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Genetic Diversity Analysis of *Palas* [*Butea monosperma* (Lam.) Taub.] Flower Variants through Random Amplified Polymorphic DNA

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Palas [*Butea monosperma* (L.) Taub.], a multipurpose leguminous tree has numerous flower variants. The genetic diversity of the flower variants (golden yellow, yellow, mustard yellow, chrome yellow, white, and scarlet) and a morphological variant, s*wadi palas* was analyzed using RAPD marker. Thirty six RAPD primers were used to analyze their genetic diversity. Similarity coefficients of the *palas* variants ranged between 0.479 for yellow and *swadi palas* and 0.705 for mustard yellow and white variant. The cluster analysis grouped the *palas* variants into 2 major clusters, first comprising of golden yellow, yellow and chrome yellow variants and the second comprising of mustard yellow, white, scarlet and *swadi palas* variants. The unique polymorphic band of 687 bp was obtained in wild scarlet *palas* with OPS 02 primer. An attempt was made to develop more locus specific marker from this fragment. The newly designed locus specific primers amplified 687 bp fragment in chrome yellow, mustard yellow, white, scarlet and *swadi palas* variants. This marker will be helpful in identifying *palas* flower variants and their conservation and use in breeding programmes.

Key Words: *Butea monosperma*, Lac host plant, Molecular marker, *Palas*, RAPD

Introduction

Palas [*Butea monosperma* (L.) Taub], is an economically important multi-purpose tree species commonly found throughout India. It has numerous uses such as timber, fuel, fodder, tannin, gums and medicinal use especially in the folk medicine. In India, it is an important host tree for lac insect, *Kerria lacca* which produces commercial lac resin (Orwa *et al*., 2009). It has beautiful flowers adorning leafless canopies during early summer and hence commonly known as 'Flame of the Forest'. Traditionally, flowers have been used as astringent, aphrodisiac, diuretic, anthelmintic, in diarrhea, gynecological and various central nervous system disorders (Kumar and Malik, 2012). *Palas* has numerous variations in flower colour, number of leaflets, leaf shape and arrangement. *Palas* with scarlet coloured flowers is the most commonly available in nature and is supposed to be the wild type. The rare yellow and white flowering trees (Sanjappa, 1987), white flowers with yellowish central portion in Madhya Pradesh, India (Kamran, 1989), golden yellow, chrome yellow and mustard flower colour *palas* in Jharkhand, India (IINRG, Annual Report 2011-12) and rare variant with unifoliate leaves in Jharkhand (Kumar *et al*., 2006) have been reported in literature. *Swadi palas*, a morphological variant is found mainly in Jharkhand; the size of leaf and flower of which is larger than the normally occurring *palas*. Leaflets of *swadi palas* are acuminate in shape, and that of normal *palas* is obtuse. Flower colour of *swadi palas* is orange as compared to scarlet in normal *palas* (Lohot, 2011).

Considering the economical importance of this tree species and its medicinal values, the genetic diversity needs to be studied for genetic improvement and germplasm conservation in the wake of deforestation. Molecular markers have numerous merits over morphological markers as they are stable and detectable in all tissues despite any growth and development (Agarwal *et al*., 2008). Among different molecular methods available to study genetic diversity, RAPD (Random Amplified Polymorphic DNA) is the most simple, handy, inexpensive, rapid and powerful technique where sequence information of the target organism is lacking. RAPD is a PCR (Polymerase Chain Reaction) based marker and uses random decamer primers to bring out the polymorphism in the genotypes being compared (Williams *et al*., 1990; Welsh and McClelland, 1990). It has been widely used in the identification and genetic relationship analysis of a number of plant and animal species. Although the reproducibility of the RAPD marker is at stake in some cases, it can be converted

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to much more specific marker called SCAR (Sequence Characterized Amplified Region) marker. These SCAR markers generally reveal higher levels of polymorphism owing to higher annealing temperatures and longer primer sequence specificity (Kumla *et al*., 2012). The main advantage of SCARs is that they are quick, easy to use and require only low quantities of template DNA. In addition, SCARs have a high reproducibility and are locus-specific.

Genetic diversity of *palas* has been studied by previous workers from different agro-ecological zones using RAPD (Vaishali *et al*., 2008). DNA profiling of elite lac host *palas* trees with the help of RAPD was carried out (Vashishtha *et al*., 2014); genetic structure and diversity of natural populations of *palas* from different geographical regions were studied employing RAPD, ISSR (Inter Simple Sequence Repeats) and SRAP (Sequence Related Amplified Polymorphism) (Vashishtha *et al*., 2013). *Palas* flower colour variants were also analyzed by ISSR (Kandasamy *et al*., 2013). However, no attempt has been done so far in the development of SCAR in *palas* flower variants. Hence, RAPD marker was selected in this study to estimate the genetic diversity of *palas* flower variants and an attempt was also made to develop SCAR marker for *palas* variants from the polymorphic band obtained from the wild type *palas*.

Materials and Methods

The young leaves of *palas* flower variants were collected from different parts of Jharkhand during January 2013, transported in ice bags and stored at -80 °C until further use. The place of collection of *palas* variants and their geographical co-ordinates are given in Table 1. The study was conducted at Biotechnology laboratory of Lac Production Division of ICAR-IINRG, Ranchi from January to June 2013.

Genomic DNA was isolated from young *palas* leaves of the variants mentioned above using CTAB method (Doyle and Doyle, 1990) with minor modifications. One gram of leaf sample was weighed and crushed in liquid nitrogen. Finely ground leaf sample was transferred to a tube containing 10 ml CTAB. It was incubated at 55 °C for 1.5 hours and centrifuged at 12000 rpm for 5 min. Supernatant was taken in a 50 ml tube and equal volume of phenol: chloroform: isoamyl alcohol [25:24:1] was added. It was centrifuged at 12000 rpm for 15 min. The aqueous layer was transferred to another tube and 1/10th volume of 7.5 M ammonium acetate and 2-2.5 volume of ice cold absolute ethanol was added. It was incubated at -20 °C for an hour for better DNA precipitation. After that, it was centrifuged at 12000 rpm for 15 min. DNA pellet was washed with two changes of ice cold 70% ethanol. The residual ethanol was removed by air drying the DNA pellet. It was dissolved in TE buffer containing RNase (100 µg/ml). After suspending the DNA, it was incubated at 37 °C for 30 min. and stored at -20 °C. To purify the DNA, 1.5 volume of diluted binding buffer of HiPurA plant genomic DNA miniprep purification kit (cat. no. MB507, HiMedia, India) was added to 1 volume of DNA and mixed thoroughly. The mixture was loaded to mini preparation spin column and centrifuged at 8000 rpm for 2 min. After discarding flow through, the column was washed twice with wash solution. Then the genomic DNA was eluted with elution buffer in 1.5 ml tube. Purity and quantity of DNA was checked using Nanodrop 2000 spectrophotometer (Thermo scientific, USA).

RAPD Analysis

Forty random decamer primers were used for the amplification of genomic DNA by PCR. Primers used were of OPS and OPH series. The volume of the final reaction was of 25 µl. It was made up of 1X buffer,

Table 1. *Palas* variants used in the study and their place of collection

S.No.	Variant	Place of collection	Latitude	Longitude
1.	Golden yellow	Khakhikalan, Giridih, Jharkhand	$23^0$56’53.6”N	$86^0$01’48.3”E
2.	Yellow	Khakhikalan, Giridih, Jharkhand	$23^0$56’55.37”N	$86^0$01’49.07”E
3.	Mustard yellow	Chainpur, Giridih, Jharkhand	$24^0$00’41.59”N	$86^0$01’58.50”E
4.	Chrome yellow	Bahadurpur, Bokaro, Jharkhand	$23^0$39’38.44”N	$85^0$57’10.49”E
5.	White	IINRG Research farm, Ranchi, Jharkhand	$23^0$19’51.2”N	$85^0$22’06.3”E
6.	Scarlet	IINRG Research farm, Ranchi, Jharkhand	$23^0$19’51.2”N	$85^0$22’06.3”E
7.	*Swadi palas*	Putadag, Angara, Jharkhand	$23^0$25’.746”N	$85^0$22’.478”E

4 mM $MgCl_2$, 0.1 mM dNTPs, 10 pico mole primers, 1.25 U *Taq* DNA polymerase (MBI Fermentas, USA) and 10 ng of template DNA. Amplification was carried out in labcycler (Sensoquest, Germany) with an initial denaturation period of 2 min at 94 °C, followed by 40 cycles of 30 sec at 94 °C, 30 sec annealing at 35 °C, 2 min extension at 72 °C and a final extension step of 10 min at 72 °C. The PCR products were run on 1% agarose gel and DNA bands were visualized by ethidium bromide staining and saved in Gene Genius Bioimaging system (Syngene, UK). Each PCR was repeated twice to confirm reproducibility.

Scoring of RAPD Data and Construction of Dendrogram

Only the clear, unambiguous and reproducible bands present across the DNA samples were used for scoring. With reference to molecular weight marker, at particular band size, the presence of bands was scored '1'; while the absence of bands or very faint bands was scored '0'. Information content of the RAPD primers was estimated through Diversity Index (DI), Marker Index (MI) and Resolving Power (RP). Marker Index was calculated using the following formula MI = Diversity index (DI) X Effective Multiplex Ratio (EMR). DI of a primer is defined as $1 - \Sigma P_i^2$, where P_i is the band frequency of the *i*th allele. EMR is the product of number of polymorphic bands (*i.e.* absence of band at least in one genotype at a particular locus) per primer and the fraction of polymorphic bands (Milbourne *et al*., 1997). Resolving power was calculated as per the following formula $Rp = \sum I_b$. Band informativeness, $I_b = 1-[2 X |0.5-p|]$, where p is the proportion of the seven genotypes containing the band (Prevost and Wilkinson, 1999). The binary data were analyzed using NTSYSpc software version 2.02i. The similarity matrix was constructed based on Jaccard's coefficient. Clustering was done by UPGMA using SAHN module and the dendrogram was created subsequently.

Cloning of Polymorphic RAPD Locus of Wild Scarlet Palas

Unique and polymorphic band of around 700 bp was obtained from the wild scarlet *palas*. It was eluted from the gel using QIAquick gel extraction kit (Qiagen, Germany) following manufacturer's instructions. The eluted fragment was ligated with pGEM Teasy vector (Promega, USA). *E. coli* DH5α cells were transformed with the ligation mix and the recombinant clones were selected on the LB agar plate containing ampicillin, X-gal and IPTG. Plasmids were isolated from the white colonies and the size of the inserts was confirmed by PCR and restriction digestion with *EcoRI* enzyme. The plasmids having correct size insert were sequenced using T7 and SP6 primers at the sequencing facility of Xcelris Labs Ltd. Ahmedabad, India. The sequence was analyzed using Geneious software ver. 6.1.6. BLAST analysis was also carried out to find homologues for the sequence obtained (http://blast.ncbi.nlm.nih.gov/Blast).

Designing of Locus Specific Primers

The sequence obtained was used to design sequence characterized amplifiable region (SCAR) primers. The primer sequences are given below:

Sc.F2: 5' CTG ACT GTC ATA ATA AGT TCT A 3'

Sc.R2: 5' CCT CTG ACT GGG CAA AGC 3'

The primers were used to amplify all *palas* variants to check their specificity. The volume of the final reaction was of 25 µl. It was made up of 1X buffer, 2 mM $MgCl_2$, 0.1 mM dNTPs, 10 pico mole primers, 1.25 U *Taq* DNA polymerase and 25 ng of template DNA. Amplification was carried out in Sensoquest thermal cycler with an initial denaturation period of 2 min at 94 °C, followed by 35 cycles of 30 sec at 94 °C, 30 sec annealing at 60 °C, 2 min extension at 72 °C and a final extension step of 10 min at 72 °C. The PCR products were run on 1% agarose gel and DNA bands were visualized by ethidium bromide staining and saved in Gene Genius Bioimaging system (Syngene, UK).

Results and Discussion

The present investigation was carried out to study the genetic diversity in *palas* variants using RAPD markers. Genomic DNA was isolated from six flower variants and a morphological variant, *swadi palas*. The purity index of genomic DNA isolated from the *palas* variants ranged from 1.8 to 2.0 showing the DNAs obtained were of good quality, without any contamination.

A total of 40 arbitrary decamer primers (OPS and OPH series) were used for initial screening. Out of 40 primers used, 36 could generate good PCR profile and reproducible results. Vaishali *et al.* (2008) used 30 RAPD primers for *B. monosperma*, of which only 12 generated reproducible results. The selected 36 primers were used for RAPD analysis of 7 *palas* flower variants. The present RAPD analysis generated 450 bands across all 7 variants. A representative picture for RAPD profiling

of *palas* variants with OPS 10 primer is shown in Figure 1.

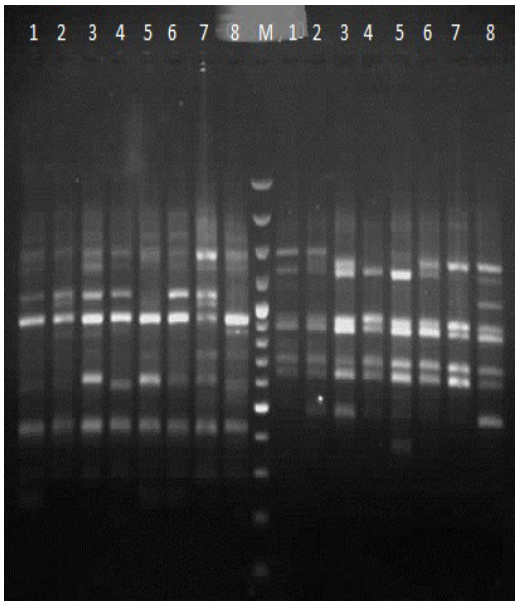

Fig. 1. RAPD profile of *palas* variants using OPS 10 primer. Lane M- 100 bp ladder, 1-golden yellow, 2-yellow, 3-mustard yellow, 4-chrome yellow, 5-white, 6-scarlet flower colour variants and 7-s*wadi palas* variant

It has been reported that the ability to resolve genetic variation may be more directly related to the degree of polymorphism detected by the marker system. Primers OPH 01, OPS 05, and OPS 16 showed 100% polymorphism while least polymorphism was shown by OPS 03 (43.75%). The average polymorphic band percentage was found to be 70.44%. However, a lesser level of polymorphism (66.3%) was obtained for elite lac host *palas* trees (Vasishtha *et al*., 2014) and higher level of polymorphism (86%) for *palas* collected from different agro-ecological zones of India (Vaishali *et al*., 2008). Since the amplification depends upon the sequence of RAPD primers, coverage of genome by the primers and genetic nature of genotypes used, the rate of polymorphism tend to vary. A wide range of polymorphism occurs in the tree crops from as low as 10.48% in *Pongamia pinnata* (L), Panigrahi (Kesari *et al*., 2010) to as high as 99.39% in *Grevillea* species with RAPD markers (Pharmawati *et al*., 2004). Average number of bands per primer was found to be 12.5, which was higher than the RAPD analysis of *B. monosperma* by Vaishali *et al*. (2008) and Vasishtha *et al*. (2013). On an average each primer produced nine polymorphic bands, which was also more than the one (5.6) reported by Vasishtha *et al*. (2013) for RAPD analysis for *palas* trees. A maximum of 21 scorable bands were recorded with primer OPH 12, whereas OPS 06 showed a minimum of five scorable bands.

An efficient marker system must be capable of distinguishing different genotypes. Resolving Power (RP) depends on the distribution of the alleles within genotypes and estimates the capacity of discrimination of each primer. The resolving power of primers used in the study ranged from 1.42 to 8.57. OPS 05 and OPS 15 were the most informative primers having the highest RP of 8.57 and OPS 01 showed the least RP of 1.42. A very high range of RP from 3.85 to 19.14 with an average of 10.46 per primer was reported for 14 *Thymus* accessions (Yousefi *et al*., 2015). OPS 15 primer was able to differentiate 6 *palas* flower variants.

The Diversity Index (DI) value of the primers ranged from 0.69 to 0.99. The Marker Index (MI), which is the product of DI and EMR, was used to evaluate the overall utility of each marker system. The parameter, MI was first used by Milbourne *et al.* (1997) to compare different molecular markers in genetic relationship studies and the MI was defined as a general measure of efficiency of the primers in detecting polymorphism. MI was calculated for all the RAPD primers in this study and found to vary from 1.25 (OPS 06) to 13.75 (OPS 05). However, MI varied between 2.9 and 15.85 with an average of seven for ISSR primers used in the genetic diversity analysis of *palas* flower colour variants (Kandasamy *et al*., 2013).

Similarity matrix was constructed for *palas* variants using Jaccard's co-efficient and tabulated in Table 2. The similarity co-efficient values ranged from 0.479 (between yellow and *swadi palas*) to 0.705 (white and mustard yellow). Jaccard similarity matrix based on RAPD binary data was used to cluster the *palas* variants following Unweighted Pair Group with Arithmetic Mean (UPGMA) method. The dendrogram (Fig. 2) revealed that *palas* variants could be clustered into two major groups — first group consisted of three variants: golden yellow, yellow and chrome yellow colour variants of the *palas* while second group consisted of four variants: mustard yellow, white, scarlet colour variants and *swadi palas*. This result is comparable with the ISSR studies of flower colour variants of *palas* (Kandasamy *et al*., 2013), wherein golden yellow and yellow variants were in one cluster and rest of the variants were in another cluster.

Table 2. Similarity coefficients of *palas* variants

Variant	Golden yellow	Yellow	Mustard yellow	Chrome yellow	White	Scarlet	*Swadi Palas*
Golden yellow	1.000						
Yellow	0.659	1.000					
Mustard yellow	0.606	0.540	1.000				
Chrome yellow	0.656	0.633	0.613	1.000			
White	0.587	0.538	0.705	0.602	1.000		
Scarlet	0.597	0.541	0.671	0.629	0.683	1.000	
Swadi Palas	0.559	0.479	0.675	0.565	0.673	0.661	1.000

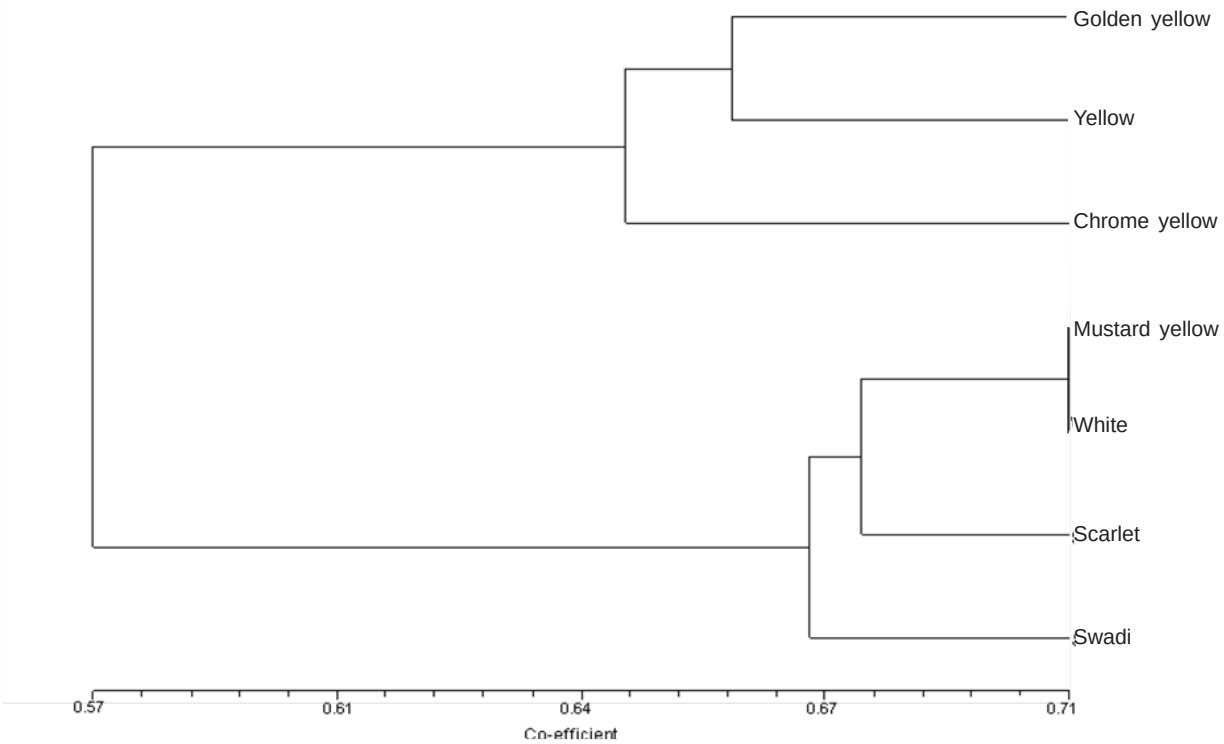

Fig. 2. Dendrogram of *palas* variants based on RAPD data

The primer, OPS 02 generated a unique, locus specific polymorphic fragment of 687 bp size with wild type scarlet *palas* (Fig. 3). This fragment was cloned and sequenced. The sequence obtained was of 687 bp and is given below. The OPS 02 primer sequence is shown in lower case.

c c t c t g a c t g T C A T A A T A A G T T C T A G T T A T G G G C T A A T G A T G C C T A C T T T T A G C T T A G T C
A T A A T T T G T T T G T T G T G C C C A A T T A G G G G C T T T T T A G T T T T G T A T T T A A A C A C C A A
C T G T A A T A C T T C A A G T G T G T G T A A A A G T T G T G T G T G A A A A A C T A A A A T A C A T T A A
A T G A G T T T G T G C T C T T T T C T C C T T T T T T G T A T G T A T G G T T G T C A T T T T C C T T A A A G C C
T T T T T G C T A G T T T A G T C C A A T A G C T T T G T T G C T G A G T C T A A T A G T T T T C C A T C A A G
T C T A A T A A C T T T T T C A A T A G T T T A A A A A C C T G T T C T A T T T T T C A A C A G A T A T C T A A A
A G C A G A A A A G T T A G A G A A G T G A G G A A T A C T T A A T A A C A A T G G C C T G G G C C T G T A
G G C T G G G G G G A G G T T C A T C T C C C T A T C A A T A A A G A G C A T T C C T T A T C A T G T A T T T
T T A A T G G C T A T T A C A G C A G A C A G T G G C C A A A T G T T A T G C C A A A A A A T G A G G A G T
G T T G G A A A T T G A G A T T T T T C C A A C T A T C A T A A T A A A A T A T T T T A A T G T C T C T G T A G
T T T T C T A C T T G C T T G T A A G T T T G G T T A A A G G T T G T T T A T G T T T A G A A A A A G C T T G G
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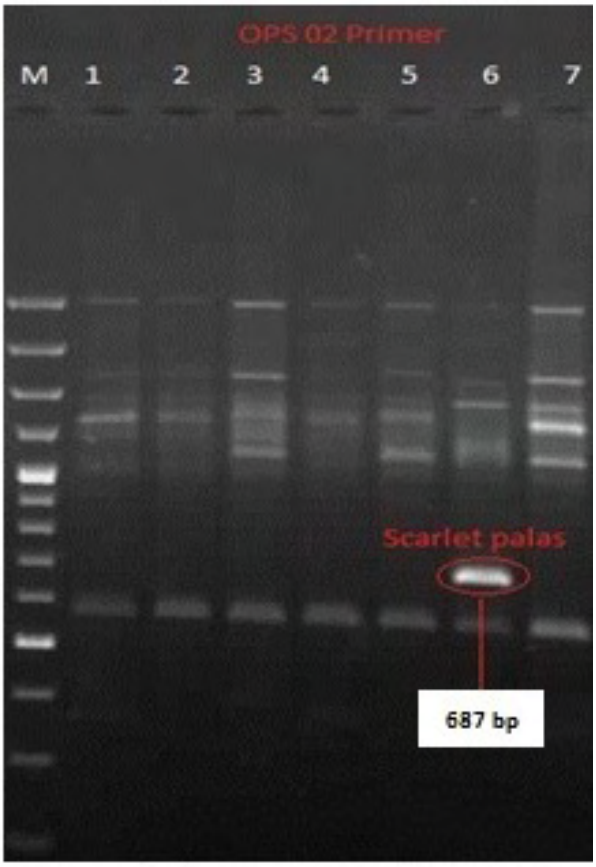

Fig. 3. RAPD profile of palas variants using OPS 02 primer. Lane M- 100 bp ladder, 1-golden yellow, 2-yellow, 3-mustard yellow, 4-chrome yellow, 5-white, 6-scarlet colour variants and 7-swadi palas variant

When the sequence was used for homology BLAST search in NCBI GenBank, it revealed that 120 bp of the query sequence (from 549 to 669 nucleotides) shared 80 % identity with *Glycine max* uncharacterized (LOC100790248) transcript variant X5, ncRNA with E value of 2e-13. Hence it could be considered as some uncharacterized region form the *palas* genome.

Based on the sequence of 687 bp, longer locus specific primers (Sc.F2 and Sc.R2) were designed. The newly designed primers were tested against *palas* flower variants. The primers amplified all *palas* variants except golden yellow and yellow flower colour variants (Fig. 4). Although it was anticipated that the primers have to amplify specifically scarlet *palas,* the primers amplified chrome yellow, mustard yellow, white, scarlet and *swadi palas*. However, it did not amplify golden yellow and yellow, which is in a different cluster of the dendrogram. In RAPD, primers compete for binding site and might have missed a particular locus of 687 bp in other *palas* variants as obtained in scarlet *palas* with OPS 02 primer. However, in locus specific primers there is no competition for the primers and could have amplified the same locus in other variants including mustard yellow, chrome yellow, white and *swadi palas* besides scarlet *palas*. In natural conditions, mostly yellow *palas* and golden yellow *palas* trees are found in nearby locations. The locus specific primer set (Sc. F2XSc.R2) could be used for differentiating yellow and golden yellow *palas* variants from other flower variants even before flowering, which will be highly useful in selection of trees for plantation or for medicinal uses.

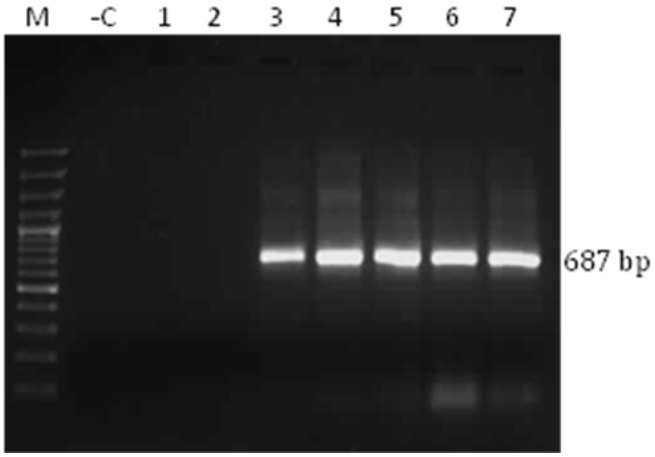

Fig. 4. PCR profile of *palas* variants using locus specific primers Sc.F2XSc.R2. Lane M- 100 bp ladder, -C-negative control, 1-golden yellow, 2-yellow, 3-mustard yellow, 4-chrome yellow, 5-white, 6-scarlet colour variants and 7-s*wadi palas* variant

Palas trees are under heavy exploitation due to its medicinal values and continuous threat of deforestation. The genetic base may be eroded because of these reasons and conservation of its genetic base is very essential. RAPD markers used in the study were highly efficient and suitable for diversity analysis of *palas* flower variants generating sufficient polymorphic data and to identify flower variants for future genetic improvement of this tree. Similarly, the SCAR marker which is amplifying non-yellow and golden yellow flower variants will also be helpful in conservation strategies and in genetic improvement programmes. However, the specific SCAR makers for flower variants may be developed by screening more number of RAPD primers, which would be much more informative and useful in the future breeding programmes.

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