RESEARCH ARTICLE



Development of competitive allele specific PCR based SNP genotyping assays for breeding applications in castor

Manmode Darpan Mohanrao¹, M. Sujatha¹, S. Vanisri¹, B. Vidya Sagar¹, Y. Rushwanth Reddy¹, K. Divya Sravanthi[#] and S. Senthilvel^{*}

Abstract

Castor is an industrially important oilseed crop, which provides raw material to many industries. Despite its industrial importance, the genomic resources available for molecular breeding applications are limited. In the present study, a set of 135 SNP loci polymorphic between two castor inbred lines namely, JC-12 and RG-1963 were targeted to develop competitive allele specific PCR assays for genotyping purposes. The assays were validated in the F₂ population of JC-12 × RG-1963 cross. Out of 135 assays designed, 124 were found to be successful in discriminating three expected genotypes in the F₂ population. Using the SNP genotyping data, a genetic linkage map representing 10 haploid chromosomes of castor was constructed, which corresponded very well with the physical map. This set of validated SNP markers is a useful resource for application in molecular breeding of castor.

Keywords: Ricinus communis, KASP assay, molecular markers, linkage map

Introduction

Castor (*Ricinus communis* L.) is an important oilseed crop supplying raw materials for manufacturing industries like lubricants, cosmetics, varnishes and paints (Ogunniyi 2006; <u>Mutlu</u> and Meier 2010). India produces 1.2 million tonnes castor seed per annum catering 85 per cent of the world demand (FAOSTAT 2019). Despite being an important industrial crop, castor is still a neglected crop in terms of availability of genomic resources for research.

Availability of genetic markers is an important requirement in molecular breeding. Among several classes of DNA markers, the marker system based on SNP is preferred because of its abundance in genome, high level of polymorphism and amenability to high throughput techniques. The first report of using SNP markers in castor was that of Foster et al. (2010), who used 48 SNP markers in assessing genetic diversity. Later, <u>Senthilvel</u> et al. (2019) developed a genotyping array comprising of 6000 SNP loci discovered through whole genome sequencing of 14 diverse castor lines. Recently, a chromosome level genome assembly has been published (Xu et al. 2021). The SNP markers linked to wilt resistance (Shaw et al. 2021) and a few agronomic traits (Xu et al. 2021) have been identified. The information on genome-wide SNPs and marker-trait associations can be effectively used in improvement of castor provided a suitable genotyping method is available for routine use in breeding programme like marker assisted selection (MAS), genetic purity testing of hybrids etc.

SNP genotyping is performed using different methods (Rasheed et al. 2017). Among various methods for SNP genotyping, the high throughput techniques such as SNP arrays or Genotyping-by-sequencing (GBS) are most popular. However, these techniques are ideal only when hundreds of samples are to be genotyped with thousands of SNPs. Other than discovery applications, use

*ICAR-Indian Institute of Oilseeds Research, Rajendranagar, Hyderabad 500 030, Telangana, India

¹⁵Professor Jayashankar Telangana State Agricultural University, Rajendranagar, Hyderabad 500 030, Telangana, India

Acharya N.G. Ranga Agricultural University, Lam, Guntur 522 034, Andhra Pradesh, India

***Corresponding Author:** S. Senthilvel, ICAR-Indian Institute of Oilseeds Research, Rajendranagar, Hyderabad 500 030, Telangana, India, E-Mail: senthilvel.senapathy@icar.gov.in

How to cite this article: Mohanrao M.D., Sujatha M., Vanisri S., Sagar B.V., Reddy Y.R., Sravanthi K.D. and Senthilvel S. 2022. Article Title. Indian J. Genet. Plant Breed., **82**(3): 350-354.

Source of support: ICAR-IIOR, Hyderabad

Conflict of interest: None.

Received: Dec. 2021 Revised: May 2022 Accepted: July 2022

[©] The Author(s). 2022 Open Access This article is Published by the Indian Society of Genetics & Plant Breeding, NASC Complex, IARI P.O., Pusa Campus, New Delhi 110012; Online management by www.isgpb.org

of SNP arrays or GBS is limited due to the low efficiency of customization, less flexibility and high cost of equipment. A modest breeding programme requires a simple, flexible and affordable method of genotyping to utilize the available SNP information in routine breeding applications. The competitive allele specific PCR based assays such as KASP[™] (He et al. 2014) and Amplifluor like SNP genotyping (Jatayev et al. 2017) largely meet the requirements of breeding programmes. Allele Specific PCR based genotyping has several advantages compared to other genotyping methods. It is convenient and does not require expensive equipment. The assay design is flexible. Any useful SNPs can be converted into genotyping assay without the need for availability of restriction enzyme recognition sites. In this context, the present study was aimed at developing PCR based assays for a set of polymorphic SNP loci and mapping them onto the castor genome.

Materials and methods

Plant material and DNA extraction

The F_2 population developed from the cross JC-12 × RG-1963 was raised in the research farm of ICAR-Indian Institute of Oilseeds Research (ICAR-IIOR), Hyderabad, India during 2018 rainy season (June - September). Leaf samples from 119 F_2 plants were collected and genomic DNA was extracted using NucleoSpin Plant II, Mini kit (Macherey-Nagel) following manufacturer's instructions. The quantity and quality of DNA was evaluated using 0.8% Agarose gel electrophoresis. Working DNA samples were prepared with a final DNA concentration of 4-5 ng/µL.

Genotyping assays

A set of 135 SNP loci polymorphic between JC-12 and RG-1963 were selected from the list of SNPs reported by Senthilvel



Fig. 1. Snapshot depicting different clustering pattern of SNPs (Successful assays A: Rc_30130-24525, B: Rc_29729-297813,C: Rc_56125-257; Failed assays D: Rc_29739-99683, E: Rc_30147-3420431,F: Rc_28583-16847)

et al. (2019) for developing genotyping assays. The assays were developed based on KASP[™] genotyping technology from LGC Genomics, UK. The KASP assay comprises of two components *viz.*, assay mix (primer-mix) and master-mix. The assay mix is specific to each SNP locus and contains two different allele specific forward primers (one for each SNP allele) with a unique unlabelled tail sequence at the 5' end and common primer (reverse). The master-mix is the universal component in the system, which contains FRET cassettes and optimized *Taq* polymerase.

Out of 135 SNP loci, assay mix for 86 loci were ordered from LGC genomics, UK; whereas the primer sets (two allele specific primers and common reverse primer) for the remaining 49 SNP loci were designed in-house using WASP tool available at https://bioinfo.biotec.or.th/WASP/ (Wangkumhang et al. 2007) with the option 'no mismatch' at the penultimate position of primers. The allele specific primers with targeted SNP at 3'end were added with the standard FAM (5' GAAGGTGACCAAGTTCATGCT 3') and HEX (5' GAAGGTCGGAGTCAACGGATT 3') tails at 5'end.

PCR set up and data collection

PCR was performed in 384-well plates (Genaxy). The PCR mixture was prepared by adding 2.5 µL KASP[™] master-mix, 0.07 µL assay mix (all provided by LGC) and 2.5 µL DNA (~ 5 ng/µL). For the assays designed in-house, assay mix (20X) was prepared by adding 1-µL of each allele specific primers (100 pmole/µL), 15 µL of common reverse primer (100 pmole/µL) and 183 µL of distilled water. The PCR mixture was prepared by adding 2.25 µL DNA (~ 5 ng/µL), 2.5 µL KASP[™] master-mix (supplied by LGC Genomics) and 0.25 µL of 20X Assay mix.

PCR was performed with one cycle of initial denaturation step at 94°C for 15 min followed by 10 cycles of denaturation at 94°C for 20 s and annealing and extension step at 61°C for 1 min (drop 0.6°C per cycle) followed by 26 cycles of denaturation at 94°C for 20 s and annealing and extension at 55°C for 1 min. After completion of PCR, the plates were read using FRET-capable plate reader (Victor-III, Perkin Elmer) and the fluorescence readings were analysed using KlusterCaller[™] software (LGC Genomics).

Construction of linkage map

Linkage map was constructed using QTL IciMapping software (Meng et al. 2015). The markers were assigned to linkage groups at the logarithm of the odds (LOD) threshold of >3. Markers within each linkage group were ordered using the 'Record' function. Map distances between markers were calculated using the Haldane mapping function. The linkage map generated in this study was compared with the previously published linkage map (Senthilvel et al. 2019) and physical map (Xu et al. 2021) of castor using 'Mapfuser', a shiny application available at https://plantbreeding. shinyapps.io/mapfuser.'Mapfuser' uses Genetic Map Comparator (Holtz et al. 2017) module for visualization of genetic map comparison.

Table 1.	Detail o	f linkage	map	constructed

Chromosome	No. of markers	Map Length (cM)	Average distance between markers (cM)
1	12	144.0	12.0
2	6	77.9	13.0
3	10	100.8	10.1
4	16	121.3	7.6
5	20	163.8	8.2
6	8	82.5	10.3
7	9	82.9	9.2
8	15	118.3	7.9
9	10	120.6	12.1
10	18	142.0	7.9
Total	124	1154.1	9.3



Fig. 2. Linkage map for JC-12 × RG-1963 F₂ population

103.5

Rc 27568-142589

- Rc_29729-297813

Rc_29841-43060

- Rc_29588-179206

70.0

82.9

Rc_29773-77448

79.0

352

-Rc_29718-44038

Rc_30073-109361

-Rc_30148-457688

-Rc_30128-354465

697 774

86.8

92.3

118.4

- Rc_29172-81277

- Rc_29983-431016

91.0

120.6

Results and discussion

A set of 135 SNP loci from across 10 chromosomes of castor was selected to develop the genotyping assays. The selected set of SNPs was used to genotype 119 F_2 individuals of the cross JC-12 × RG-1963. Out of 135 SNPs, 124 differentiated the expected three genotypic classes (two parental alleles and heterozygote). The genotype profiles of few successful SNP loci are depicted in Fig. 1A, B and C. The remaining 11 assays failed to discriminate the three expected clusters (Fig. 1D, E and F).

The successful assay conversion rate was 91.9%, which is comparable to 93.8% in groundnut (Khera et al. 2013) and 92.0% in maize (Jagtap et al. 2020). As the SNPs used in this study are true SNPs (pre-validated with Illumina Infinium genotyping), the possibility of failure due to sequencing artefacts is ruled out. The failure of assays might be due to improper amplification of alleles. Re-designing the primers or optimizing the PCR conditions might improve the performance of those assays.

Genotyping data from 119 F2 individuals at 124 SNP loci was used to construct the linkage map. Out of 124 markers,



Fig. 3. Comparison of genetic map constructed in the present study (JC-12 × RG-1963 F2) with the linkage map of JC-12 × 48-1 RILs (Senthilvel et al. 2019) and physical map (Xu et al. 2021)

The marker orders in the map were comparable with the previously published linkage map (Senthilvel et al. 2019) and physical map (Xu et al. 2021) with a very few local inconsistencies (inversions or rearrangements) especially in regions where markers were closely spaced. The comparison of maps is depicted in Fig 3. Cloutier et al. (2012) also observed local rearrangements of markers spanning over short interval particularly in closely spaced markers. To ascertain the reason for local discrepancies in marker orders, we scrutinized the genotyping data for data typing errors, missing values and segregation distortion. The missing values in the genotyping data appear to be the major factor causing local rearrangement of markers. Hacket and Bradfoort (2003) observed that the missing values in genotyping data can cause local rearrangements of markers particularly in closely placed markers with marker separation less than 2 cM.

This study added six more SNP loci viz., Rc_30010-75456, Rc_29093-59915, Rc_28567-12962, Rc_29939-142290 Rc_30061-182901 and Rc_29747-445740 to the linkage map constructed by Senthilvel et al. (2019) out of which Rc_28567-12962, Rc_29939-142290 and Rc_30061-182901 were mapped to the region in chromosome-8, where cluster of SNPs were found to be putatively linked to Fusarium wilt resistance in the genome-wide association analysis (Shaw et al., 2021). Thus, the assays developed in the study can be readily used for marker-assisted selection in addition to other breeding applications such as genetic purity/ identity analysis, QTL mapping and allele mining that require genotyping data ranging from a few to several hundred SNP loci per sample. The sequences of primer sets designed for 135 SNP can be accessed from http://krishi.icar.gov.in/jspui/ handle/123456789/67773.

Authors' contribution

Conceptualization of research (SS, MDM), Designing of the experiment (SS., MDM), Contribution of experimental materials (SS), Execution of experiments and data collection (MDM, YRR, KDS.), Analysis of data and interpretation (MDM, SS, SV), Preparation of manuscript (MDM, SS, MS, SV, BVS).

Acknowledgement

The first author is thankful to the Indian Council of Agricultural Research (ICAR), New Delhi for providing research fellowship during Ph.D. programme.

References

- Cloutier S., Ragupathy R., Miranda E., Radovanovic N., Reimer E., Walichnowski A., Ward K., Rowland G., Duguid S. and Banik M. 2012. Integrated consensus genetic and physical maps of flax (*Linum usitatissimum* L.). Theor. Appl. Genet., **125**: 1783–1795.
- FAOSTAT. 2019. Online Database of Food and Agriculture Organization of the United Nations, web: http://www.fao. org/faostat/en/#data/QC (accessed 06.07.2021).
- Foster J.T., Allan G.J., Chan A.P., Rabinowicz P.D., Ravel J., Jackson P.J. and Keim P. 2010. Single nucleotide polymorphisms for

assessing genetic diversity in castor bean (*Ricinus communis*). BMC Plant Biol., **10**: 13.

- Hackett C.A. and Broadfoot L.B. 2003. Effects of genotyping errors, missing values and segregation distortion in molecular marker data on the construction of linkage maps. Heredity, **90**: 33–38.
- He C., Holme J., Anthony J., 2014.SNP Genotyping.The KASP assay. In: Crop Breeding, Methods in Molecular Biology (Methods and Protocols). (eds. D. Fluery and R. Whitford), Springer, New York, USA: 75-86.
- Holtz Y., David J.L. and Ranwez V. 2017. The genetic map comparator: a user-friendly application to display and compare genetic maps. Bioinformatics, **33**: 1387–1388.
- Jagtap A.B., Vikal Y. and Johal G.S. 2020.Genome-Wide Development and Validation of Cost-Effective KASP Marker Assays for Genetic Dissection of Heat Stress Tolerance in Maize. Int. J. Mol. Sci., **21**: 7386.
- Jatayev S., Kurishbayev A., Zotova L., Khasanova G., Serikbay D., Zhubatkanov A., Botayeva M., Zhumalin A., Turbekova A., Soole K., Langridge P. and Shavrukov Y. 2017. Advantages of Amplifluor-like SNP markers over KASP in plant genotyping. BMC Plant Biol., **17**: 254.
- Khera P., Upadhyaya H.D., Pandey M.K., Roorkiwal M., Sriswathi M., Janila P., Guo Y.F., Mckain M.R., Nagy E.D., Knapp S.J., Leebens-Mack J., Conner J.D., Ozias-Akins P. andVarshney R.K. 2013. Single Nucleotide Polymorphism-based Genetic Diversity in the Reference Set of Peanut (Arachis spp.) by Developing and Applying Cost-Effective Kompetitive Allele Specific Polymerase Chain Reaction Genotyping Assays. The Plant Genome, 6.
- Meng L., Li H., Zhang L. and Wang J. 2015. QTL IciMapping: Integrated software for genetic linkage map construction and quantitative trait locus mapping in biparental populations. The Crop J., **3**: 269–283.
- Mutlu H., Meier M.A.R. 2010. Castor oil as a renewable resource for the chemical industry. Eur. J. Lipid Sci. Technol. 112, 10–30.
- Ogunniyi D.S. 2006. Castor oil: A vital industrial raw material. Bioresour. Technol., **97**: 1086–1091.
- Rasheed A., Wen W., Gao F., Zhai S., Jin H., Liu J., Guo Q., Zhang Y., Dreisigacker S., Xia X. and He Z. 2016. Development and validation of KASP assays for genes underpinning key economic traits in bread wheat. Theor. Appl. Genet., **129**: 1843–1860.
- Senthilvel S., Ghosh A., Shaik M., Shaw R.K.and Bagali P.G. 2019. Development and validation of an SNP genotyping array and construction of a high-density linkage map in castor. Sci. Rep., **9**: 3003.
- Shaw R.K., Shaik M., Prasad M.S.L., Prasad R.D., Mohanrao M.D. and Senthilvel S. 2021. Genomic regions associated with resistance to Fusarium wilt in castor identified through linkage and association mapping approaches. Genome, 65: 123-136.
- Wangkumhang P., Chaichoompu K., Ngamphiw C., Ruangrit U., Chanprasert J., Assawamakin A. andTongsima S. 2007. WASP: a Web-based Allele-Specific PCR assay designing tool for detecting SNPs and mutations. BMC Genomics, 8: 275.
- Xu W., Wu D., Yang T., Sun C., Wang Z., Han B., Wu S., Yu A., Chapman M.A., Muraguri S., Tan Q., Wang W., Bao Z., Liu A. and Li D-Z.
 2021. Genomic insights into the origin, domestication and genetic basis of agronomic traits of castor bean. Genome Biol., 22: 113.