Marker assisted detection of seed sex ratio in palmyrah palm (*Borassus flabellifer* L.)

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The palmyrah palms are slow-growing dioecious perennials and have no distinguishing features to identify the sex until flowering. The palm commences flowering only after 12–15 years of maturity. The seed sex ratio (primary sex ratio) was estimated by germinating seeds and growing seedlings under favourable conditions with minimal mortality until flowering. Early sex determination was done to assess the seed sex ratio of one-, two- and three-seeded fruits with a male-specific random amplified polymorphic DNA marker. The seed sex ratio (M : F) of seedlings raised from one-, two- and three-seeded fruits were 57 : 43, 35 : 65 and 61 : 39 respectively. There was no correlation between the number of seeds in a fruit and sex of the seedlings raised from them. The overall percentage of M : F was 52 : 48, which was almost 1 : 1 ratio. While comparing the sex ratio of one-, two- and three-seeded fruits individually, the sex-ratio of two-seeded fruits was 0.350 with probability 0.263. This indicated a female-biased sex ratio (M : F) in two-seeded fruits (35 : 65).

Keywords: Apocolon, pyrenes, random amplified polymorphic DNA, secondary sex ratio, seed sex ratio.

PALMYRAH is a dioecious palm and is considered to be a native of tropical Africa. It is distributed in Africa, India, Burma and Sri Lanka. The palm belongs to the family Arecaceae, subfamily Borassoideae and genus *Borassus*. The palmyrah palms are slow-growing dioecious perennials and have no distinguishing features to identify the sex until flowering. The palm commences flowering only after 12–15 years of maturity. Among the four sugar-yielding palms, viz. *Borassus flabellifer* (palmyrah), *Phoenix dactylifera* (date), *Caryota urens* (sago) and *Cocos nucifera* (coconut); palmyrah palm ranks first in yielding sugar[1].

The palm can be propagated only through seeds and there is no vegetative method of propagation. In palmyrah, the germination is tubular, the embryo is straight and the cotyledon extends so that the plumular portion of the seedling is carried away from the seed through the elongation of the proximal portion of the cotyledon, forming a structure widely termed as 'cotyledonary tube' or 'apocolon'[1,2]. The expansion of the distal portion of the cotyledon inside the seed functions as a haustorium[3]. The palm seed started to germinate within 45–60 days and the eophyll (first leaf) emerged out after a period of 150 days of seed sowing.

A male-specific random amplified polymorphic DNA (RAPD) marker was identified in palmyrah to determine the sex at the seedling stage itself[4]. This marker is a useful tool for the estimation of seed sex ratio or else it would be difficult in a palm with a long juvenile phase.

The seed sex ratio (primary sex ratio) was estimated by germinating seeds and growing seedlings under favourable conditions with minimal mortality[5]. Many theoretical studies have suggested that, under the control of genes, the seed sex ratio of dioecious species in a well-mixed population can be expected to be 1 : 1 ratio[6]. Paulas and Padmanabhan[7] reported that in palmyrah fruits, single-seeded fruits gave rise to female palms, one seed each from the two-seeded fruit produced one female and male palm each and the smallest seed from the three-seeded fruit produced one female and two males. Our communication has validated this hypothesis with a male-specific RAPD marker. Hence, the objective of the study was to examine the seed sex ratio of one-, two- and three-seeded fruits in palmyrah palms.

Mature fruits were harvested from 10 palmyrah palms at the Central Plantation Crops Research Institute (CPCRI) campus, Kasaragod during April 2006. The numbers of one-, two- and three-seeded fruits were recorded. A total of 100 seeds from 50 fruits were sown in three groups, viz. group 1: 15 fruits from one-seeded set, group 2: 20 fruits from two-seeded set and group 3: 15 fruits from three-seeded set in the polybags measuring 65 cm × 40 cm and labelled for checking the seed sex ratio. The observations were recorded at monthly intervals till the germination was completed (i.e. after the first bladed leaf appeared). The genomic DNA was extracted from immature leaf samples of seedlings raised from one-, two- and three-seeded fruits separately using sodium dodecyl sulphate (SDS) method and polymerase chain reaction (PCR) amplification was performed with male-specific RAPD marker OPA-06_{600} reported by Jiji *et al.*[4].

In palmyrah palms, four types of fruits were observed (one-, two-, three- and four-seeded fruits). A total of 187 nuts were harvested out of which, it was observed that 18.2% were one-seeded, 38% two-seeded, 43.3% three-seeded and 0.05% four-seeded fruits (Figure 1 *a*). In some three-seeded fruits, two seeds were separated by a thin carpel (Figure 1 *b*).

Palmyrah fruit is a globose drupe, dark purple to black, mesocarp thick, fibrous, yellowish and the endocarp usually comprising three hard pyrenes (Figure 1 *c*). The apocolon emerged through the micropylar region of the seed. The anterior segment of the apocolon showed a smaller diameter (about 2 cm), whereas the posterior portion had a larger diameter (about 13 cm). When growth continued the tuberous part set forth roots. The apocolon reached a length of about 33 cm in 100 days after being sown in

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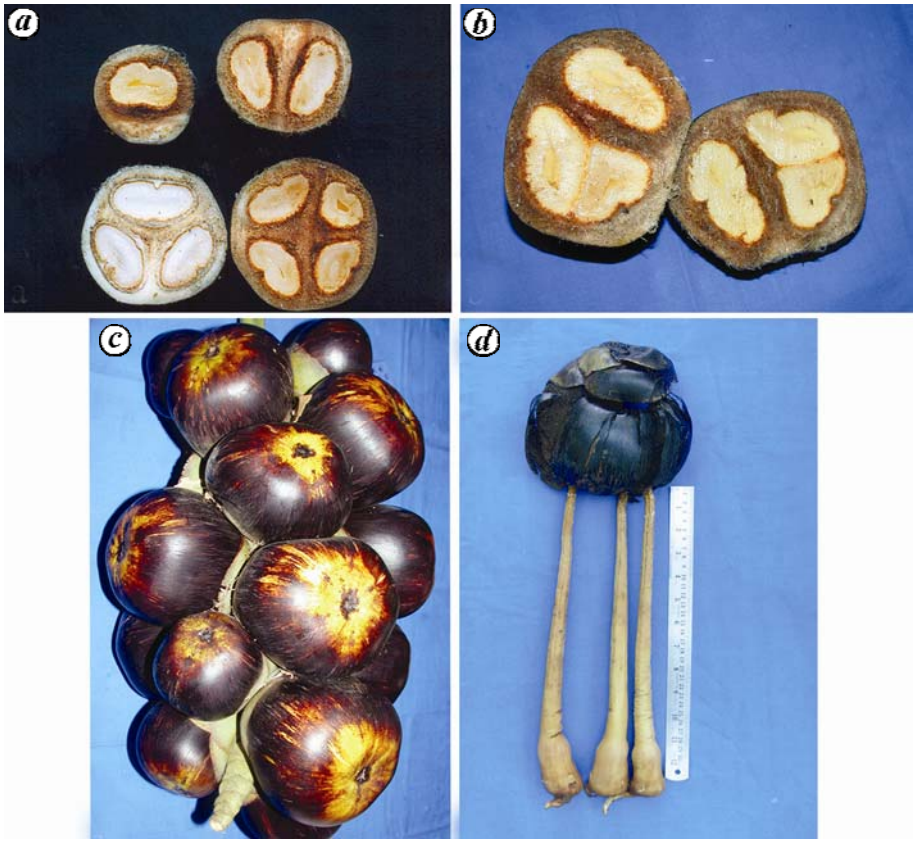

Figure 1. Different types of palmyrah fruits showing carpel distribution and seed germination. ***a***, Cross-section of one-, two-, three- and four-seeded fruits; ***b***, Cross-section of three-seeded fruit; two seeds were separated by thin carpel; ***c***, A bunch of palmyrah fruits; ***d***, Elongation of apocolons from three-seeded fruit during tubular germination.

polybags (Figure 1 *d*). The seedlings emerged from polybags after a period of about 150–170 days.

All seeds from group 1 were taken into consideration only if they germinated. From group 2, those seedlings in which both the seeds of a respective fruit germinated were considered. Similarly from group 3, those seedlings in which all the three seeds of a respective fruit germinated were considered and finally this reduced the sample size to 63 seedlings from 100 seeds (Table 1).

PCR analysis was done for sex determination of seedlings raised from one-, two- and three-seeded fruits with a male-sex specific RAPD marker $OPA\text{-}06_{600}$ (5′GGTCCCTGAC3′)[4]. The RAPD banding profile is shown in Figure 2. The seed sex ratio (M : F) of seedlings raised from one-, two- and three-seeded fruits was 57 : 43, 35 : 65 and 61 : 39 respectively. Overall percentage of male : female was 52 and 48 respectively (Figure 3) which was almost in 1 : 1 ratio. Morphological characters such as length and breadth of the seeds from one-, two- and three-seeded fruits were recorded which was then compared with the sex of the seedlings raised from them. The results showed that there was no relationship between size of the seeds and sex of the seedlings raised from them (data is not included).

The sex ratio of one-seeded fruits showed that the chance of occurrence of male : female was almost equal and there was no stringent bias towards either sex. The sex ratio of two-seeded fruits showed a female bias (0.350) whereas in three-seeded fruits, a male bias (0.611) was shown. The values of binomial test and chi square test in palmyrah seeds of one-, two- and three-seeded fruits were $p = 1.00$ and $\chi^2 = 0.143$, $p = 0.263$ and $\chi^2 = 1.80$ and $p = 0.243$ and $\chi^2 = 1.77$ respectively. While comparing the overall male and female seeds in palmyrah fruits, the values obtained in binomial test were $p = 0.801$ and $\chi^2 = 0.143$ (Table 1).

Kovoor[8] reported that an abnormal number of seeds (one, two or four) is more often seen in *Borassus aethiopum* whereas trilocular fruit is frequent in *B. flabellifer*. In this study, four-seeded fruits were also rarely observed in *B. flabellifer*.

The result of sex determination of seedlings raised from one-, two- and three-seeded fruits showed that there was no stringent relationship between sex and number of seeds in a fruit. This is contradictory to the report of Paulas and Padmanabhan[7] that single-seeded fruit gives rise to female palm, one seed each from the two-seeded fruit produces female and male sex each and the smallest seed

Table 1. Number of male and female seedlings of *Borassus flabellifer* raised from one-, two- and three-seeded fruits

Type of fruits	Male	Female	*p*	Sex ratio	Percentage of male/female	χ^2
One-seeded	4	3	1	0.571	57 : 43	0.143
Two-seeded	7	13	0.263	0.350	35 : 65	1.80
Three-seeded	22	14	0.243	0.611	61 : 39	1.77
Total	33	30	0.801	0.476	52 : 42	0.143

The *p* value denotes the two-sided significance compared to a fraction 0.5 males in a binomial test; seed sex ratio and percentage of male : female seedlings raised from each type of fruit.

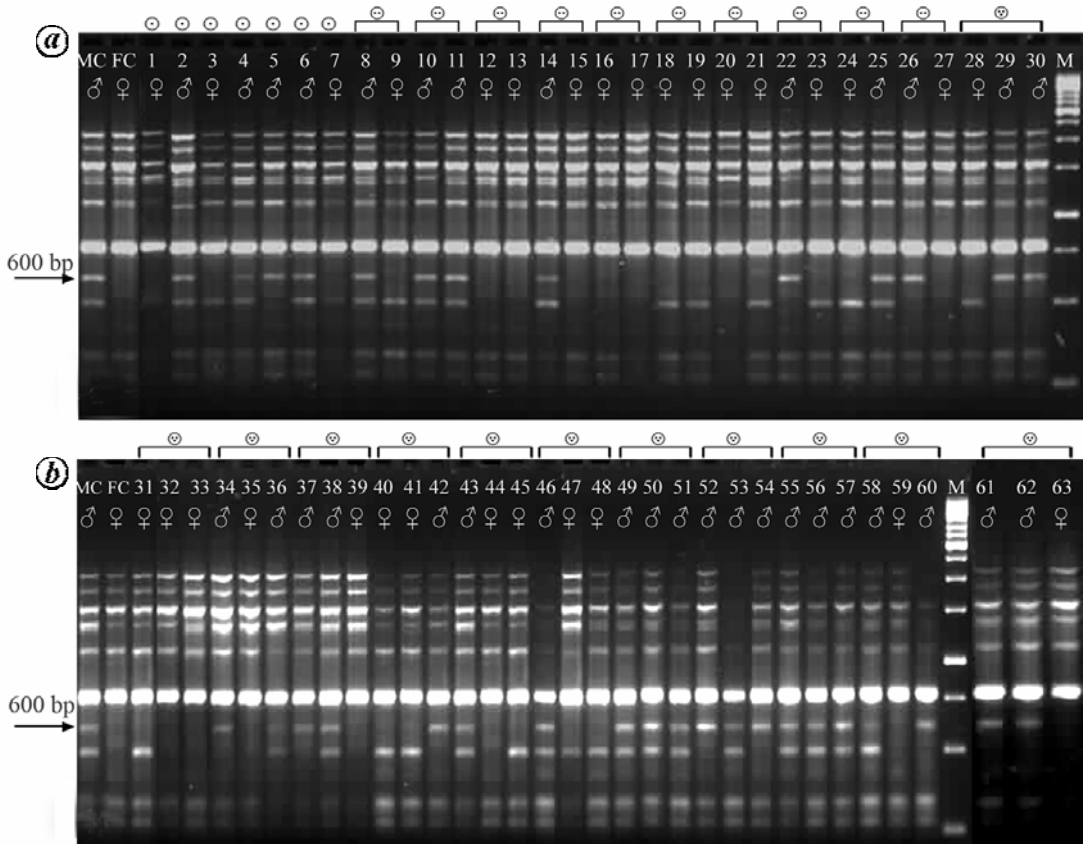

Figure 2 *a, b*. RAPD banding profile of palmyrah seedlings of one-, two- and three-seeded fruits using primer OPA 06. The lanes marked ♂ and ♀ represent DNA amplified from individual male and female plants. The symbols ⊙, ⊙ and ⊙ represent seedlings of one-, two- and three-seeded fruits, respectively. The 600 bp male-specific band is indicated with an arrow, MC & FC were male and female control, M: 1 kb ladder.

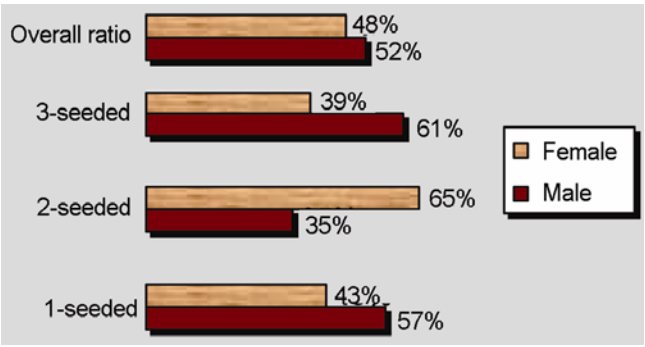

Figure 3. Seed sex ratio of one- two- and three-seeded fruits of palmyrah.

from the three-seeded fruit produces female and the two are males.

The overall seed sex ratio (M : F) obtained from seedlings of one-, two- and three-seeded fruits was 52 : 48, which was almost equal to 1 : 1. Tkatchenko[9] conducted a survey in Kampuchea in 1938 for calculating secondary sex ratio, out of 1,531,100 palms, the male : female ratio was 52.5 : 47.5. This indicated that there was no significant variation in secondary sex ratio and seed sex ratio in palmyrah palms.

Under the control of nuclear genes, the seed sex ratio of a sexually dimorphic species in a well-mixed population can be expected to be close to 1 : 1 in most cases[6].

According to classical genetic concepts, a cross between a heterogametic male and a homogametic female, which is almost a universal occurrence in dioecious species, would segregate in a 1 : 1 ratio. The number of homogametic ovules in a flower cannot therefore conceivably affect the sex of the progeny which is solely dependent on segregation of the Y chromosome or sex gene during the meiosis which gives rise to the particular pollen grain that successfully competes in fertilization.

However, wide deviations from the expected sex ratio have been recorded in a number of angiosperm species. The selective elimination of the minority sex (usually the male) could occur at various stages: meiosis, pollen-tube growth, zygote, embryo or seedling. The particular stage and causes of the distortion are unknown in most species, except for a few where Y pollen have been observed to grow slower than tubes of X pollen. Dense pollination and the consequent pollen-tube competition would thus favour a preponderance of females in the progeny[8].

When comparing the sex ratio of one-, two- and three-seeded fruits individually, the sex ratio of two-seeded fruits was 0.350 with probability 0.263. This result clearly indicated a female-biased sex ratio (M : F) in two-seeded fruits (35 : 65). Female palms are superior in producing good quality and quantity of neera and other valuable products[10]. But there was no easy method to determine the sex of the palmyrah palm before flowering and it takes 12–15 years of maturity. A male-specific RAPD marker was identified in palmyrah by Jiji *et al.*[4] but this was expensive from the farmer's point of view. In addition, due to its tubular germination, seeds are sown directly in the field to avoid difficulty in transplantation. Ross *et al.*[11] reported on the seed variation and seedling establishment in *Manicaria saccifera*, that double-seeded fruits have achieved a balance of better optimum viability than one- or three-seeded fruits. Seeds from fully ripened palmyrah fruits can be separated with bare hands and there is no need of any equipment. Thus, it is better to select two-seeded fruits as planting material. However, the results presented here are based on only limited sample size. Therefore, it is only indicative particularly with reference to number of seeds per fruit and sex ratio. For generic applications, the result should be validated in a large number of samples.

In dioecious species, the resource investment in reproductive effort is usually greater for females than for males[12,13]. This reproductive investment can affect growth rate, making it lower in the females than in the males. In extreme cases, the greater reproductive effort can lead to a higher mortality rate in females resulting in populations with male-biased sex ratios[14]. Antolin[15] reported that seed sex ratio is assumed to be under maternal control and a single nuclear gene in the mother with two alleles controls the sex ratio of the seeds.

Using molecular markers, Eppley[16] found a male bias in a seagrass, even before plants had flowered. Studies on the seed sex ratio of long-lived perennial plants are incomplete, probably due to the time and effort that needs to be put in for growing plants until maturity with minimal mortality. Sex-linked molecular marker is a useful tool to learn the seed sex ratio of such plants in juvenile stages.

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