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A SCAR marker based method for sex determination in dioecious betel vine (*Piper betle*)

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Betel vine (*Piper betle* L., family Piperaceae), native to Malaysia is an important commercial horticultural crop cultivated in South East Asian Countries. Due to the strong pungent aromatic flavour of betel leaves, it is widely used as masticatory agent. The leaves are reported to possess antioxidant, anti inflammatory, anticarcinogenic, carminative, digestive, stimulant, antibacterial, antifungal and nematocidal properties (Sawangjaroen *et al*. 2006). The essential oil extract is added in skin care products (http://www.tcff-thailand.com/betal.html). Mature female spikes are highly valued for their use in the treatment of upper respiratory tract diseases in traditional and ayurvedic medicines.

The plant is vegetatively propagated through stem cuttings and through tillers arising from the base of mature plants. In spite of its dioecious nature, the gender distinction and determination has not been well studied (Verma *et al.* 2004). Lack of morphological descriptors to distinguish male and female plants makes their identification difficult before flowering, which takes several years from the date of planting. Moreover, flowering occurs only under stringent climatic conditions and the males and females are distinguished based only on their spike morphology with former characterized by longer spikes. Gender based differences due to difference in major bioactive principles like total phenol and thiocyanate content have been reported (Usha *et al.* 2009). Maiti *et al.* (1992) reported the presence of sexual dimorphism associated with economic traits such as disease resistance, leaf quality etc. of betel leaves. Thus a method for identifying the sex of plants at younger stage is essential for breeding purposes and also when the plant is grown for its female spikes.

Significant efforts have been made in the past for identifying and characterizing molecular markers involved in plant dioecy (Kafkas *et al.* 2001). Several RAPD markers have been converted into SCAR markers (Paran and Michelmore 1993) to improve the efficiency of detection of sex in different crops (Liao *et al.* 2009). Molecular marker based efforts towards sex determination in *P. betle* has been conducted through RAPD (Usha *et al.* 2009, Samantaray *et al.* 2012) and sex specific markers were identified. However, the possibility of their conversion into SCAR markers was not explored. Khadke *et al*. (2012) reported development of SCAR marker for sex determination in dioecious betel vine using ISSR markers. However, there is no report on sex specific SCAR markers derived from RAPD markers in betel vine. Both these markers may be utilized for sex determination in betel vine.

The plant materials (Table 1) were obtained from the Central Horticultural Experimental Station (CHES), Hirehalli, a substation of Indian Institute of Horticultural Research (IIHR), Bangalore, India. Twenty six accessions were taken for the study that includes nine males, ten females and seven sex blinded samples (Table 1). Using the bulk segregant approach (Michelmore *et al*. 1991) two separate bulks for males and females were prepared by pooling aliquots of 5 µg DNA from 9 male and 10 females individually (Table 1). Genomic DNA was isolated from the leaves of individual plants by the standard CTAB method (Doyle and Doyle 1987) using the modified extraction buffer [3% cetyl trimethyl ammonium bromide (CTAB) buffer, 0.2% β- mercaptoethanol and 2% poly vinyl pyrrolidine] and quantified spectrophotometrically.

The random amplification was performed following an optimized protocol (Sheeja *et al*. 2008). PCR mixture contained *Taq* assay buffer with 1.5 mM $MgCl_2$ (1X), dNTPs (0.2 mM), primer (10 pmol- OPERON Technologies Inc), template DNA (50 ng) and *Taq* DNA polymerase (1U) (Bangalore Genei, India) in a total reaction volume of 25 µl. The PCR was performed in a thermal cycler (EP gradient S, Eppendorf, Germany) with an initial denaturation at 94°C

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for 4 minutes followed by 35 cycles of denaturation at 94°C for 1 minute, annealing at 37° C for 1 minute and extension at 72°C for 1 minute and a final extension at 72°C for 15 minutes. The PCR products were separated by electrophoresis in 1.5% agarose gels.

A total of 82 RAPD primers were used to generate 527 fragments in the male and female bulk DNA samples. Random primers of the series OPA-1 to 20, OPB-1 to 20, OPD-1 to 20, OPE-1 to 20 and OPJ-12 and OPJ-18 were used for screening. Three prominent polymorphic bands were identified in the bulked DNA analysis. One male specific band of ~600 bp was produced by primer OPE-11 and female specific bands of size 400 bp and 488 bp with primers OPE-1 and OPB-20 respectively. These markers were first screened individually in all the 19 genotypes (9 male and 10 female plants), to obtain one specific fragment (OPB-20_{488}) consistently in females only. The band generated by OPB-20 (5'-GGACCCTTAC-3') was selected for SCAR development, since it was more prominent and consistent than those by other two primers. The fragment was eluted from the agarose gel using the Perfectprep Gel Cleanup Kit (Eppendorf), cloned into a TOPO TA cloning vector (Invitrogen, USA) and transformed into competent DH 5α cells. The transformed colonies were confirmed by colony PCR and custom sequenced (Bioserve Biotechnologies, Hyderabad) using M13 forward and reverse primers. The G+C content was 26%, which on BLAST analysis (Altschul *et al.* 1997) showed 87% identity with a portion of the *Piper cenocladum* chloroplast genome (DQ887677, E value = 2e-121).

Based on the nucleotide sequence, two SCAR primers, viz. Pibet-20 F-5' GGACCCTTACCTATACTAAATGATGA 3' and Pibet-20 R-5' GGACCCTTACATTCAAACCC 3' were designed that contained RAPD primer sequences at the 5' end followed by 10-16 bases of the amplicon. The reaction mixture was same as before except that 2.5 pmol of each primer was used in 25 μl reaction volume with an initial denaturation at 94°C for 5 minutes followed by 35 cycles of denaturation at 94°C for 30 sec, annealing at 65° C for 1 minute, extension at 72°C for 1 minute and a final extension at 72°C for 15 minutes. The amplification products were separated in a 1.8% agarose gel.

The effectiveness of the SCAR marker 'Pibet-20' was confirmed by screening in male and female samples individually along with sex blinded samples. The comparison of results of molecular marker identification with the actual gender showed 100% agreement, which further verified the specificity of the SCAR, Pibet-20 to female sex determination. The sequence has been deposited in NCBI (GenBank Accession No. JN228255).

SUMMARY

We report for the first time, development of a female sex specific RAPD-SCAR marker from betel vine. The maximum identity of the marker developed in the present study with the chloroplast genome of a related member of the genus (*Piper cenocladum*) in BLAST analysis indicates the uniparental (maternal) inheritance of the isolated loci and provides further support to the association of the marker with female sex in *P. betle*. The results in the present study showed that the reliable SCAR marker (Pibet-20) would be useful for the sex determination in betel vine plants at an early developmental stage. This method is simple and marker can be used to screen plants at very early developmental stages to support breeding programs. On the other hand for the growers it is desirable to know the sex of all plants before field planting.

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