 2003).

## **3. MATERIALS AND METHODS**

### **3.1. Plant material**

Two commercially important Brazilian *C. papaya* cultivars, grown in the state of Espírito Santo, were analyzed: cv. 'Golden' of 'Solo' group and cv. 'Rubi' of 'Formosa' group, kindly provided by Caliman Agrícola S.A. and Instituto Capixaba de Pesquisa, Assistência Técnica e Extensão Rural (Incaper), respectively.

Leaf samples from seven female and seven hermaphrodite mature plants of each cultivar were harvested. Male samples were not used in our experiments since farmers do not cultivate them for commercial purposes. Following collection, the leaf tissues were frozen in liquid nitrogen and stored at  $-80^{\circ}\text{C}$  till further use in both PCR and FISH techniques.

All the procedures were carried out at the Laboratório de Citogenética e Citometria Vegetal, of the Departamento de Biologia Geral, Universidade Federal de Viçosa (UFV).

### **3.2. Genomic DNA extraction**

Genomic DNA of the samples was extracted by using GenElute™ Plant Genomic DNA Miniprep kit (Sigma®). DNA concentration and purity were determined spectrophotometrically, and its integrity was further checked by agarose-gel electrophoresis.

### **3.3. PCR amplification with SCAR primers**

Seven SCAR markers from previous studies were investigated regarding the sex discrimination potential in those above papaya cultivars, by PCR assays. The papain gene marker was used as positive control of PCR conditions.

Firstly, annealing temperatures for each primer pair and PCR conditions were optimized. Some information about the molecular markers used is summarized in Table 4. Each PCR mixture consisted of 100 ng genomic DNA, 0.2 mM dNTPs, 2% dimethyl sulfoxide, 2 U AccuTaq™ LA DNA Polymerase (Sigma®), 1X Polymerase buffer, 0.4 μM of each primer (forward and reverse), in 20 μL-final volume. Amplification reactions were performed on thermocycler Mastercycler Gradient (Eppendorf®) in two rounds. The first round consisted of preheating at 95°C for 5 min, 15 cycles of 94°C for 1min, temperature 1°C below the annealing temperature ( $T_m$ , specific for each primer) for 30 sec, a transition time of about 2 min until reaching  $T_m$ ,  $T_m$  for 1 min, and 68°C for 1 min. The second round was 30 cycles of 94°C for 45 sec,  $T_m$  for 1 min and 68°C for 1 min, followed by a final extension at 68°C for 5 min.

Table 4. Information of the molecular markers used

Marker name	Primer sequence (5' → 3')	Product size	Reference	$T_m$ (°C)*
NAPF-2	F-Napf-76: GAGGATCCCTATTAGTGTAAG R-Napf-77: GAGGATCCCTTTTGCACCTG	0.83 kb	Parasnis et al. (2000)	59.2
T12	T12-F: GGGTGTGTAGGCACTCTCCTT T12-R: GGGTGTGTAGCATGCATGATA	0.8 kb	Deputy et al. (2002)	63.9
SCARpm	SDP-2: GGATAGCTTGCCCAGGTCAC SDP-3: GGTAAGAGTTTTTCCCAAGC	347 bp	Urasaki et al. (2002b)	61.8
T1	T1-F: GTCTCTCGTATAGTTCTCGT T1-R: ACGTCTCCACTCGCTTCCAT	1.3 kb	Magdalita & Mercado (2003)	58.7
W11	W11-F: ATCTCGGTGTGTGCGTAGTC W11-R: TCATCTACTAGTGCAGTAGTC	0.8 kb	Magdalita & Mercado (2003)	56.7
SDSP	CFw: AAACCTACCGTGCCATTATCA CRv: AGAGATGGGTTGTGCACTG	369 bp	Chavez-Bedoya & Nuñez (2007)	59.2
SDSP2	CFw: AAACCTACCGTGCCATTATCA CRv2: TTGCGGAGCTACCAGGTTTG	369 bp	Chavez-Bedoya & Nuñez (2007)	63.5
Papain	P5': GGGCATTCTCAGCTGTTGTA P3': CTCCTTGAGCGACAATAAC	221 bp	Urasaki et al. (2002b)	62

\* Annealing temperature, experimentally determined.

PCR products were separated by electrophoresis in 1.5% agarose gels and visualized under UV light after Nancy-520 (Sigma®) staining. The images were digitally photographed.

### **3.4. Selection of SCAR marker and probe labeling**

The SCAR marker showing the best polymorphic banding pattern was chosen for testing signal specificity in FISH analyses. The probe was constructed by PCR-based direct labeling reaction. Four Polymerase enzymes were tested for labeling efficiency: AccuTaq™ LA DNA Polymerase (Sigma®), AmpliTaq Gold® 360 DNA Polymerase (Applied Biosystems®), Thermo Sequenase™ DNA Polymerase (GE Healthcare) and Platinum® Taq DNA Polymerase High Fidelity (Invitrogen™). In addition to DNA Polymerases, the labeling reactions consisted of 200 ng PCR products, 0.2 mM dATP, dCTP and dGTP each, 0.1 mM dTTP, 0.1mM tetramethylrhodamine-5-dUTP (Roche Diagnostics®), 1X Polymerase buffer, 0.4 µM of each primer (forward and reverse), in 20 µL-volume. These reactions were performed on termocycler Mastercycler Gradient (Eppendorf®) in two steps too: 10 cycles in the first step and 20 cycles in the second step, at the same conditions described above. The labeled probes were quantitated at the Qubit® 2.0 Fluorometer (Invitrogen™), and evaluated by electrophoresis in 1.5% agarose gels.

### **3.5. FISH**

For FISH procedure, we developed a protocol for obtaining nuclei suspension from the leaf tissues frozen and stored at -80°C, which was further used in slide preparations. For adequate recovering, thawing of frozen tissues should occur as fast as possible. Thus, the leaves were incubated into a 40°C water bath for 5 min. Soon after, the leaves were fixed in 3% formaldehyde solution with 1X Tris-HCl (pH 7.5), at 4°C for 20 min, and washed three times with 1X Tris-HCl (pH 7.5), at 4°C for 5 min each. Thereafter, the leaves were submitted to nuclei extraction by chopping procedure (GALBRAITH et al. 1983) in 0.2 mL of the OTTO I lysis buffer (OTTO 1990) containing 0.1 M citric acid, 0.5% Tween 20 and 2.0 mM dithiothreitol, pH 3. After 5 min, 0.2 mL of the same buffer was added and the suspension was filtered in a 20 µm-mesh nylon filter (Partec®). Three

choppings executed apart were joined in one microcentrifuge tube (Eppendorf®), gently vortexed and incubated for additional 5 min. Then, the suspension was centrifuged at 1100 rpm for 5 min. The supernatant was discarded, resuspended in 100 µL fresh methanol : acetic acid solution (3:1), and stored at -20°C for 10 min. Nuclei suspension was dropped onto very clean slides. Slides were immediately dehydrated in a cold ethanol series (twice in 70% and twice in 96%) for 5 min each.

Prior to hybridization, nuclei slides were pretreated with pepsin at 37°C for 10 min. After washing in phosphate-buffered saline solution twice, for 2 min each, fixing with 1% formaldehyde solution at 4°C for 10 min and washing again, the slides were dehydrated in a cold ethanol series (70%, 85% and 100%). The hybridization mixture consisted of 50% formamide, 2X saline-sodium citrate (SSC) buffer, 10% dextran sulfate, 0.01 µg/µL C<sub>0</sub>t-1 DNA, 3 ng/µL DNA probe, and was denatured at 99°C for 10 min. Hybridization was performed at 37°C for 20 h, followed by washes in 50% formamide in 2X SSC, 2X SSC, and 2X SSC with Triton 1% at 42°C for 5 min each. Subsequently, the slides were dehydrated in 70%, 85% and 100% ethanol, and nuclei were counterstained with 4',6-diamidino-2-phenylindole (DAPI).

Images of nuclei were captured by a DP71 video camera (Olympus™) attached to a BX-60 fluorescence microscope (Olympus™), with a 100x objective lens, WG filters for FISH analysis and WU filter for DAPI staining. The frame was digitized using the Image Pro®-Plus 6.1 software (Media Cybernetics™). The merging and final adjustments of the images were done with the tools of the same software.

## 4. RESULTS

### 4.1. PCR assays with SCAR markers

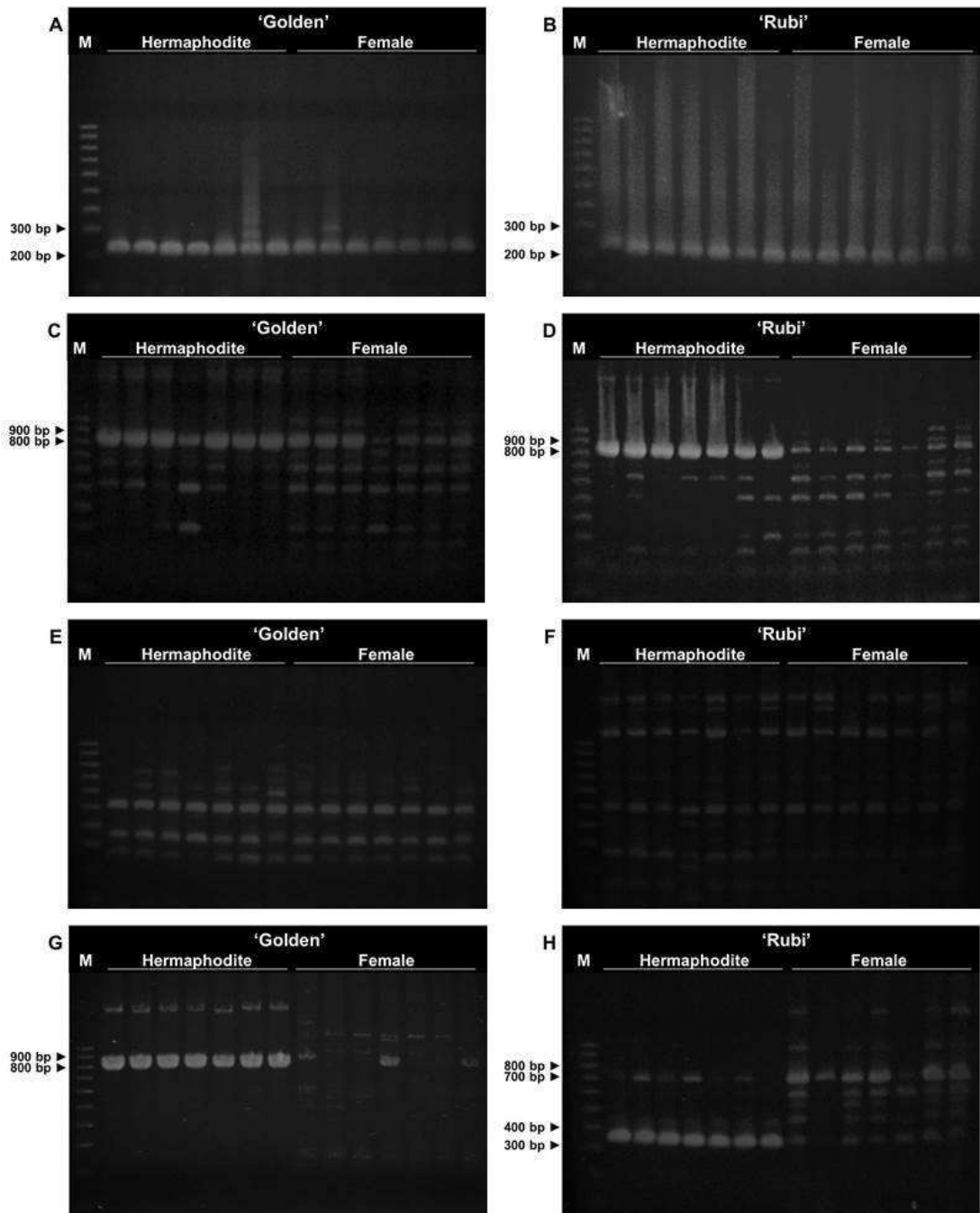
Papaya SCAR markers were explored regarding their potential to discriminate hermaphrodite from female individuals of the Brazilian cultivars 'Golden' and 'Rubi'. A pair of primers used for amplifying a partial sequence of the papain gene (gender-neutral) served as positive control of PCR conditions. A fragment between 200 and 300 bp was amplified in all samples of both cultivars (Figure 1a, b).

Each of the seven sex-specific SCAR markers investigated in this study showed very similar banding patterns, containing fragments of varying lengths, when comparing the two cultivars. Therefore, the considerations made for each marker will serve for both cv. 'Golden' and 'Rubi', excepting only one which showed PCR profiles slightly different between them (T12 marker). In this particular case, it could be noticed a more meaningful production of 800 – 900-bp fragments in 'Rubi' hermaphrodite samples, while there is a more homogeneous production of fragments within that range in all 'Golden' samples (Figure 1c, d).

Among the remaining SCAR markers, three of them (T1, W11 and SDSP2) amplified different PCR products regardless the sex, while others (NAPF-2, SDSP and SCARpm) generated at least one consistently polymorphic band, clearly differentiating hermaphrodite from female samples. Two representative PCR profiles show no difference on the sex-specific banding behavior for the two sex forms of the two cultivars studied: SDSP2 amplification in 'Golden' samples (Figure 1e) and W11 amplification in 'Rubi' samples (Figure 1f).

Likewise in the amplification of T12 primers, NAPF-2 primers produced hermaphrodite-specific 800 – 900-bp fragments in an expressive quantity but interestingly in both cultivars (Figure 1g). Products of that same length were also detected in some female samples, but in a very low intensity. Another discrete hermaphrodite-specific band of high-molecular-weight fragments was also seen. PCR of the SDSP marker amplified

hermaphrodite-specific fragments of nearly 400 bp long and female-specific fragments of slightly above 400 bp long. Besides, a low intensity high-molecular-weight band was observed in female samples, which was absent in hermaphrodites (data not shown). Amplification profile of the SCARpm marker evidenced different sex-specific bands: hermaphrodite-specific 300 – 400-bp and female-specific 700 – 800-bp fragments, both in high intensity, and three other female-specific bands (<700 bp long) in lower intensity (Figure 1h).



**Figure 1.** Representative PCR profiles after amplification of papain primers and SCAR primers tested in this study, in *Carica papaya*. *A* and *B*: PCR amplification of the partial sequence of the papain gene. *C* and *D*: PCR profiles from amplification of T12 primers. *E* and *F*: PCR profiles showing no sex-specific polymorphic banding pattern – amplification of SDSP2 primers in 'Golden' samples (*E*); amplification of W11 primers in 'Rubi' samples (*F*). *G* and *H*: PCR profiles showing sex-specific polymorphism – amplification of NAPF-2 primers in 'Golden' samples (*G*); amplification of SCARpm primers in 'Rubi' samples (*H*). M: 100 bp ladder (Sigma®).

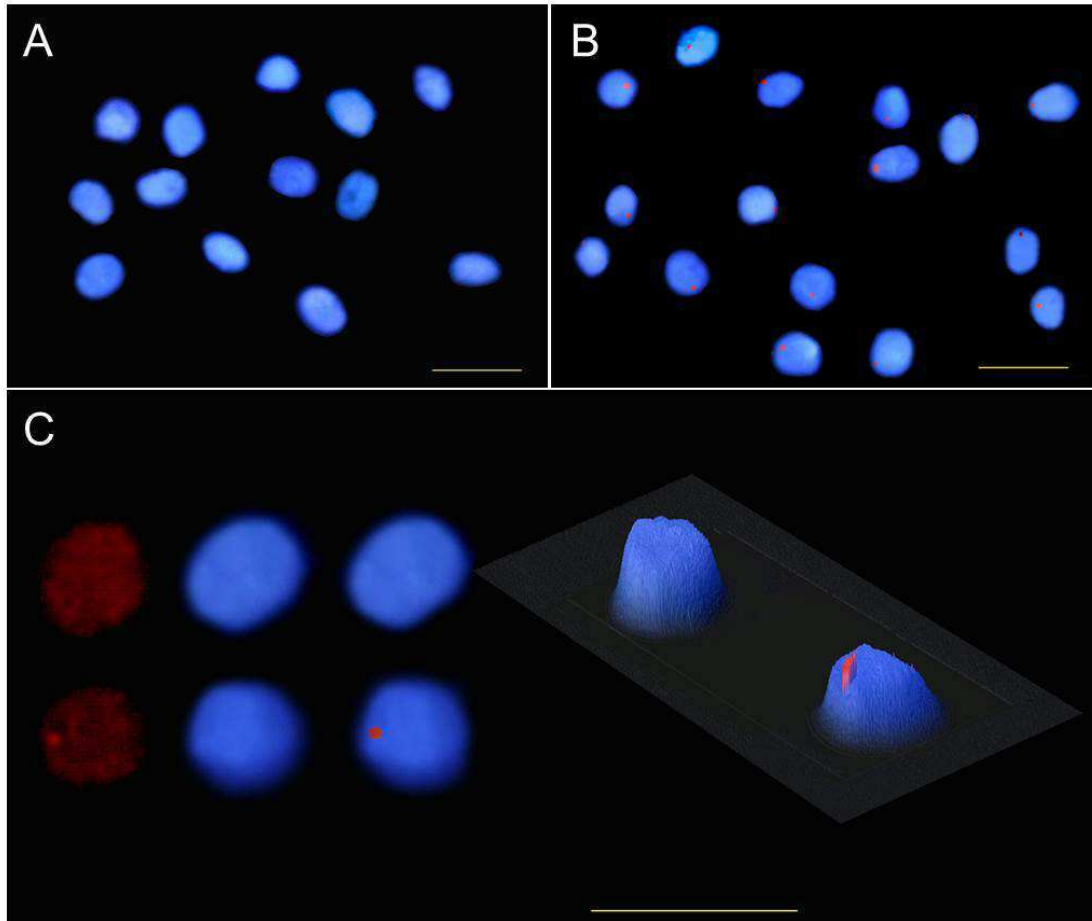
## 4.2. FISH

Based on the PCR amplification profiles of the SCAR molecular markers which showed sex-specific polymorphism, the NAPF-2 marker was selected for evaluating signal specificity in FISH analyses, considering its highly contrasting banding pattern. For this, the PCR products obtained were reamplified in a labeling reaction by testing four DNA polymerases. Although the probes have been quantified by fluorometer and qualified by agarose-gel electrophoresis, the labeling efficiency is in fact assessed on FISH analyses.

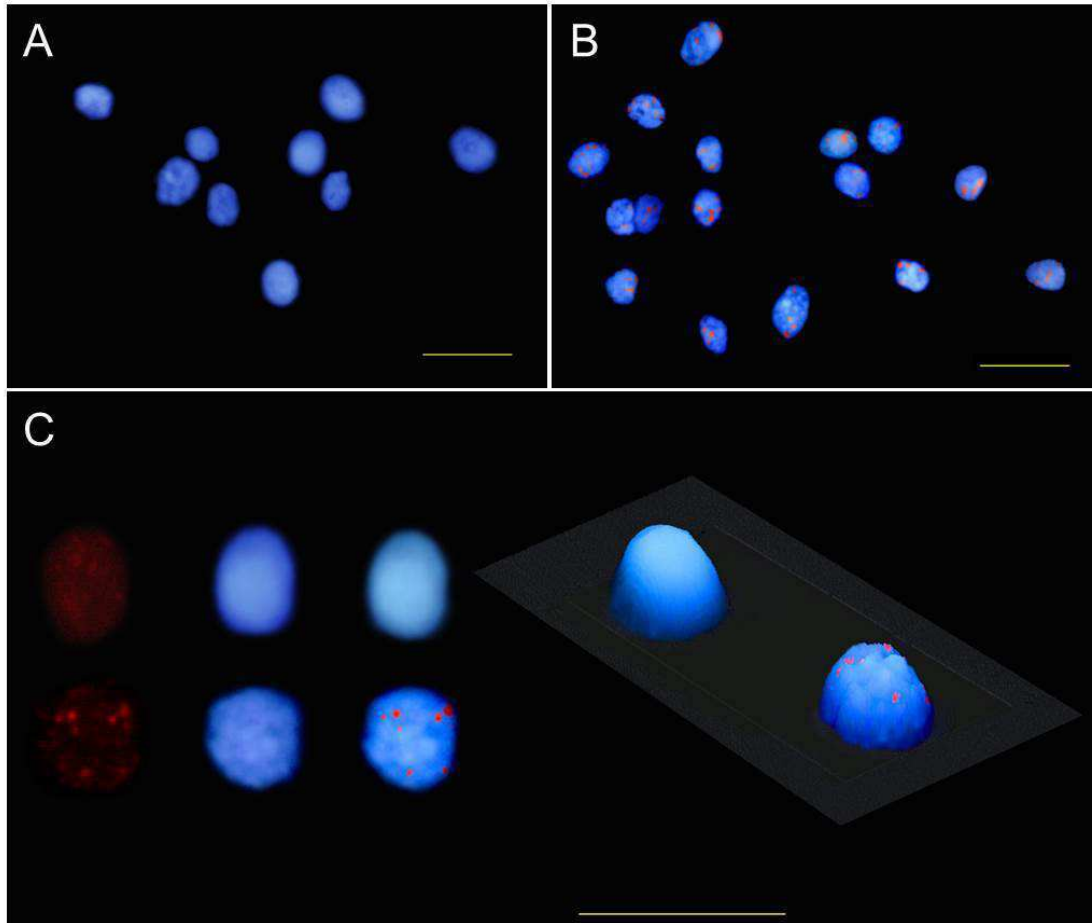
It is noteworthy that the protocol developed for obtaining nuclei suspension from frozen leaf tissues was successful as intact and well-individualized nuclei, with no cytoplasmic background noises, were gathered in a great quantity per slide. These are important aspects for better reaching of probes to nuclear DNA and further visualization of hybridization.

Thus, with adequate slide preparations, the labeling efficiency of each DNA polymerase on probe construction could be evaluated by FISH. The probe synthesized with Thermo Sequenase<sup>TM</sup> DNA Polymerase provided the best FISH result, giving strong and unambiguous fluorescence signals, and, therefore, it was employed in the subsequent analyses.

FISH analyses in *C. papaya* by using NAPF-2 marker as probe showed the same qualitative results in both cultivars 'Golden' and 'Rubi': fluorescence signals present in hermaphrodite nuclei, and no detectable or very low intensity fluorescence signal in female nuclei. In the hermaphrodite slide of the cv. 'Golden', all the nuclei exhibited a single strong fluorescent signal, most of them located at the nuclear peripheral region. Some smaller and diffuse fluorescent spots, however, were also visualized in few nuclei. On the other hand, many strong fluorescent signals spread on hermaphrodite nuclei were evident in the cv. 'Rubi'. The FISH results of the two sex types of the cv. 'Golden' and 'Rubi' are demonstrated in Figures 2 and 3, respectively.



**Figure 2.** *C. papaya* cv. 'Golden' nuclei after FISH by using sex-specific NAPF-2 marker as probe. A: nuclei from female leaf tissue. None fluorescence signal was detected. B: nuclei from hermaphrodite leaf tissue, showing a single strong fluorescent signal. C: on the left side, a comparative image of representative nuclei rhodamine-stained, DAPI-stained and merging them (from left to right), from female (above) and hermaphrodite (below) material; on the right side, a 3D density profile of the merged nuclei to highlight the fluorescence signal. Bars = 10  $\mu$ m.



**Figure 3.** *C. papaya* cv. 'Rubi' nuclei after FISH by using sex-specific NAPF-2 marker as probe. A: nuclei from female leaf tissue. None fluorescence signal was detected. B: nuclei from hermaphrodite leaf tissue, showing a number of fluorescent spots. C: on the left side, a comparative image of representative nuclei rhodamine-stained, DAPI-stained and merging them (from left to right), from female (above) and hermaphrodite (below) material; on the right side, a 3D density profile of the merged nuclei to highlight the fluorescence signals. Bars = 10  $\mu$ m.

## 5. DISCUSSION

### 5.1. PCR assays with SCAR markers

Sex-linked DNA molecular markers have been developed for *C. papaya* species over the years, and applied in early prediction of sexual type (MING et al. 2007). This practice plays an important role on papaya fruit production improvement, of great interest by farmers (MAGDALITA & MERCADO 2003, CHAVES-BEDOYA & NUÑEZ 2007). Nevertheless, considering that most of the markers come from arbitrary sequences, the identification of markers conserved on papaya genome and specific for each sex form regardless the genotype becomes a hard task. Moreover, sex system in papaya is hypothesized to be determined by one genic locus with three alleles, thus dominant markers like RAPD eventually would be linked to the recessive allele, which is present in all sex types (OLIVEIRA et al. 2007). So, it is necessary to investigate the potential sex-specific markers for each papaya cultivar or variety of interest.

In this study, seven sex-specific SCAR markers were tested for discriminating at molecular level hermaphrodite from female plants of two commercially important Brazilian cultivars (male plants were not evaluated because they are not cultivated for commercial purposes in Brazil), in addition to the SCAR marker for papain gene used as positive control of PCR conditions. In the latter case, the 200 – 300-bp fragment obtained after amplification, in all samples, corroborates the expected fragment size of 221 bp (URASAKI et al. 2002b, RIMBERIA et al. 2005), indicating that the PCR conditions adopted were suitable. Among the seven SCAR markers studied, six of them (excluding T12 marker) showed very similar banding patterns between the cultivars. This fact suggests a conservation of their sequences in these two Brazilian cultivars. Conversely, PCR results for the T12 SCAR marker demonstrate the presence of polymorphism intra- (between hermaphrodite and female plants of cv. 'Rubi') and inter-cultivars. The production of 800 – 900-bp fragments in 'Rubi' hermaphrodite samples was also found by DEPUTY et al. (2002) in hermaphrodite and male plants from

Hawaiian cultivars. The RAPD-PCR fragment designated T12 had been previously characterized and mapped on *SEX1* locus (SONDUR et al. 1996).

In relation to the T1 SCAR marker, whose RAPD-PCR fragment was also linked to the *SEX1* locus (SONDUR et al. 1996), similar results to ours were reported by DEPUTY et al. (2002). These authors observed a single product of ~1300 bp in all plants, with no sex segregating pattern. However, MAGDALITA & MERCADO (2003) found a 1300-bp PCR product in both females and hermaphrodites, while male had no band. About W11 SCAR marker, these three studies showed different results. While no segregating band was detected for both Brazilian cultivars in the present study, a polymorphic band (~800 bp) was produced in hermaphrodite and male plants from Hawaiian cultivars (DEPUTY et al. 2002), and in hermaphrodites only (MAGDALITA & MERCADO 2003).

Among the SCAR markers that generated sex polymorphism in our analyses, the SDSP marker produced a hermaphrodite-specific fragment of nearly 400 bp long, which is consistent with the amplification of a 369-bp product in hermaphrodites and males from Colombian genotypes (CHAVES-BEDOYA & NUÑEZ 2007), and the SCARpm marker produced a 300 – 400-bp fragment in hermaphrodites, also in line with a hermaphrodite and male-specific 347-bp band obtained by URASAKI et al. (2002b). Also it can be inferred that each of these sequences are conserved among their respective cultivars analyzed. On the other hand, female-specific PCR products were also detected for both SCAR markers, SDSP and SCARpm, but these findings were not reported by CHAVES-BEDOYA & NUÑEZ (2007) neither by URASAKI et al. (2002b), respectively.

NAPF-2 was considered by our analyses the best sex-specific SCAR marker for the two cultivars, 'Golden' and 'Rubi', producing a consistently polymorphic band of 800 – 900 bp long. PARASNIS et al. (2000), who selected and tested this molecular marker, found a 831-bp fragment in male plants, but absent in females. Only dioecious papaya varieties were tested so that there were no hermaphrodite plants. The fact that the NAPF-2 marker produced a ~800-bp fragment in hermaphrodite and male plants by these distinct studies could be explained by the probable location of this sequence

on the MSY region, where nearly identical DNA sequences are shared by genomes of these two sex types, as proposed by LIU et al. (2004).

This is the first work that investigated the segregating potential of the SCAR markers mentioned above in the Brazilian commercial papaya cultivars 'Golden' ('Solo' group) and 'Rubi' ('Formosa' group). However, a previous report evaluated some of these markers in sexing of papaya plants from other four Brazilian commercial varieties: 'Sunrise' and 'JTA' of 'Solo' group, and 'Tainung n°1' and 'Calimosa' hybrids of 'Formosa' group (OLIVEIRA et al. 2007). Any of the markers tested (SDSP, NAPF-2, SCARpm and W11) was validated for the true sex identification of these varieties, revealing the presence of false-positives and -negatives in the analyses.

## **5.2. FISH**

The more common cytological targets in the FISH technique are mitotic metaphase chromosomes mainly used for cytogenetic mapping (JIANG & GILL 2006). Optionally, application of FISH in interphase nuclei has been a valuable tool to investigate the nuclear architecture, chromatin organization and spatial organization of DNA sequences (TIRICHINE et al. 2009). In the present work, we proposed to apply the FISH technique in papaya interphase nuclei as a new and relatively faster diagnostic method for sex type identification. This method is based on qualitative analysis of fluorescence signals: in this case, presence of the signal representing the hermaphrodite material and absence, the female material.

The low sensitivity of FISH characterized by inconsistent and not reproducible fluorescent signal is not uncommonly found in plants preparations. This is due to the presence of rigid cell walls and cytoplasmic debris and, when chromosomes are the target, a more pronounced condensation of metaphase chromosomes (JIANG et al. 1995) that may negatively affect probe DNA accessibility, and the limited number of cell at the relevant stages (ZHANG & FRIEBE 2009). The protocol developed here for obtaining nuclei suspension from frozen plant leaf tissues was successful,

providing both a satisfactory nuclear morphology and, hence, a very good probe penetration, as verified by the FISH results. An advantage of this protocol that is worth to be pointed out refers to the time-saving procedure, since it does not require all the specific stages for chromosome preparation.

The choice of the NAPF-2 marker to be used as FISH probe was based on its polymorphic banding profile, which would probably result in the desired qualitative analysis. In fact, the FISH images obtained for both cultivars demonstrated the good choice of it as molecular marker and the efficiency of this new FISH-based diagnostic method for sex identification of these specific papaya cultivars. The difference in the fluorescent spots quantity between hermaphrodite nuclei of each cultivar may reflect the genomic differences among them. The FISH technique proved to be sensitive to the differential expression of this marker in both hermaphrodite materials.

## 6. CONCLUSIONS AND FUTURE PROSPECTS

*Carica papaya* species is one of the most economically important fruit crop in world scenario. Thus, its early sex identification becomes of great interest by papaya growers for mainly saving financial resources. For the first time, the sex discriminating potential of SCAR markers was investigated successfully in the Brazilian commercial papaya cultivars, cv. 'Golden' ('Solo' group) and cv. 'Rubi' ('Formosa' group). In principle, they could be applied to molecular analysis by farmers; however this is an expensive technology that becomes unviable in the practice. Then, a new, reliable and efficient FISH-based diagnostic assay for early sexing in papaya was proposed for being an alternative less costly and relatively faster than PCR approach. This assay could be considered feasible in order to be applied in large-scale cultivation.

The molecular biology and cytogenetics approaches described in this study open other opportunities of studies in genetic research in papaya. Cloning and sequencing of the PCR products obtained from those selected markers would provide a better characterization of their sequences, which could be further applied in physical and cytogenetic mapping on the papaya chromosomes. Hence, these genomic data should improve the knowledge about the papaya sex determination system. In addition, BAC clones containing known sex-specific sequences could be employed as probe in fluorescence *in situ* hybridization in suspension (FISHIS) approach for a large-scale screening by flow cytometry of sex types from interest cultivars.

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