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Microsatellite $(GATA)_n$ reveals sex-specific differences in Papaya

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Abstract Papaya, an economically important fruit plant, is polygamous in nature. The sex of dioecious papaya plants can be deduced only after they attain reproductive maturity (6–8 months). Normally, 50% of the population in a field is composed of unfruitful male plants and almost 45% of these have to be uprooted at the flowering stage. This unnecessary cultivation of unwanted males leads to wastage of resources, which can be avoided if the sex of the plant is determined at juvenile stage. Morphological and cytological studies conducted so far have failed to differentiate between the various sex forms of papaya. Its dioecious nature, occasional sex-reversal of male flowers and the absence of a heteromorphic pair of sex chromosomes make papaya an interesting system to study sex determination at the molecular level. In the present study, highly informative microsatellite and minisatellite probes were employed to identify sex-specific differences in papaya. Among these, only the microsatellite probe $(GATA)_4$ demonstrated sex-specific differences in all the cultivars analysed. The diagnostic potential of this microsatellite marker was exploited to sex papaya plants at the seedling stage. This study also indicates that the genetic material of the X and Y chromosomes of papaya is diverging in a sex-specific manner and hence they are in the process of differentiation.

Key words Dioecious plants · *Carica* · Papaya · Seedling sex diagnosis · Microsatellites · An Indian patent pending

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Introduction

Carica papaya is a native of tropical America and a member of the family Caricaceae. Species of the genus *Carica*, other than papaya, are strictly dioecious. However, papaya is polygamous and three main sex types exist, namely pistillate or female, staminate or male, and hermaphrodite or bisexual (Storey 1941). Papaya is widely cultivated for its edible fruits and milky latex. Papain, a proteolytic enzyme and carpain alkaloids obtained from the latex of papaya are of great commercial importance. Papain shows a very broad spectrum of activity and therefore is used in food, textile, dairy, pharmaceutical and perfume industries (Jones and Mercier 1976). Generally, the dioecious papaya cultivars are preferred for extraction of papain all over the world, because the yields and proteolytic activity of the crude papain obtained from the female fruits are greater than that of hermaphrodites (Madrigal et al. 1980).

Conventionally, papaya plants are propagated through seeds. In the case of dioecious cultivars, the seeds are sown in seedbeds and 1–2 month-old seedlings are transplanted to the field. Among the seedlings, 50% of the plants are male and 50% are female. However, the sex of these plants cannot be deduced from the external morphology of embryonic or juvenile forms. Also, extensive cytological and genetic studies on papaya have failed to detect any heteromorphic pair of sex chromosomes (Hofmeyr 1939; Muthukrishnan and Irulappan 1995). Therefore, usually 2–3 seedlings are planted in one pit so as to obtain the desired number of fruit-bearing female trees in the field. The sex of the seedlings is known only after the plants attain reproductive maturity, i.e. after 5–8 months. Most of the male plants (45%) are unnecessary and need to be uprooted from the field after maturity, which leads to wastage of resources. If the sex of the dioecious plants is to be identified at the seedling stage, prior to their transplantation to the field, a desired ratio of male and female plants (5% males: 95% females) can be achieved and resources like planting space, fertilisers and water could be devoted to the cultivation of female

plants. A single female papaya plant normally produces as many as 100 fruits in its life cycle and about 250 g of crude dried papain in a year (Singhal 1996). Thus, an increase in the number of fruit-bearing trees per hectare of land would directly increase the yield of fruit and papain, making papaya cultivation more profitable. Therefore, it is of immense agricultural importance to identify sex of papaya plants at juvenile stage.

Microsatellites or simple sequence repeats are short tandem repeats, dispersed in the genome. They have been shown to be extremely useful as markers for the DNA fingerprinting of eukaryotes, including plants (Epplen et al. 1991; Ramakrishna et al. 1995). Sex-specific differences were detected by using micro- and minisatellites such as $(GATA)_4$ and $(GACA)_4$ in guppy fish (Nanda et al. 1990, 1992) and mice (Schafer et al. 1986a), $(TG)_{10}$ in cattle (Kashi et al. 1990), a human minisatellite probe pV47 in brown skua (Millar et al. 1992), and Jeffrey's 33.15 probe in striped backed wrens (Rabenold et al. 1991). Thus, the sex-specific distribution of these repeats in evolutionarily diverse organisms is of particular interest; however, no such data are available in plants.

In the present study, we report the use of microsatellite repeats for the identification of sex-specific DNA markers in papaya and further demonstrate the diagnostic application of this marker for the sex identification of papaya seedlings (2-months old). A possible role of microsatellite repeats in the evolution of the sex chromosomes of papaya is also discussed.

Materials and methods

Seeds of commonly grown and commercially important papaya cultivars [Dioecious – Coimbatore (CO)-1,CO-2, CO-4, CO-5, CO-6, MF-1, Pant-1, Washington, Pusa-giant, Pusa-dwarf; gynodioecious – Disco and Sunrise, and a wild species *C. cauliflora*] were obtained from breeding stations in India and grown at the regional fruit research station (MPKV), Pune, India. The varieties Disco and Sunrise are characterised as hermaphroditic in nature, whereas the variety Washington is grown all over the world for its good-quality fruits. The male plants of the dioecious cultivars showed occasional sex reversal in response to extreme climatic conditions.

Mature plants were identified in the field and young leaves were harvested at the flowering stage for DNA isolation using the CTAB method (Rogers and Bendich 1988). In order to obtain a suitable enzyme-probe combination, the DNA samples were digested with various restriction enzymes such as *Alu*I, *Hae*III, *Hin*fI, *Taq*I, *Eco*RI, *Eco*RV, *Msp*I, *Mbo*I, *Sau*3AI and *Dra*I. Subsequent gel electrophoresis, gel drying, Southern blotting, and hybridizations with microsatellite [$(TG)_{10}$, $(CAC)_5$, $(GAA)_6$, $(GGAT)_4$, $(GATA)_4$ and $(GACA)_4$] and minisatellite (pV47 and M13) probes were carried out as described by Ramakrishna et al. (1995). RNA isolations were performed according to the protocol of Jones et al. (1981). The RNA gel-electrophoresis and hybridisations were done as reported by Schafer et al. (1986a).

Two-month-old seedlings of the dioecious papaya cultivars CO1, CO2, CO4, CO5, CO6, Washington, Pusa-giant, MF1, Pant1 and Ranchi, and the wild species *C. cauliflora*, were numbered 1–50 in the field. A single leaf of each seedling was harvested, frozen in liquid nitrogen and used for DNA isolation. DNA samples were digested with the restriction enzyme *HinfI* and further hybridisation with probe $(GATA)_4$ was performed as described earlier.

Results and discussion

The microsatellites $(GATA)_4$ and $(GAA)_6$ reveal male specific hybridisation in papaya

Microsatellites and minisatellites were explored for their potential to serve as sex-specific markers in papaya. Of the various probes employed, the microsatellite repeats $(GATA)_4$ and $(GAA)_6$ detected sex-specific differences in *Hin*fI or *Hae*III digests while microsatellite repeats $(TG)_{10}$, $(CAC)_5$, $(GGAT)_4$ and $(GACA)_4$ and the minisatellites PV47 and M13 failed to detect any sex-specific patterns.

Figure 1 shows hybridisation of the microsatellite $(GATA)_4$ to *Hin*fI-digested DNA isolated from different

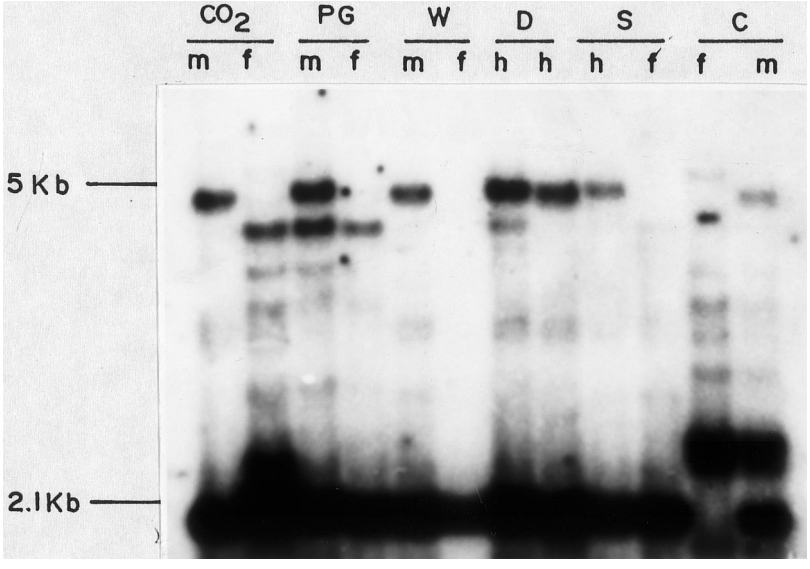

Fig. 1 Southern hybridization of *Hin*fI digested papaya DNA to radioactively end-labelled oligonucleotide $(GATA)_4$, demonstrating the presence of a sex-specific band at 5 kb in the males (indicated by an *arrow*). *m* – male; *f* – female; *h* – hermaphrodite. *CO2* – Coimbtore 2; *PG* – Pusa-Giant; *W* – Washington; *D* – Disco; *S* – Sunrise, *C* – *C. cauliflora*. *CO2, PG, and W* represent commonly grown dioecious clutivars. *D and S* are gynodioecious varieties having only hermaphrodite and female individuals, and *C. cauliflora* is a wild species of *Carica*

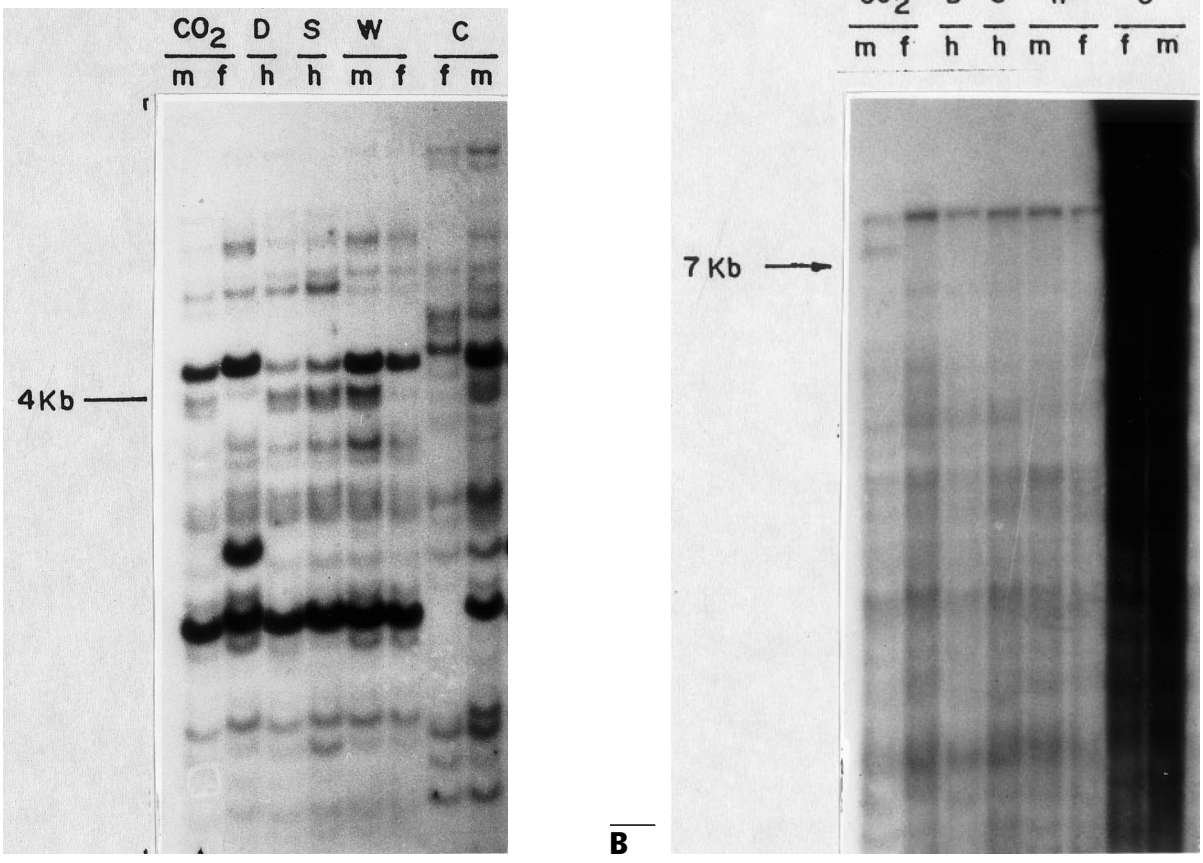

Fig. 2A Southern hybridisation of *Hae*III-digested papaya DNA to $(GATA)_4$, showing the presence of a sex-specific band at 4 kb in the male individuals (indicated by an *arrow*). *m* – male; *f* – female; *h* – hermaphrodite. *CO2* – Coimbtore 2; *PG* – Pusa-Giant; *W* – Washington; *D* – Disco; *S* – Sunrise, *C* – *C. cauliflora*. **B** Southern hybridisation of *Hae*III-digested papaya DNA to $(GAA)_6$, demonstrating the presence of a sex specific band at 7 kb in the male individuals of variety CO2 (indicated by an *arrow*). Also these repeats are present in higher copy number in the wild species *C. cauliflora* (*lanes 7 and 8*). The same gel previously hybridised with $(GATA)_4$ (Fig. 2A) was de-probed and hybridised with $(GAA)_6$. *Lanes 1–8* are as described in Fig. 2A

papaya sex forms. As is evident from this representative figure, the probe hybridised to a few prominent restriction fragments in both high- as well as low-molecular-weight regions in each lane. Interestingly, a sex-specific band of 5 kb is seen in males of three dioecious cultivars, namely CO-2, Pusa-giant and Washington (lanes 1, 3 and 5 respectively), and a dioecious wild *C. cauliflora* (lane 12) which is absent in the corresponding female DNAs (lanes 2, 4, 6 and 11). A similar sex-specific band of 5 kb is observed in hermaphrodite individuals of the gynodioecious varieties, Disco and Sunrise (lanes 7, 8 and 9), while the 5-kb band is not present in a female sample of Sunrise (lane 10). Apart from this sex specific band, highly intense low-molecular-weight bands irrespective of the sex are also observed in Fig. 1 indicating an accumulation of $(GATA)_n$ repeats in the papaya genome.

The hybridisation profile of the probe $(GATA)_4$ to the *Hin*fI-digested DNA was found to be reproducible in the different dioecious varieties of papaya analysed. The non-appearance of individual specific variations of the $(GATA)_n$- specific male band was further confirmed by analysing five males and females of a single variety.

Figure 2A shows hybridisation of the *Hae*III-digested papaya DNA probed with $(GATA)_4$. As compared to the hybridisation profile obtained with the *Hin*fI enzyme (Fig. 1), the probe detects multiple bands in *Hae*III digests. Among these a band of 4 kb is observed to be male-specific in the dioecious cultivars CO2 (lane 1), Washington (lane 5) and wild papaya (lane 8). The corresponding females (lanes 2, 6 and 7) show an absence of this band. The sex-specific band of 4 kb is also evident in hermaphrodites of the gynodioecious varieties Disco and Sunrise (lanes 3 and 4).

The microsatellite $(GAA)_6$ revealed a male-specific difference only in Coimbtore cultivars (CO2 and CO6). However, no sex-specific polymorphism was observed in other cultivars and wild species studied (Fig. 2B). Further, an unique observation was made when $(GAA)_6$ was hybridised to a wild species *C. cauliflora* (lanes 7 and 8, Fig. 2B), where $(GAA)_6$ repeats were present in unusually high copy number as compared with the cultivars. All other microsatellites investigated did not show such significant differences in copy number. Although recent reports in humans indicate $(GAA)_n$ repeat amplification in

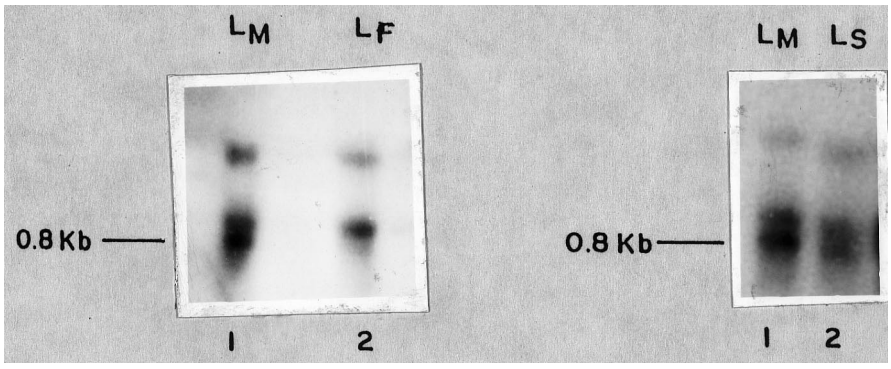

Fig. 3A, B Northern hybridisation of papaya total RNA with $(GATA)_4$. $(GATA)_n$-complementary transcripts of male leaf RNA compared to RNA of the female leaf. **A** L_M – male leaf RNA; L_F – female leaf RNA (CO2). An *arrow* indicates the male-specific transcript. **B** L_M – male leaf RNA; L_S – sex-reversed male leaf RNA (var. Washington)

a neurogenetic disorder (Campuzano et al. 1996), it is not understood why some microsatellites, such as $(GAA)_6$, preferably increased their copy number in wild species of papaya.

The sex-specific molecular markers identified in this report, support the genetic studies on the inheritance of sex forms carried out independently by Hofmeyr (1939) and Storey (1941), which conclude that in papaya, male and hermaphrodite plants are heterogamous (XY) while the female is homogamous (XX). Therefore, if genetic factors for sex are present, they should be active in males or hermaphrodites but not in females.

Northern hybridisation using a microsatellite probe $(GATA)_4$

Differential gene expression plays a major role in sex determination. Transcription of microsatellites in genes has been reported in humans and many animal species (Epplen et al. 1991). Simple $(GATA)_n$ sequences were found to be transcribed in mice, snakes and *Drosophila* (Singh et al. 1984; Schafer et al. 1986b). In case of plants, di-, tri- and tetra-nucleotide simple sequence repeats form a part of some specific genes (Gupta et al. 1996). Although their biological significance is not understood, the transcribed microsatellites have been suggested to play a role in gene regulation (Gupta et al. 1994, 1996; Gortner et al. 1996).

To investigate the presence of transcripts homologous to $(GATA)_n$ in papaya, total RNA isolated from young leaf tissue of male, female and sex-reversed male plants was hybridised with $(GATA)_4$. As seen in Fig. 3 A, two major discrete species of $(GATA)_n$ complementary transcripts are observed in male and female papaya leaf tissue. In addition, a sex-specific transcript is found in male leaf RNA at about 0.8 kb. Figure 3B presents the hybridisation of the $(GATA)_4$ microsatellite to total RNA of a normal and sex-reversed male plant of the variety Washington. A comparison of hybridisation profile reveals the presence of a male-specific transcript of 0.8 kb in both male plants. Thus, no differences in the transcripts of male and sex-reversed individuals could be detected when total RNA of leaf was probed with $(GATA)_4$. The conservation of $(GATA)_n$ repeats and their transcription in a sex-specific manner in papaya raises several intriguing questions as to whether they have accumulated on the putative Y chromosome or have a direct role in sex determination.

Diagnostic application of the microsatellite marker for sex identification of seedling papaya plants

Although, the probe $(GATA)_4$ detected sex-specific differences in the *Hae*III digests, the male specific band of 5 kb observed in the *Hin*fI digest was preferred to serve as a diagnostic marker for early sex identification of papaya plants for two reasons. First, the hybridisation of the microsatellite $(GATA)_4$ to the *Hin*fI digested papaya DNA provided a clearer profile as compared to *Hae*III digests. Secondly, unequivocal presence of the sex-specific band of 5 kb in all the male individuals examined suggested that the marker was well conserved in the population and hence would be reliable.

To establish the feasibility of the sex specific marker for early sex diagnosis, a field trial was performed on 2-month-old papaya seedlings prior to their transplantation to the field. Typically, the males were distinguished by the appearance of a distinct sex-specific marker of 5 kb, whereas female samples were marked by the absence of the sex specific band. Yet, in addition to the sex-specific marker, the multilocus probe $(GATA)_4$ also detected other non-sex-specific loci in both sexes. Particularly, a signal at 2.1 kb, common to both male, and hermaphrodite female plants, served as a standard for determining the accuracy of the procedure. The putative genetic sex of every seedling plant, determined by the molecular method, was compared with respective phenotypic sex observed in the field after flowering. The accuracy of the sex diagnostic procedure using a molecular marker was found to be 100%.

The sex-specific microsatellite marker identified in our study will be of great significance to papaya growers, seed companies, the papain industry and to papaya breeders to procure female plants at the seedling stage. In principle, this approach for early sex identification of seedlings can be applied to all other dioecious plant species, and more particularly to the commercially significant dioecious plants which take several years to attain

reproductive maturity, such as date palm, jojoba and nutmeg.

Male-specific distribution of the $(GATA)_n$ microsatellite may provide molecular evidence for a putative Y chromosome in papaya

The sex-specific organisation of simple repeats has been originally detected in female snakes as a part of the satellite DNA (Singh et al. 1981). The Bkm probe harbouring $(GATA)_n$ repeats is quantitatively associated with the specialised sex-determining W chromosome in snakes and has been shown to accumulate on the Y chromosome in mammals, the W chromosome in birds and also on the proximal region of the X chromosome of *Drosophila melanogaster* (Epplen et al. 1982; Singh et al. 1984; Chandra 1985). Comparative studies of different reptiles indicate that $(GATA)_n/(GACA)_n$ sequences play an important role in sex-chromosome evolution (Singh et al. 1981; Nanda et al. 1992). Moreover, simple repeats have allowed the detection of molecular differences between apparently homomorphic sex chromosomes, demonstrating the occurrence of a Y chromosome in guppy fish (Nanda et al. 1992) and the fly *Megaselia* (Willhoeft and Traut 1990). Hence, the differences that are observed in male and female papaya plants by using simple repeat probes such as $(GATA)_4$ and $(GAA)_6$ in the present study are highly significant. Our results may provide preliminary molecular evidence for the presence of a putative Y chromosome in papaya and offer opportunities to study the most interesting early stages of sex-chromosome evolution in plants.

The genetic material of the X and Y chromosomes of papaya is possibly diverging in a sex-specific manner

Theories for evolution predict that sex chromosomes have evolved from a homologous pair of autosomes, and during this process one of the homologues has lost most of its functional genes except for those which are crucial for sex determination (Charlesworth 1991; Singh et al. 1984). The first step in evolution may involve the restriction of recombination between genes controlling male and female sex function. Thus, mutational changes in Y-chromosomal genes cannot be corrected by recombination with sequences from the X chromosome. Therefore, differentiation of the Y chromosome can only continue and cannot be reversed (Muller 1964; Charlesworth 1991). The genetic analysis of sex determination without obvious structural differences between X and Y chromosomes indicates a recent origin of such systems. Advanced sex chromosomes are morphologically differentiated and their evolution is often associated with the accumulation of repetitive DNA sequences. Recently, an X-linked gene with a degenerate Y-linked homologue has been reported in *Melandrium album* (Gutmann and Charlesworth 1998). This X-linked gene encodes a male-specific protein but the Y-linked homologue has degenerated due to nucleotide deletion and the accumulation of repetitive sequences (Gutmann and Charlesworth 1998). Although the mode of accumulation of simple repetitive sequences on sex chromosomes is rather unclear, it is likely that these repeats are preserved on the Y chromosome as a result of crossover suppression.

Dioecious plant species represent progressive stages in the evolution of sex chromosomes. The sex chromosomes of *Asparagus officinalis* are thought to be recently evolved because the male and the female sex chromosomes are simple, almost homologous, and also carry the same set of essential genes (Galli et al. 1993; Grant et al. 1994). The sex chromosomes of *M. album* are morphologically diverged and represent a more advanced stage than the *Asparagus* chromosomes. The Y chromosomes of mammals are even more divergent from their own X chromosomes and autosomes because they are heterochromatic in nature and also carry sex-specific repeated satellite DNA sequences (Eicher et al. 1989; Parker 1990). The Y chromosome of papaya is morphologically homologous to the X chromosome and autosomes, and hence belongs to the class where sex chromosomes are primitive and in their initial stages of differentiation. However, molecular differences observed in the male and female papaya plants by using multilocus repeat probes, such as $(GATA)_4$ and $(GAA)_6$ in the present study, suggest that the genetic material of X and Y chromosomes of papaya is diverging in a sex-specific manner.

In summary, we have shown for the first time that the microsatellite $(GATA)_n$ detects sex-specific differences in diverse cultivars and a wild species of papaya. We thus, provide molecular evidence for the presence of a putative Y-chromosome in papaya based on the sex-specific distribution of the $(GATA)_4$ microsatellite. Further, we have also demonstrated the feasibility of our approach to identify the sex of commercially important papaya plants well before flowering. The sex-specific transcription of the $(GATA)_n$ microsatellite in papaya points to the need for an in-depth study to investigate its functional role and significance in sex determination. Whether selective evolutionary forces are acting on the sex-specifically conserved $(GATA)_n$ repeats can be explained only after studying their organisation in other dioecious plants.

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