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Identification of sex-specific marker in *Calamus brandisii* Becc. (Arecaceae)

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Abstract

Calamus brandisii Becc. (Family Arecaceae) is an evergreen, dioecious threatened rattan widely used for the furniture and handicraft industry. Sexing of rattan seedlings at an early stage before the establishment the plantations or seed stands can greatly enhance the long term survival and productivity. The present study is to identify molecular markers linked to sex determination using RAPD markers. Of the twenty random primers standardised, the primer OPAW 20 yielded a unique amplicon specific to male plants. It is feasible to identify sex at the early stages of plant life, which is beneficial for improving further breeding programs in *C.brandisii.*

Keywords: Sex determination, RAPD markers, Western Ghats, Dioecious rattan.

Introduction

Dioecy is generally associated with sexual dimorphism, is one of the most striking examples of evolutionary specialization. Although the majority of angiosperm species are hermaphroditic, approximately 6% are dioecious, with sexes segregated in separate individuals[1]. Early sex identification in plants is especially those species whose female representatives are more desirable in the production process include *Borassus flabellifer*[2], *Carica papaya*[3], *Hippophae rhamnoides*[4, 5], *Myristica fragrans*[6] etc. In the case of non crop plants especially in rare and threatened Non Timber Forest Products, for planning effective restoration programmes the identification of male and female genotypes are necessary to ensure sufficient large number of productive female plants with only a minimal number of male plants. *Calamus brandisii* Becc. (Family Arecaceae) is a long-living, dioecious evergreen threatened rattan endemic to the Western Ghats of India (Fig.1). This species is distributed in the evergreen forests between 1000-1500 m at Agasthyamala Biosphere Reserve and Shendurney WLS in Kerala and in Kalakkadu and Upper

Fig. 1. *Calamus brandisii* from Shendurney WLS, India.

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Kothayar in Tamil Nadu. This is an excellent small diameter rattan, extensively used in furniture and handicraft industry. Being a dioecious rattan, in which the sex of plants cannot be identified till the commencement of flowering. The existing population has highly fragmented to disjunct patches and natural regeneration is very low and cultivation and breeding programs areurgently required to ensure the survival and sustainable use of this rattan. Maintaining the sex ratio in plantations is crucial for design of seed stands to produce sufficient seeds as planting material for plantations. The number of female plants should be more and early identification of male and female individuals can help to address the limitation of dieocy. In recent years, there have been serious efforts to understand the genetic basis of sex determination in plants and to develop methods to identify sex at an early stage by using molecular markers viz. RAPD, AFLP, ISSR etc. Among these different molecular markers, RAPD markers are widely used for identifying sex linked markers in angiosperms as in *Asparagus officinalis*[7], *Simarouba glauca*[8], *Cycas circinalis*[9], *Myristica fragrans*[10], *Momordica dioica*[11] etc. Hence in the present paper, we have attempted to identify sex-specific DNA markers of this rattan using RAPD markers.

Materials and methods

Field surveys were conducted to Bonacaud and Pandimotta forest areas in Kerala part of the Western Ghats and fresh leaf samples and herbarium specimens of *Calamus brandisii* were collected from identified male and female plants.

Table 1. List of RAPD primers selected for the study

Sl. No	RAPD primers	Sequence (5'-3')
1	OPA-03	AGTCAGCCAC
2	OPA-04	AATCGGGCTG
3	OPA-09	GGGTAACGCC
4	OPA-11	CAATCGCCGT
5	OPA-12	TCGGCGATAG
6	OPA-13	CAGCACCCAC
7	OPA-15	TTCCGAACCC
8	OPA-16	AGCCAGCGAA
9	OPA-17	GACCGCTTGT
10	OPA-18	AGGTGACCGT
11	OPA-20	GTTGCGATCC
12	OPA-10	GTGATCGCAG
13	OPB-15	GGAGGGTGTT
14	OPE-02	GGTGCGGGAA
15	OPE-18	GGACTGCAGA
16	OPAU-02	CCAACCCGCA
17	OPAW-07	AGCCCCCAAG
18	OPAW-09	ACTGGGTCGG
19	OPAW-10	GGTGTTTGCC
20	OPAW-20	TGTCCTAGCC

All the herbarium specimens were deposited at Kerala Forest Research Institute (KFRI) Herbarium. Total DNA was extracted from 1g of the leaf tissues using

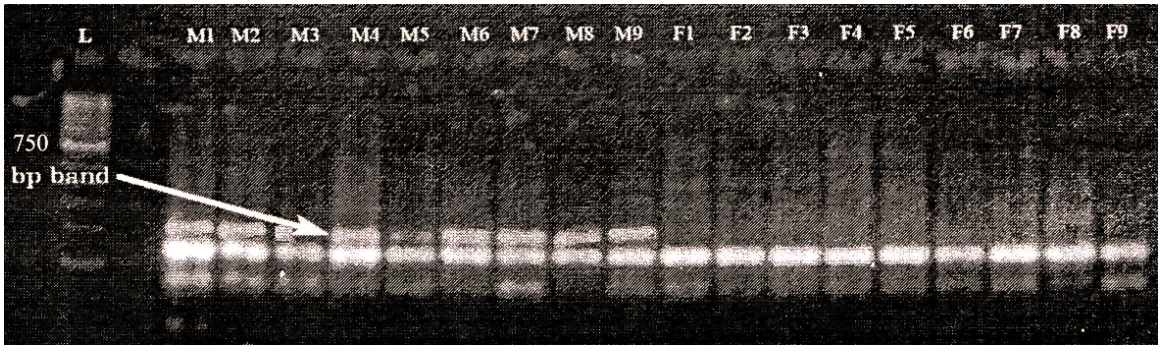

Fig.2. RAPD banding profile of male, female plants obtained by the primer OPAW 20, arrow indicates male specific 750 base pair.

the modified CTAB protocol[12] and the purified DNA was then re-suspended and stored in 100 µl TE buffer. Random primers were selected from the previous screening done on *Calamus* of the Western Ghats[13]. Only those 20 primers (OPA, OPB, OPE, OPAU, OPAW) were selected which have more than 60% polymorphism. These selected 20 polymorphic primers are listed in Table 1. PCR reactions were performed on a PTC-200 Thermocycler (MJ research., USA) in 25µl reaction volumes[14] with 50ng of template DNA, 1mM of dNTP mix, 5 picomoles of primer, 1.5 unit of Taq DNA polymerase, 10mM $MgCl_2$ and 10X Taq buffer with 15mM $MgCl_2$. The amplification was performed with an initial denaturation of 95^0 C for 4 minutes and 45 cycles of denaturation at 94^0 C for 1 minute, 1 minute annealing (36^oC), and extension at72^oC for 2 minutes. The last cycle was followed by a final extension of 72^oC for 4 minutes.

Results and discussion

Among the 20 random primers used in the present investigation, OPAW 20 yielded a unique amplicon of 750 bp only in male plants (Fig. 2).

This DNA marker is found to be consistent with a potential to develop as a male specific SCAR marker to detect male plants in *Calamus brandisii*. In rattans and other palms, sex-specific molecular markers have been identified in *Calamus simplicifolius*[15], *Borassus flabellifer*[16], *Calamus tenuis*[17] and *Phoenix dactylifera*[18,19] using RAPD and ISSR markers. Among these[15], standardisation was done in approximate 500-bp male-specific DNA fragment with the S1443 primer using RAPD markers. Molecular studies of dioecious species indicated that most of the sex-determining genes were found in male plants[20, 21]which suggests that males play a vital role in the sex determination of dioecious plants. The characteristics of male plants, including prolonged and earlier flowering cycle[22] and more frequent reproduction compared to females, might have suggested that male plants are somehow crucial for sex determination. Generally, in rattans sex of the plants becomes known only at the time of first flowering, which takes around 4-5 years. Hence, an early identification of sex can help to address the limitation of dioecy and this will be beneficial to improve restoration activities, breeding programmes and developing seed stands. In addition, we have obtained a proper RAPD protocol that is useful for other species of rattan. A RAPD marker OPAW-20_{750} band consistently appeared in male genotypes, suggesting thereby the male associated nature of this DNA marker in *C. brandisii*. OPAW-20_{750} proved to be constant reproducible under a wide variation of amplification conditions and this marker can be used for sex determination of male genotypes which can be used for screening seedlings in forest department nurseries especially for restoration programmes. Even though the reproducibility of RAPD markers are questioned and it is recommended that RAPD markers are converted to specific SCAR markers which possess several advantages over the former one, such as: (1) robust reproducible PCR amplification[23] with minimum effect of reaction conditions and (2) locus specificity amenable to easy detection/ scoring on agarose gel. In the case of *C. palustris*, a subtractive library was constructed for male floral tissue to understand the genetic mechanism for gender determination and concluded that the male floral genes may play a vital role in sex determination[24]. In the present study, the screened RAPD primer (OPAW 20) gave reproducible results for the discrimination of male plants. The generated unique bands from male plants can be sequenced and could be used further for identification of sex at early stage of seedlings. Further, more specific primers can be designed from these generated sequences which could be used for sex identification of *C.brandisii* in a more precise way at seedling stage.

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