PCR based methodology for gender identification in date palm (Phoenix dactylifera L.) of Gujarat, India

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Research Article

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Abstract

Date palm (*Phoenix dactylifera* L.) is important dioecious plant in which male and female sex flowers are on different plant. The gender identification of date palm is only possible after 4–8 years when flowers developed. The date palm fruits are produced only on female plants, therefore identification of gender in date palm plants is essential for selection of female plants at seedling stage and increasing production. Currently, there is no consistent methodology available for the sex determination in date palm. There are several DNA marker-based methods available but none can provide truthful results for date palm sex identification. Here we focused on PCR based sex determination of date palm with Glycerol-3 phosphate acetyl transferase (GPAT3) male specific primer and LOX5.1 as positive control. GPAT3 plays important role in male fertility. According to results GPAT3 amplification only occur in male date palm samples, not in female. So that, if result of PCR amplification produced two bands than male and if one band than it is female. We further validated these primers to 315 date palm samples of Kutch region of Gujarat. Out of which, from 304 samples, we can successfully identify gender of date palm with p-value of 0.809 which indicate that the technique is efficient to differentiate sex of date palm.

Key Message

The present study carried out for gender discrimination in date palm of Gujarat by targeting male specific gene. A simple PCR based analysis confirms gender of plants which further assured by sanger sequencing. Two genes identified from date palm in which Lox5.1 designed as positive control and GPAT3 is male specific primer.

1. Introduction

The Date palm (*Phoenix dactylifera* L.) is one of the chief flowering plants included in the family of Arecaceae. The date palm is a fundamental fruit crop in the Middle East, northern Africa, and India along with several tropical and subtropical regions around the globe. The environmental conditions of arid and semi-arid areas are highly favourable for growing of date palm plant (Al-Mahmoud et al. 2012). The date palm fruits are mainly used for consumption along with traditional medicinal purposes for a long time (Al-Shwyeh 2019). The different plant parts of date palm have socio economic importance like shelter, fibre, and food in India (Hiralal Jana et al. 2019). In India, the area for date palm cultivation is around 18286 ha with 171522 MT production. In India, date palm is cultivated among the major parts Kutch region of Gujarat and few regions of Rajasthan.

The date palm is a dioecious plant in which inflorescence has male and female flowers on different plant (Mathew et al. 2014). Traditionally, date palm plants are produced from seed, and because of dioecious nature, seed produced plants contain approximately the same number of male and female plants. Additionally, only female plants produced fruits, and few male plants were needed only for pollination and fertilization with female ovules. Hence, the selection of female plants is the most crucial procedure for the economic cultivation of date palms (Intha and Chaiprasart 2018). The cultivation and selection of

female date palm plants from heterogeneous gender date palm population is the most cost-effective approach and an increased number of female date palm plants per hectare potentially results in more dates production, making the plantation more profitable. The sex of seedlings can be found out only in the flowering stage and it produces mostly at the age of 4–8 years of the plant. So, the initial gender determination of date palm is very important for farmers for increasing profits as well as for plant breeders in date palm breeding experiments for crop improvement (Sarkar et al. 2017).

Several researchers have used different approaches for date palm sex determination using isoenzymes (Torres and Tisserat 1980), peroxidase (Majourhat et al.), Sequence Characterized Amplified Region (SCAR) (Al-Qurainy et al. 2018), Start Codon Targeted region (SCoT) (Adawy et al.), Random Amplified Polymorphic DNA (RAPD) (Moghaieb et al. 2010) etc. Spectroscopy, regression, and Nuclear Magnetic Resonance (NMR) were also used in a study to check sex of date palms having immature leaf (Khan et al. 2021). However, there is no currently reliable marker-based method for the identification of sex in date palms. In this study, we analysed the male-specific molecular marker glycerol-3-phosphate acyltransferase gene (GPAT3) (Torres et al. 2018) against available date palm cultivars across Gujarat. We have also used the Lipoxygenase gene (LOX5.1) as a positive control as it gives a homomorphic DNA band and identifies both the male and female plant of date palm as process control. This gene was identified from our transcriptome database of date palm and validated in date palm samples of male and female plant collected from various regions of Gujarat.

2. Materials And Methods

2.1. Plant Material

The date palm leaf samples were procured from different date palm growing areas of Gujarat at the flowering or fruiting stage to avoid any sampling mistakes. The date palm leaf samples were collected based on various genotypes or cultivars growing by farmers, institutes, and government research stations.

2.2. Genomic DNA Isolation

Leaves surface was cleaned with Tween-20 followed by rinsing with sterile water before proceeding for DNA isolation. The genomic DNA of the date palm leaves was isolated using DNeasy Plant Mini Kit by following instructions of as suggested by manufacturer (QIAGEN). The quality and concentration of DNA was determined at 260 nm using QIAxpert (QIAGEN). The elution buffer, provided in the kit, was used for DNA dilution, to reach the concentration of 30 ng/µL and stored at − 20°C.

2.3. Identification and Development of Marker

Initially, 13 primers (Table 1) were selected for the sex determination of date palm samples using PCR based amplification. The primers which can produced distinct DNA band in either in male or in female were selected for further experiments.

Table 1
List of primers used for the initial screening of date palm leaf samples.

Sr. No.	Primers	Details
1.	SCAR1	`Male specific-sequence characterized amplified region
2.	GPAT3	Glycerol 3 phosphate acetyltransferase
3.	LOG 1	Lonely Guy
4.	Dp_1	Date palm 1
5.	Dp_2	Date palm 2
6.	SRY	Sex determining region Y protein
7.	SCAR 2	Male-specific sequence characterized amplified region
8.	SCoT36	Start Codon Targeted region
9.	SCoT41	Start Codon Targeted region
10.	Op_A11	RAPD Primer
11.	Op_M11	
12.	Op_O07	
13.	Op_S07	

Polymerase Chain Reaction was carried out in 20 µl of total reaction volume which contain 10 µl of Master Mix (TAKARA), DNA template of 3 µl (30 ng/µl), forward and reverse primer of (10 pmol) 1 µl each, 1 µl of BSA (2 mg/ml) and nuclease free water of 4 µl. The PCR reactions were performed by 4 min of initial DNA denaturation at 94°C, 35 cycles (1 min of denaturation at 94°C, 30 sec of annealing at 55°C and 1 min of extension at 72°C) and 5 min of final extension at 72°C in a VeritiTM 96-well Thermal Cycler (Applied Biosystems). PCR amplified products were separated on agarose gel of 1.8% having 0.5 µg/mL of ethidium bromide in 1X TAE buffer. The gel was observed using Gel Doc XR + System (BIO-RAD).

2.4. Sequencing of PCR product amplified by GPAT3 primer

The PCR product with a male-specific band was purified using an ExoSAP-IT™ PCR product clean-up reagent (Applied Biosystem). Sequencing was done using The BigDye TM Terminator v3.1 Cycle Sequencing Kit. The sequence was analysed using the nucleotide BLAST at the NCBI database (https://www.ncbi.nlm.nih.gov/).

2.5 Data analysis

The size and number of PCR amplified product was evaluated by using Image Lab Software of Gel Doc XR + System (BIO-RAD). Samples with two bands (GPAT3 & LOX5.1) were identified as male and samples

with one band (LOX5.1) were identified as female. The data was further analysed by chi square test.

3. Results

3.1 Sample collection

Date palm leaf samples were collected from different areas of Gujarat. It includes Anand, Dantiwada, Sarangpur, Mundra, Gandhinagar and Kutch of Gujarat (Table 2).

Table 2
Sample collection from various areas.

Sr. No.	Area	No of samples/Sample code		Description of date palm plant
		Male	Female	
1	Anand	2 AM1, AM2	2 AF1, AF2	Red color fruit Variety: Local
2	Dantiwada	2 DM1, DM2	1 DF1	Fruit color: Yellow Variety: Bahree
3	Sarangpur	1 SM1	2 SF1, SF2	Fruit color: Red and Yellow Variety: Unknown
4	Mundra	2 MM1, MM2	2 MF1, MF2	Fruit color: Yellow Variety: Bahree
5	Gandhinagar	50 (G1 to G19)		Fruit color: Yellow Variety: Bahree
6	Kutch-Bhuj	209	95	Fruit color: Red and Yellow Variety: Variable varieties

3.2 PCR amplification

For date palm sex determination, initially selected thirteen primers were used to check specificity of sex in date palm genomic DNA collected from various areas of Gujarat such as Anand, Dantiwada, Sarangpur and Mundra (Table 2). From, each of the thirteen primers, none of the primers were capable of producing a unique pattern for distinguishing the sex of the date palm except one primer which is GPAT3 (Figs. 1, 2 and 3). Most of them were homomorphic or were not giving consistent band patterns in all samples. Only the GPAT3 primer was able to give the distinct male-specific band of 450 bp (Figs. 1, 2 and 3). We have also designed a primer for Lipoxygenase gene (LOX5.1) and used for date palm male and female plant DNA samples as a positive control as it produced amplification of 156 bp among both male and female samples (data not shown). Hence, LOX5.1 selected as positive control along with GPAT3 primer. The 450

bp band was visible in male plants, and it was absent in female plants. These two primer combinations were further used for more date palm samples for sex determination.

Table 3
Primer Sequences and their references

Primer	Sequence	Amplicon size (bp)	Reference
GPAT3	Forward- AGAAAACCTGATATGCTCTCTG	450	(Torres et al. 2018)
	Reverse - TGTGATGCACTTGGTAACTACT		
LOX5.1	Forward- CTACACCGCAGAGTTTGTCG	156	-
	Reverse - AGATTGGACCCATGAGTTGC		

More than 315 date palm leaf samples were collected from the Kutch area (Table 2) of Gujarat considering availability of variable cultivars grown by farmers. Out of 315 date palm leaf samples, these two primers can efficiently distinguish male and female plants from 304 plants (Fig. 4). After date analysis, the chi square value is 0.26 and p-value is 0.809.

3.3 Sequence Analysis-

We have also checked the amplified product by GPAT3 primers with date palm samples collected from Anand, Mundra, Dantiwada and Sarangpur by sequencing. The DNA sequence was analysed using 3500 Bioanalyzer (Applied Biosystem). After getting sequence data, it was analysed by BLAST analysis against NCBI Nucleotide Database. The BLAST analysis of sequence data shows that the amplified products from *Phoenix dactylifera* with *Phoenix dactylifera* glycerol-3-phosphate acyltransferase gene (Table 4). This enzyme is mainly responsible for male reproductive organs and male fertility.

Table 4
BLAST analysis of sequencing data produced GPAT3 primer amplified product

Sample	Per. Identity	Query Cover	Accession No.	Organism	Prediction
Male Anand	100%	99%	XM_03921660.1	*Phoenix dactylifera*	*Phoenix dactylifera* glycerol-3-phosphate acyltransferase
Male Dantiwada 11	100%	99%	XM_03921660.1	*Phoenix dactylifera*	
Male Sarangpur 3	100%	97%	XM_03921660.1	*Phoenix dactylifera*	
Male Mundra 1	99.77%	99%	XM_03921660.1	*Phoenix dactylifera*	

4. Discussion

Traditionally, gender identification of date palm can be done by phenotypic characters of flowers of date palm. Additionally, for developing flowers, date palm plant takes 4–8 years for flower development depending on environmental conditions and after appearance of flower, male and female separation can possible. The shape of spathe is swollen or bloated near the distal end and larger and shorter than female date palm before emergence of the inflorescence. The male flower also having noticeable petals, stamens and prolific pollen and producing fragrance at the pollen release time.

For the economic production of date palm, farmers have to maintain desired population of gender specific date palm plants. For this, early sex identification is crucial step. There are many researchers tried hard to solve the complex puzzle by using various methodology. Siljak-Yakovlev et al (Siljak-Yakovlev et al. 1996) used cytological methods, but it needs advanced instrument facilities. Abdul et al (Khan et al. 2021), used nuclear magnetic resonance (NMR) spectroscopy, Near-Infrared Reflectance Spectroscopy (NIRS) and Fourier transform infrared attenuated total reflectance (FTIR/ATR) methods to identify the sex differentiation in immature date palm leaves. However, for performing this experiment, also required sophisticated costly instruments. The PCR based molecular technique of date palm sex determination is not affected by age, environment and reproductive stage of plant. Same technique also used by Intha and Chaiprasart (Intha and Chaiprasart 2018), for gender identification of date palm of cv. KL1 of Thailand.

The gender identification of date palm by gender specific marker was also tried by Al-Mahmoud et al (Al-Mahmoud et al. 2012) and Solliman et al (EL-Din Solliman et al. 2019), but they did not include Gujarat date palm cultivars. Additionally, we also used SRY primer suggested by Solliman et al (EL-Din Solliman et al. 2019), but it cannot distinguish sex of date palm of Gujarat cultivars. This experiments, hence, showing its importance on various date palm cultivars grown by farmers.

The sex determination of seedlings at early stage could help to improve breeding efforts by generating experimental gender specific genetic pools that will promote date palm genetic improvement (Bekheet and Hanafy 2011). It was reported that GPAT, Glycerol-3-Phosphate Acyltransferase 3 (OsGPAT3), shows a central role in rice male fertility. Mutation in GPAT (osgpat) leads to defective anther cuticle and pollen exile formation in Rice (Men et al. 2017). The repeatability of the GPAT and LOX5.1 primer were tested in 315 date palm samples from Gujarat with known gender. After data analysis, chi square value is 0.26 which shows a difference between observed and expected value and p-value is 0.809 which shows that observed and expected results are almost same. In this experiment, GPAT3 and LOX5.1 primer can distinguish male and female date palm samples based on the presence of a 450 bp and 156 bp sized amplified band. If only 150 bp sized band is present, then its female and if both 450 bp and 156 bp bands are present then it is male sample. Hence, the presence or absence of the GPAT3 amplified band could allow male and female plants to be identified at the seedling stage.

5. Conclusion

The GPAT3 primer can be used to identify gender from the seedling stage to save time as date palms take 5–8 years to attain reproductive maturity. This technique has the advantage of allowing for the early

detection of male plants, thereby reducing the costs associated with the cultivation of non-productive males on the plantation. When tested repeatedly, the approach proved simple to use and accurate. As a result, plant breeders can use this marker to identify the gender of date palm seedlings before they are planted in fields.

Declarations

Ethics approval and consent to participate: Not applicable

Consent for publication: All authors agreed with the content and that all gave explicit consent to submit. Publication has been approved by all co-authors.

Availability of data and materials: All data generated or analysed during this study are included in this published article.

Competing interests: The authors declare no competing financial interests.

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Author Contributions: Chaitanya Joshi, Madhvi Joshi: Conceptualization, Methodology, Funding acquisition. **Fenil Patel:** Investigation, Supervision**,** Reviewing. **Haidar Abbas**: Data curation, Visualization, Resources. **Mansi Jani**: Writing- Original draft preparation, and Editing.

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References

1. Adawy SS, Jiang J, Atia Adawy SS, Jiang J, Atia MAM Identification f Novel Sex-Specific PCR-Based Markers to distinguish the genders in Egyptian Date Palm trees
2. Al-Mahmoud ME, Al-Dous EK, Al-Azwani EK, Malek JA (2012) DNA-based assays to distinguish date palm (Arecaceae) gender. Am J Bot 99:e7–e10. https://doi.org/10.3732/ajb.1100425
3. Al-Qurainy F, Al-Ameri AA, Khan S, et al (2018) SCAR marker for gender identification in date palm (Phoenix dactylifera L.) at the seedling stage. Int J Genomics 2018:. https://doi.org/10.1155/2018/3035406
4. Al-Shwyeh H (2019) Date palm (*Phoenix dactylifera* L.) fruit as potential antioxidant and antimicrobial agents. J Pharm Bioallied Sci 11:1. https://doi.org/10.4103/JPBS.JPBS_168_18
5. Bekheet SA, Hanafy MS (2011) Towards Sex Determination of Date Palm. In: Date Palm Biotechnology. Springer Netherlands, pp 551–566
6. EL-Din Solliman M, Mohasseb HAA, Al-Khateeb AA, et al (2019) Identification and sequencing of Date-SRY Gene: A novel tool for sex determination of date palm (Phoenix dactylifera L.). Saudi J Biol

Sci 26:514–523. https://doi.org/10.1016/j.sjbs.2017.08.002

7. Hiralal Jana and Debabrata Basu (2019) Importance of Date Palm cultivation in India. https://doi.org/10.24327/ijcar.2019
8. Intha N, Chaiprasart P (2018) Sex determination in date palm (Phoenix dactylifera L.) by PCR based marker analysis. Sci Hortic (Amsterdam) 236:251–255. https://doi.org/10.1016/j.scienta.2018.03.039
9. Khan AL, Al-Harrasi A, Numan M, et al (2021) Spectroscopic and molecular methods to differentiate gender in immature date palm (Phoenix dactylifera l.). Plants 10:1–15. https://doi.org/10.3390/plants10030536
10. Majourhat K, Bendiab K, Medraoui L, Baaziz M Diversity of leaf peroxidases in date palm (Phoenix dactylifera L.) as revealed in an example of marginal (seedling derived) palm groves PII: S 0 3 0 4–4 2 3 8 (0 2) 0 0 0 3 2–8
11. Mathew LS, Spannagl M, Al-Malki A, et al (2014) A first genetic map of date palm (Phoenix dactylifera) reveals long-range genome structure conservation in the palms
12. Men X, Shi J, Liang W, et al (2017) Glycerol-3-phosphate acyltransferase 3 (OsGPAT3) is required for anther development and male fertility in rice. J Exp Bot 68:513–526. https://doi.org/10.1093/jxb/erw445
13. Moghaieb REA, Abdelhadi AA, Gamal A, Sief Eldein E (2010) Genetic diversity and sex determination in date palms (Phoenix dactylifera L.) based on DNA markers Microbial metabolism of short-chain alkenes View project Developing Abiotic Stress Tolerant Wheat Using Gene Editing View project
14. Sarkar S, Banerjee J, Gantait S (2017) Sex-oriented research on dioecious crops of Indian subcontinent: an updated review. 3 Biotech 7
15. Siljak-Yakovlev S, Benmalek S, Cerbah M, et al (1996) Chromosomal sex determination and heterochromatin structure in date palm. Sex Plant Reprod 9:127–132. https://doi.org/10.1007/BF02221391
16. Torres AM, Tisserat B (1980) Leaf Isozymes as Genetic Markers in Date Palms
17. Torres MF, Mathew LS, Ahmed I, et al (2018) Genus-wide sequencing supports a two-locus model for sex-determination in Phoenix. Nat Commun 9:. https://doi.org/10.1038/s41467-018-06375-y

Figures

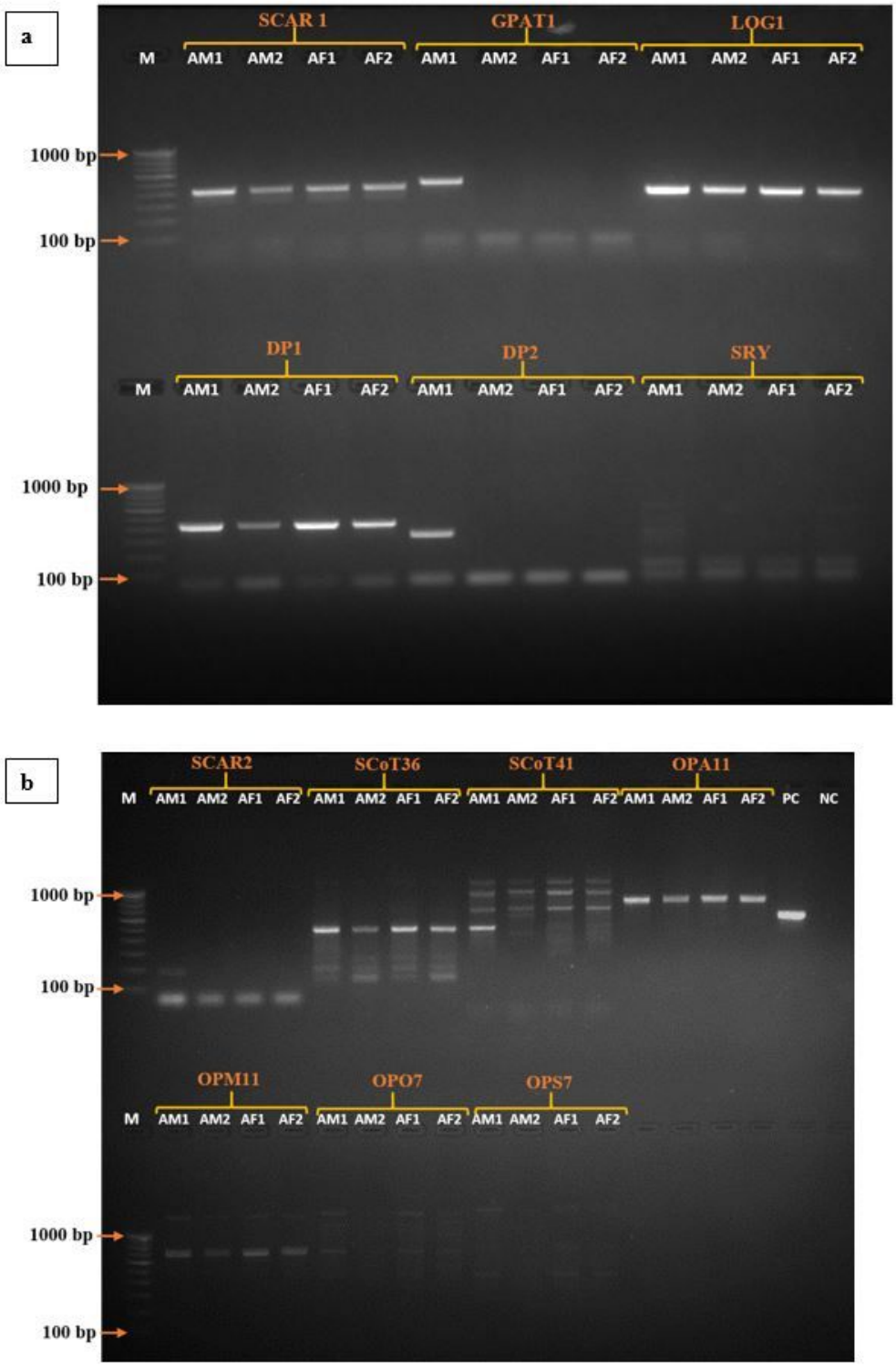

Figure 1

PCR amplification profiles of known date palm male and female leaf samples collected from Anand, Gujarat region as mentioned in Table 2. **a** Results for primer number 1-6 listed in Table 1 **b** Results for primer number 1-6 listed in Table 1. M= 100 bp marker

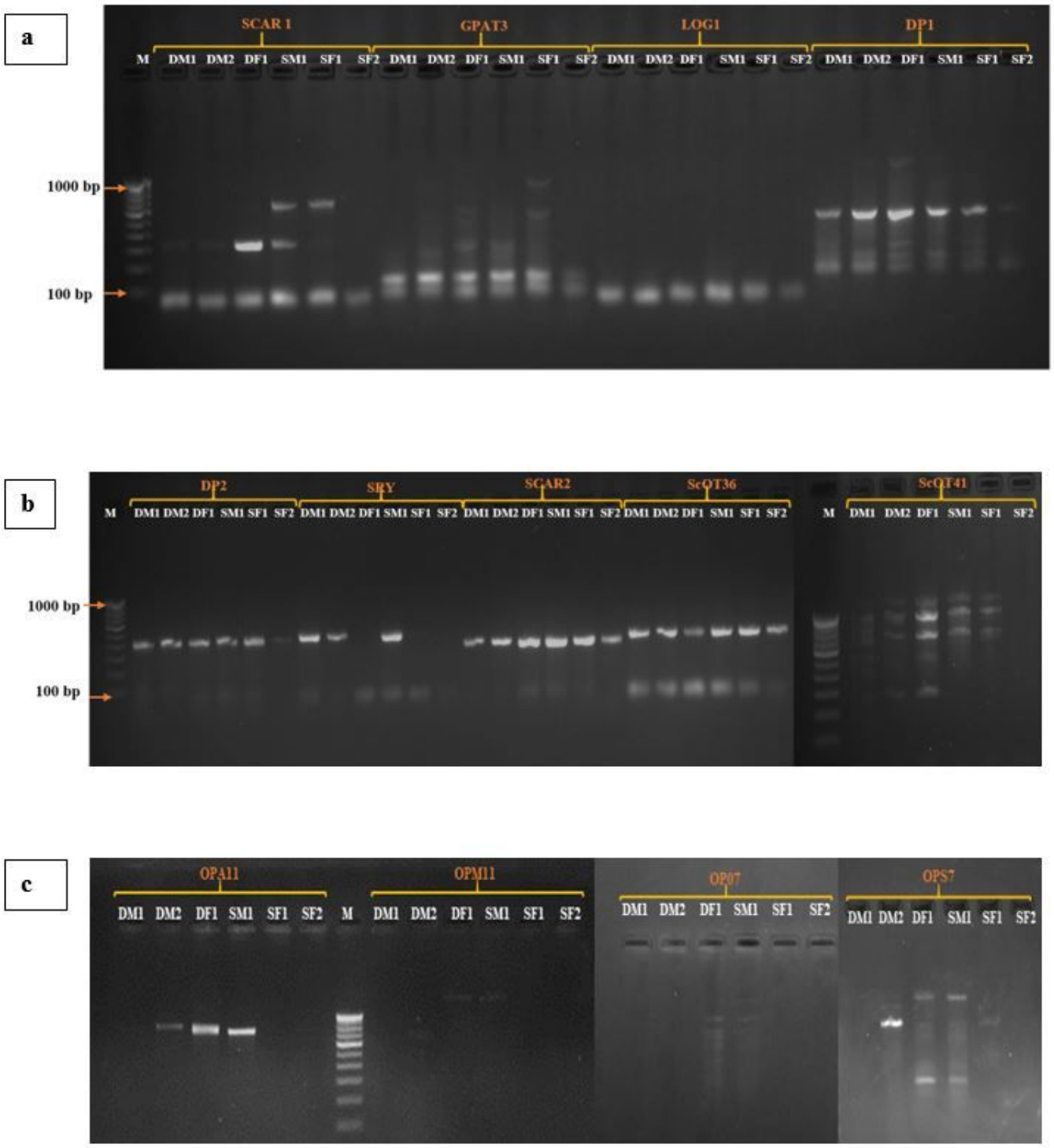

Figure 2

PCR amplification profiles of known date palm male and female samples collected from Dantiwada and Sarangpur region of Gujarat as mentioned in Table 2. **a** Results for primer number 1-4 listed in Table 1. **b** Results for primer number 5-9 listed in Table 1 **c** Results for primer number 10-13 listed in Table 1. M= 100 bp marker

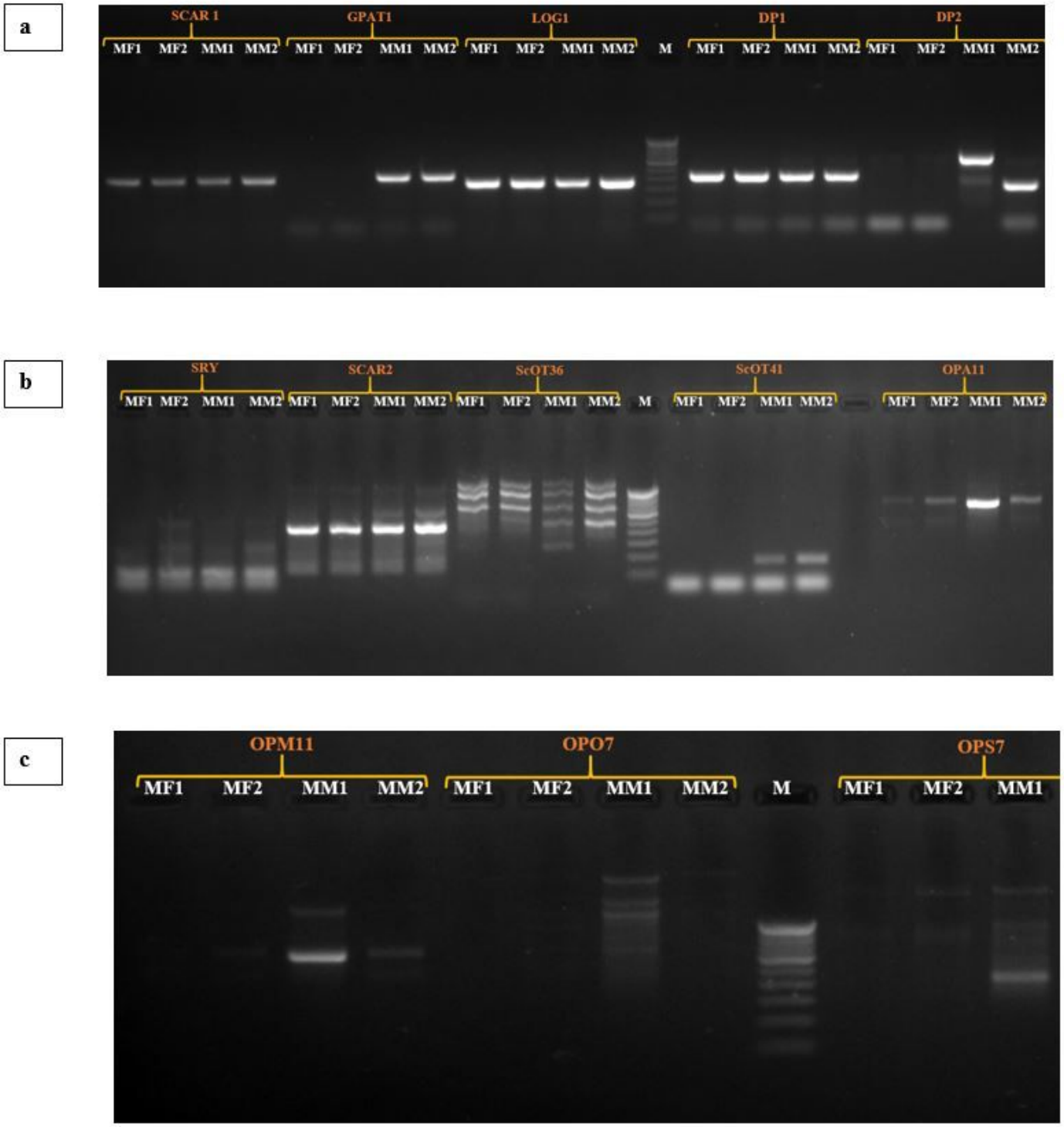

Figure 3

PCR amplification profiles of known date palm male and female samples collected from Mundra, Gujarat region as mentioned in Table 2. **a** Results for primer number 1-4 listed in Table 1. **b** Results for primer number 5-9 listed in Table 1 **c** Results for primer number 10-13 listed in Table 1. M= 100 bp marker

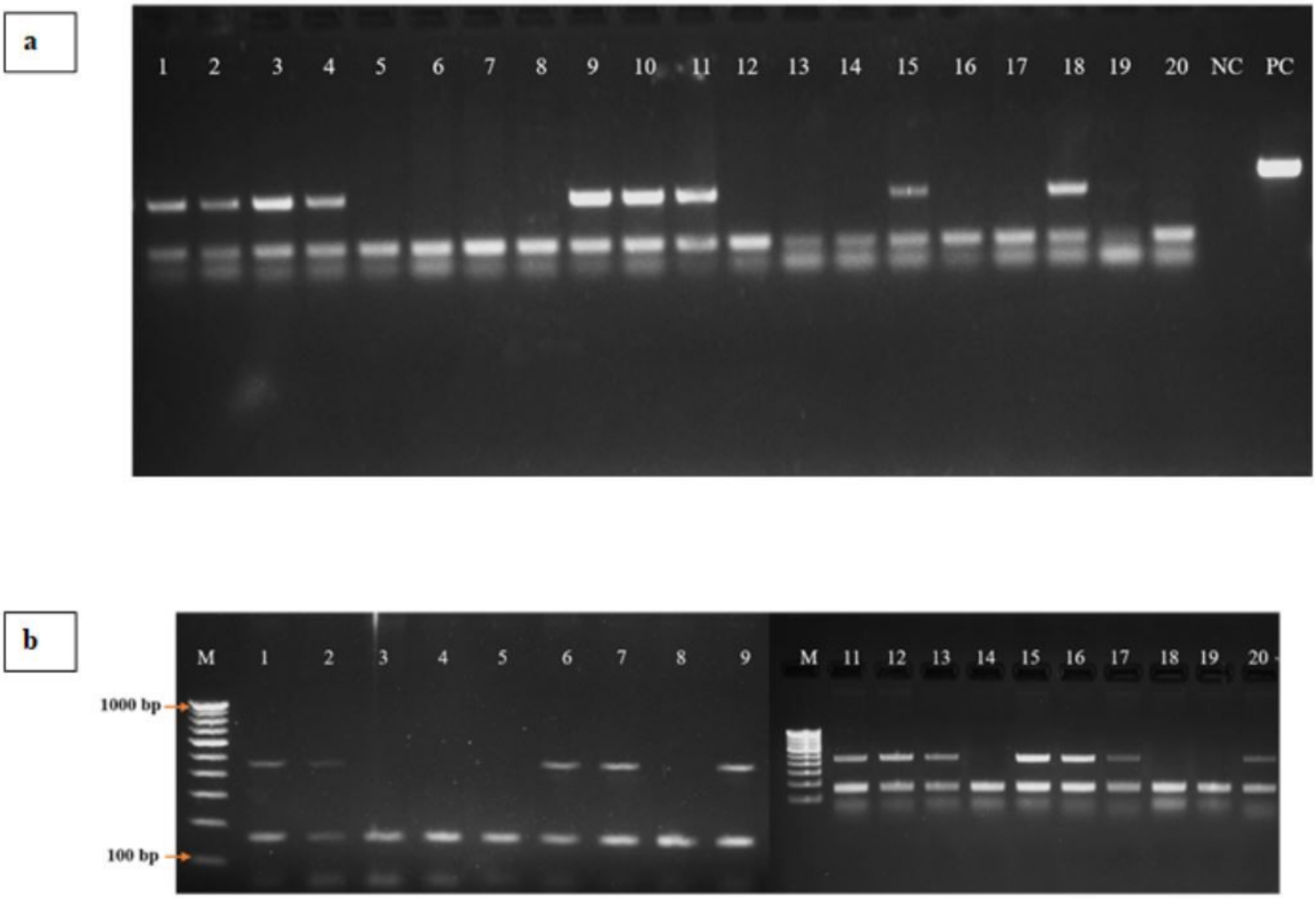

Figure 4

PCR amplification profiles for GPAT3 and LOX5.1 primer for date palm male and female samples collected Kutch, Gujarat region as mentioned in Table 2. **a** Representative image of date palm leaf samples collected from Kutch region. Male Sample number: 1, 2, 3, 4, 9, 10, 11, 15, 18, remaining are female **b** date palm leaf samples collected from Gandhinagar region. Male sample number: 1, 2, 6, 7, 9, 11, 12, 13, 15, 16, 17, 20, remain are female. M= 100 bp marker, NC=Negative control, PC=Positive control

Supplementary Files

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