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Exploration of various markers in segregation of male and female in palmyra palm (*Borassus flabellifer* L.)

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Abstract
Borassus flabellifer L. popularly called karpaga viruksham i.e,. "Multipurpose tree" in Tamil culture is an economically significant plant with its vast range of application in food and medicine. Especially, the quality of food produces from female palmyra attracts high economical value which encourages the farmers to grow trees with more female to male sex ratio. But Palmyra has a long juvenile period of 12-15 years and is difficult to segregate them before inflorescence. In the recent past, very few attempts had been made in the arena of molecular markers to distinguish the sex of palmyra and most of the work states that markers couldn't be relied strongly or it fails to segregate them. Yet OPA-06 marker was found to be reliable sex discriminating marker. Thus this study focus on examining the reliability of four different markers including OPA-06, M1 & M2, F1 & F2, F3 & F4 in sex discrimination of palmyra from three different locations (Coimbatore, Ramnad, Viluppuram) of Tamil Nadu. Thus the outcome of the evaluation of the above mentioned molecular markers used as tool for the identification of sex in palmyra palm is presented.

Keywords: Asian palmyra palm, sex-linked markers, sex determination, dioecious

Introduction
The Asian palmyra palm (*Borassus flabellifer*) is a perennial dioecious plant belonging to the Arecaceae family, with many commercially important palm species, more specifically Oil palms (*Elaeis oleifera*) and Date palm (*Phoenix dactylifera*). The origin of Asian palmyra palm is claimed to be tropical Africa, mainly distributed in Southeast Asia through India, Sri Lanka, Burma, to New Guinea. In India, palmyra palm is adorning to the dry areas of the semiarid regions in Tamil Nadu, Andhra Pradesh, Orissa, West Bengal, Bihar, Karnataka, and Madhya Pradesh. Amongst, India has the highest count (85.9 million) of palmyra and more particularly Tamil Nadu holds the major share of 51.9 million palmyra trees.

Asian palmyra palm (*Borassus flabellifer* L, 2n= 36) is a slow growing perennial dioecious tree that can be propagated only through seeds. They start to germinate after 45- 60 days of sowing. The first leaf emerges on 150th day and produces first inflorescence after 10-15 years and attains maturity. One's matured, they continued to produces fruits and flowers throughout its lifespan. It is declared as Tamil Nadu state tree because of its multipurpose use. Because, the tubers, fruits, inflorescence, leaves and trunk wood of palm, have advantages in the preparation of value-added products (palm candy, preserved nungu, palm fruit jam and palm chocolate, palmyra tender ice cream,) toddy, sugars, fibre, fuel and construction material respectively. Further, the tap root system, stores huge amount of water increasing the Ground water level in a particular locality. Palm gur or karupatti is the best alternative for white sugar as a sweetener, since the white sugar manufacturer's use sulphur dioxide to bleach cane sugar, which in-turn become the source of acid rain, haze, and drought. Meanwhile karupaati was prepared only by boiling, solidifying the unfermented sap of palmyra. Indeed, it also has less glycaemic index than cane sugar that propitiate the precedents health benefits in preventing diabetes and obesity. Consequently, the nutritional richness of karupatti (vitamin B1, B2, B3 and C) and fermented sap (Vitamin A, Calcium, Iron, Zinc, and Copper) provides consistent and long-lasting energy and increases quantities of enzymes cofactor including Thiamine, Riboflavin, and Niacin.

The fruit bearing capacity and quality of value-added products including sugar and toddy will be comparatively elevated in female tree than male. Though, it's been considered as cash crop, farmers prefer optimal male and female ratio to have better crop production. But its long maturation period and inability to distinguish male and female during their initial growth stage diminishes its area of production and productivity. Thus, the discrimination of female tree from male tree at the early stage becomes significant. Till date very few attempts were made to discriminate the sex of palmyra palm. Rather sex discrimination, most of the work attempt to identify the sex of palmyra palm at its early stage which majorly comprises of seed sex ratio analysis using marker assisted identification. George *et al.* (2007) [9] screened almost 180 RAPD markers in discriminating the sex of male and female but, only three markers (OPBE-12, OPBA-13 and OPA-6) showed polymorphism. OPBE – 13 produces an amplicon of 500 bp and is present only in female palms whereas OPBA -12 and OPA – 6 produces amplicons of 1100 bp and 600 bp only in male plants. They also stated that OPA-6 was found to be tightly linked to male sex locus rather than OPBE-12 and OPBA-13. Further, Seed to sex ratio was analysed in one, two and three seeded fruits through OPA-6 marker and germination test. The seed sex ratio of M:F of one seeded, two and three seeded palmyra palms were 57:43; 35:65; and 52:48 respectively which is almost 1:1. Thus, there was no direct and proper correlation between number of seeds and the sex of palmyra palm (George *et al.*, 2007) [9]. The developed 130 ISSR markers and analysed 20 different accessions of palmyra for their polymorphic character and only 65 markers showed polymorphs with 47.94%. Yet, none of the 130 ISSR markers were able to discriminate the sex of palmyra palm. Direct cloning of subtraction subtractive hybridization of cDNA, DNA finger printing and transcriptome sequencing were attempted to develop sex-linked markers for Asian palmyra. But none of these techniques were able to obtain a single sex-linked marker in Asian palmyra which states the very small sex determining region of palmyra palm and the complexity in discrimination of sex.

Thus, this study attempt to use four different sex-linked markers in sex discrimination of male and female palmyra palm from three different locations such as north, south and Southwestern region of Tamil Nadu.

Materials and Methods

Plant materials

For isolating DNA, young leaves are collected from the matured palmyra plants of known sexes from three regions of Tamil Nadu.

Nucleic acid isolation

Total genomic DNA was extracted by using CTAB method, In which 1g of known mature leaf sample is was grinded into a fine powder using liquid nitrogen. Then transfer into micro centrifuge tube and add 500 µl of CTAB extraction buffer. This mixture is subjected to incubation for 1h @ 60 °C in water bath and transfer the aqueous phase. For phase separation add 750 µl of 24:1 chloroform: isoamyl alcohol mixture. The supernatant was transferred into a new tube and on addition of 500 µl of ice-cold isopropanol, DNA is precipitated, then keep the precipitated DNA in overnight incubation @-20 °C, thus the DNA containing tube is centrifuged and the pellets are washed with 70% ethanol and air dried, then dilute the DNA with 50 µl of ultrapure water and store at -20 °C. To assess the purity of the DNA, the extracted genomic DNA was placed onto a 0.8% agarose gel and stained with ethidium bromide then the gel was run at 101V for 40 minutes. Bands were observed and recorded in the gel documentation system (BIO-RAD documentation unit)

PCR Amplification

PCR was performed in Eppendorf master cycler with PCR mixture containing 10 µl of master mix, 1 µl each of forward and reverse primer, 1 µl of DNA and add 7 µl of ultrapure water to make up the volume 20 µl. For discriminating the sex of palmyra, we studied various set of markers, which follows its unique PCR amplification condition such as OPA-06 marker as mentioned in (*George et al.*, 2007) [9] which is tightly linked to the male sex locus, which has initial denaturation at 94 °C for 5 min along with 40 cycles of denaturation at 94 °C for 1 min, annealing at 42 °C for 1 min and extension at 72 °C for 1min and final extension with one cycle at 72 °C for 7 min. Another set of male markers M1 & M2 reported in (Pipatchartlearnwong *et al.*, 2019) [10] which follows PCR amplification condition as initial denaturation at 94 °C for 5 min, denaturation at 94 °C, annealing 60 °C for 30 sec followed by 35 cycles, elongation 72 °C for 30 sec and final elongation at 72 °C for 10 min. Then from the report (Pipatchartlearnwong *et al.*, 2019) [10] we withdrawn two sets of marker F1 & F2, F3 & F5 (female specific marker), which has its PCR amplification condition as, pre denaturation at 94 °C for 5min, denaturation at 94 °C for 30 sec, annealing 60 °C for 45 sec along with 35 cycles, elongation at 72 °C for 45 sec, and final elongation 72 °C for 10 min. The PCR products were run on 1% agarose gel at 101V for 1hours 30 min to ensure the complete separation of bands. The gel was scanned through the gel documentation system (BIO-RAD gel documentation unit).

Results and Discussion

Based on the literature survey to discriminate sexes of Palmyra, three gene specific primer and one RAPD based markers were selected. The RAPD based marker (OPA-6) was found to be a reliable sex discriminating marker as stated by (George *et al.*, 2007) [9] The other three markers were selected randomly as reported (Pipatchartlearnwong *et al.*, 2019) [10] to examine their reliability in our samples collected from three different locations of Tamil Nadu (Coimbatore, Ramnad, Viluppuram). The experiment was carried out with five tree replicates of male, female from each locations. The DNA is isolated using CTAB method was confirmed for its presence and PCR amplified with all the primers and analysed for the presence or absence of band using auto documentation. This study focuses on screening and discriminating of male and female from three different location through RADP analysis. The OPA-06 was used for the sex discrimination based on the amplification through polymerase chain reaction. The OPA-06 primer produced relatively higher amplification fragments in Coimbatore than in Ramnad and Viluppuram. It produced nine DNA amplicons of size ranges from 250 bp to 1500 bp. All the bands are Based on the literature survey on the gene specific primer (or) marker assisted discrimination of male from female palmyra through three distinct primers OPA-06(RAPD marker) M1 & F1 (male and female specific) markers. The primer showed different amplification pattern

within males/females and between males and females.

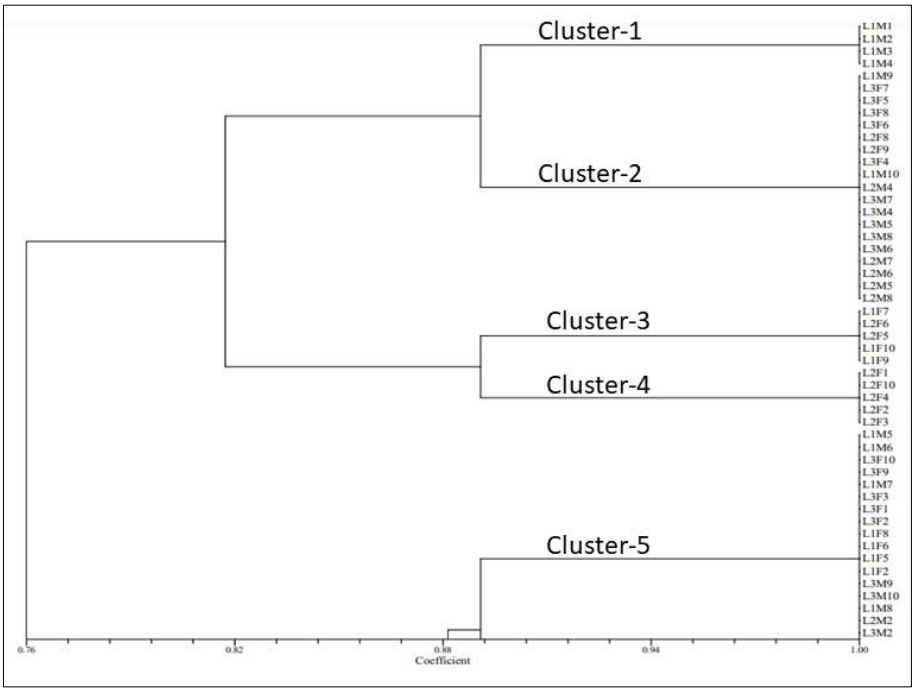
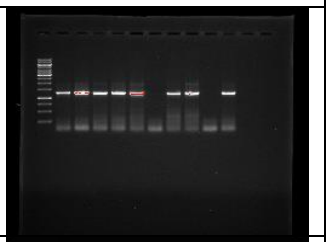
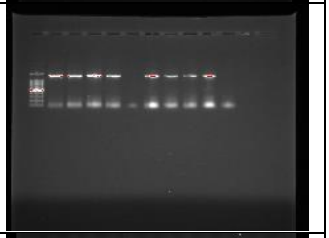
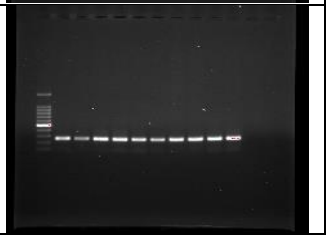
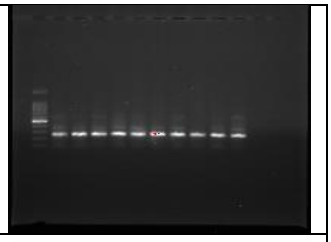
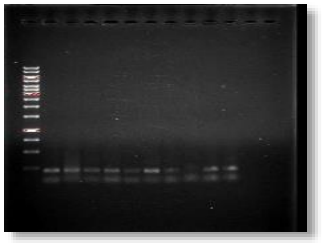
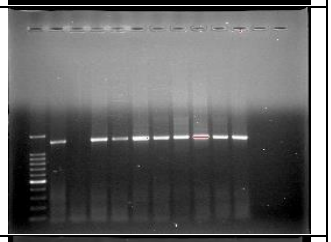
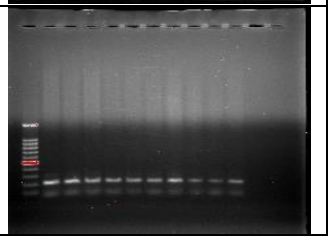


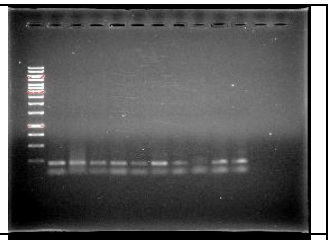
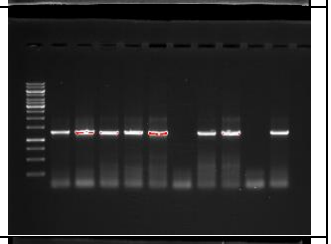
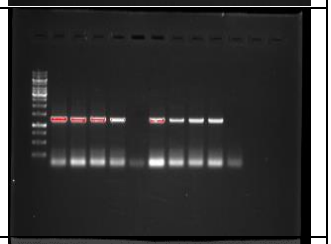
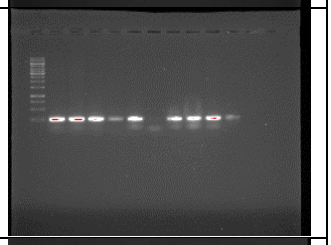
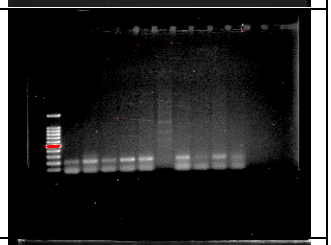
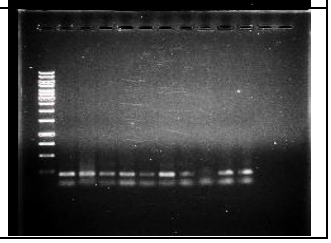
Fig 1: Dendrogram of Male and Female of Palmyra palm analysed using OPA-06 Marker Collected from Three Different Location

Corresponding dendrogram shows the cluster (fig.1). Among the five-cluster formed, the cluster 2 contains male samples from location 1 and female samples from location 2 & 3. The cluster 3 & 4 contains female sample from location 2 & 1. While the cluster 1 contains male sample from location 1. The cluster 5 carriers both the male and female samples from all the location. Through the cluster 1, 3 & 4 contains only male or female respectively, the cluster 2 & 5 contains both male and female samples of palmyra. The problem with this OPA-06 marker might be it is not completely linked to the either of the sex discriminating locus. Yet, a contrasting report on the tightly linkage of OPA-06 marker to male locus was reported by (George *et al.*, 2007) [9].

Table 1: A Comparatively Analysis of Male and Female palmyra samples collected from Coimbatore, Ramnad, Viluppuram using Three different Sex Discriminating Markers (M1 & M2, F1 & F2, F3 & F4)

Location	Sample	Primer	Linked trait	Product size	
Coimbatore	Female	F1-fp-5'GGGAGGAGGGATTCAAAGAC F2-rp-5'AGGAGGCCCTTGTTGAT	Female specfic marker	1250 bp	
Coimbatore	Male	F1-fp-5'GGGAGGAGGGATTCAAAGF2-rp-5'AGGAGGCCCTTGTTGAT	Female specfic marker	1250 bp	
Coimbatore	Female	F3-fp-5'GGTGGAGGAGTATGGCAAAA F4-rp-5'GCAAACATTGCAGAAACGGA	Female specific marker	250 bp	

Coimbatore	Male	F3-fp-5'GGTGGAGGAGTATGGCAAAA F4-rp-5'GCAAACATTGCAGAAACGGA	Female specfic marker	250 bp	
Coimbatore	Male	M1-fp-5'CCTTAAACCACCGATGTCGT M2-rp-GTGCCAGATAGGCAAACCAC	Male specfic marker	300 bp	
Coimbatore	Female	M1-fp-5'CCTTAAACCACCGATGTCGT M2-rp-GTGCCAGATAGGCAAACCAC	Male specfic marker	300 bp	
Ramnad	Female	F1-fp-5'GGGAGGAGGGATTCAAAGAC F2-rp-5'AGGAGGCCCTTGTTGAT	Female specfic marker	1250 bp	
Ramnad	Male	F1-fp-5'GGGAGGAGGGATTCAAAGAC F2-rp-5'AGGAGGCCCTTGTTGAT	Female specfic marker	1250 bp	
Ramnad	Female	F3-fp-5'GGTGGAGGAGTATGGCAAAA F4-rp-5'GCAAACATTGCAGAAACGGA	Female specfic primer	250 bp	
Ramnad	Male	F3-fp-5'GGTGGAGGAGTATGGCAAAA F4-rp-5'GCAAACATTGCAGAAACGGA	Female specfic marker	250 bp	
Ramnad	Male	M1-fp-5'CCTTAAACCACCGATGTCGT M2-rp-GTGCCAGATAGGCAAACCAC	Male specfic marker	300 bp	

Ramnad	Female	M1-fp-5'CCTTAAACCACCGATGTCGT M2-rp-GTGCCAGATAGGCAAACCAC	Male specfic marker	300 bp	
Viluppuram	Female	F1-fp-5'GGGAGGAGGGATTCAAAGAC F2-rp-5'AGGAGGCCCTTGTTGAT	Female specfic marker	1250 bp	
Viluppuram	Male	F1-fp-5'GGGAGGAGGGATTCAAAGAC F2-rp-5'AGGAGGCCCTTGTTGAT	Female specfic marker	1250 bp	
Viluppuram	Female	F3-fp-5'GGTGGAGGAGTATGGCAAAA F4-rp-5'GCAAACATTGCAGAAACGGA	Female specfic marker	250 bp	
Viluppuram	Male	F3-fp-5'GGTGGAGGAGTATGGCAAAA F4-rp-5'GCAAACATTGCAGAAACGGA	Female specfic marker	250 bp	
Viluppuram	Male	M1-fp-5'CCTTAAACCACCGATGTCGT M2-rp-GTGCCAGATAGGCAAACCAC	Male specfic marker	300 bp	
Viluppuram	Female	M1-fp-5'CCTTAAACCACCGATGTCGT M2-rp-GTGCCAGATAGGCAAACCAC	Male specfic marker	300 bp	

Thus, the table shows that the female marker (F3F4 & F1F2) produces specific bands at 250 & 1250 bp respectively. However, both the bands are present in all the male samples collected from three different regions of Tamil Nadu. Subsequently, the male specific marker M1 & M2 showed distinctive amplicon at 300 bp for the male sample from Coimbatore. Whereas in female plant the 300 bp amplicon was absent in all the tree replicates. Yet the replication of the experiment didn't provide the consistent result of both the amplicons (300 bp & 250 bp) in the male palmyra as indicates in the Table 1. The absence of 300 bp in two of samples shows the in consistency of markers (or) non-specific binding of primer.

Conclusion

This research demonstrates that the promises of marker-assisted sex determination in Palmyra palm have significant difficulties. This is due to the complexity and small sex determining regions of Asian palmyra palm and also the molecular data of Asian palmyra palm is limited. which led to

the lack of universal marker, which have made an challenging avenue in the development of a modern technique such as nanotechnology for the early sex determination of Asian palmyra palm.

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