Current Botany 2024, 15: 70-75
doi: 10.25081/cb.2024.v15.8844
https://updatepublishing.com/journal/index.php/cb

Research Article

ISSN: 2220-4822

Discovery of a nuclear haplotype potentially useful for the identification of medicinal rice Njavara (*Oryza sativa* L.)

R. Dinesh Raj[1]*, U. Suresh Kumar[2], M. R. Vinitha[3], B. C. Patra[4], O. N. Singh[4], George Thomas[3]

[1]Post Graduate Department of Botany and Biotechnology, Bishop Moore College, Mavelikkara-690110, Kerala, India, [2]Regional Facility for DNA Fingerprinting, Rajiv Gandhi Centre for Biotechnology, Thiruvananthapuram-695014, Kerala, India, [3]Plant Disease Biology and Biotechnology, Rajiv Gandhi Centre for Biotechnology, Thiruvananthapuram-695014, Kerala, India, [4]Division of Crop Improvement, Central Rice Research Institute, Cuttack-753006, Odisha, India

ABSTRACT

The present study report the development of an amplified fragment length polymorphism (AFLP)-derived sequence characterized amplified region (SCAR) marker for reliable identification of Njavara (*Oryza sativa* landrace Njavara), a rice landrace in India used extensively in health foods and Ayurveda treatments. The SCAR locus, named $OsN_{SCAR131}$ after *O. sativa* landrace Njavara, is located on chromosome 3 between nucleotide positions 7793982 and 7794108, and yields a 131-bp allele in Njavara accessions and a 127-bp allele in other rice cultivars due to a 4-bp Insertion-Deletion (InDel) mutation at nucleotide position 7794026. We standardized the SCAR assay to be like those used for detecting microsatellite markers by using fluorescently (6-FAM) labeled primers and separating the alleles by capillary electrophoresis. As an alternative, we further adapted the method so as to allow allele detection by polyacrylamide gel electrophoresis (PAGE). A single rice grain can be tested for authentication by this PAGE assay. The SCAR marker developed here has great utility in authenticating Njavara grains in both the health food and pharmaceutical sectors.

KEYWORDS: Medicinal rice, Njavara, Ayurveda, SCAR, Health rice

Received: February 27, 2024
Revised: May 11, 2024
Accepted: May 13, 2024
Published: May 22, 2024

***Corresponding Author:**
R. Dinesh Raj
Email: dineshrajr@gmail.com

INTRODUCTION

Njavara (*Oryza sativa* L. landrace Njavara), called *shashtika* in Sanskrit, is an Indian rice landrace used as a health food and in Ayurveda treatments (Deepa *et al.*, 2008; 2010; Simi & Abraham, 2008; Sreejayan *et al.*, 2011; Disket *et al.*, 2013; Reshmi & Nandini, 2018). Several descriptions about the medicinal and nutritive features of Njavara can be found in ancient Sanskrit treatises of Ayurveda such as Charakasamhita and Susrutasamhita (circa. 300-700 BCE). Scientific research shows significantly higher levels of nutrients, anti-oxidants and anti-inflammatory molecules in Njavara as compared to other rice cultivars (Deepa *et al.*, 2008; Rao *et al.*, 2010; Mohanlal *et al.*, 2011, 2013; Lal preethi *et al.*, 2022). The ayurvedic industry has been utilizing several health-conducive applications using Njavara grains, like *Njavavarakizhi* and *Njavara theppu* (*shashtika anna lepa*), both claimed as therapies for neural, circulatory and rheumatic disorders (Deepa *et al.*, 2008, 2010; Simi & Abraham, 2008; Sreejayan *et al.*, 2011; Reshmi & Nandini, 2018). With the recent upsurge of interest among people in alternative therapeutic approaches, Ayurvedic traditions have recently witnessed a revival in both India and abroad (Patwardhan *et al.*, 2005; Simi & Abraham, 2008). Selection of accurate germplasm in compliance with traditional knowledge is essential for ensuring consistency, quality and efficacy in traditional practices (Patwardhan *et al.*, 2005).

Njavara cultivation is currently localized to Kerala state. We previously characterized accessions of Njavara (Sreejayan *et al.*, 2011) and found that Njavara is morphologically and genetically distinct from syntopic traditional cultivars (Varghese *et al.*, 2013), but no trait was identified that is suitable to authenticate Njavara grains in the commercial sector. Deepa *et al.* (2009) reported two molecular polymorphisms between Njavara and two other cultivars, Jyothi and IR 64, but the variants were not validated in a larger sample set or converted into commercially amenable markers. Scientific methods are essential to authenticate Njavara grains in the health food and pharmaceutical sectors in order to prevent intentional fraud (Njavara is two to three times costlier than the rice used for regular consumption) or inadvertent mixing due to improper identification by farmers or physicians.

Among the different molecular markers used for the identification of cultivars and medicinal species, SCAR markers have several advantages as they are locus-specific, less sensitive to PCR conditions, easy to assay and reproducible across laboratories (Kiran *et al.*, 2010; Sarwat *et al.*, 2011, Zheng *et al.*, 2021; Kethirun *et al.*, 2023). SCAR markers are generally derived from species- or cultivar-specific random amplified polymorphic DNA (RAPD) markers (Bautista *et al.*, 2002; Ohtsubo & Nakamura, 2007; Jain *et al.*, 2008; Moon *et al.*, 2010; Marieschi *et al.*, 2011) or AFLP markers (Shan *et al.*, 1999; Shirasawa *et al.*, 2004). However, the success rate of SCAR development from AFLP markers is generally low (Brugmans *et al.*, 2003; Shirasawa *et al.*, 2004). AFLPs detect Insertions-Deletions (InDels) and single nucleotide polymorphisms (SNPs) (Brugmans *et al.*, 2003; Shirasawa *et al.*, 2004), and the polymorphic nature of an ancestral AFLP fragment is usually lost when amplicons are generated from target genomic DNA by using primers designed to anneal internally to the terminal *Mse*I and *Eco*RI primer sequences (Shan *et al.*, 1999; Brugmans *et al.*, 2003). Efficiency in the conversion of AFLPs to SCARs can be improved by using primers that anneal to sequences flanking the AFLP (Brugmans *et al.*, 2003). In particular, Shirasawa *et al.* (2004) successfully developed several SCAR markers able to differentiate the two major Japanese rice cultivars, Nipponbare and Koshihikari, by designing AFLP-flanking primers based on alignments of rice genomic sequences. In the present study, using the method of Shirasawa *et al.*, (2004), we explored the possibility of developing SCAR markers for authentication of Njavara using the predominantly Njavara-specific AFLPs reported earlier (Sreejayan *et al.*, 2011).

MATERIALS AND METHODS

Plant Materials

The plant materials used in the study included: 73 accessions of Njavara, covering the complete geographic range of its cultivation in Kerala; 85 traditional rice cultivars from Kerala, comprising most of the cultivars currently grown in Kerala; 107 traditional cultivars randomly chosen from 13 other rice-cultivating states in India; and 21 improved hybrid varieties usually cultivated in Kerala (Supplementary Table S1).

DNA Isolation

Genomic DNA was isolated from 100 mg of tender leaf tissues from a single plant using a GenElute™ Plant Genomic DNA Miniprep Kit (Sigma) following the manufacturer's instructions. Genomic DNA from a single rice grain was isolated using a NucleoSpin® Plant II (Macherey-Nagel) kit according to the protocols provided by the manufacturer. Final elution was in 15 µL of elute buffer and the whole amount was used for PCR amplification.

Gel elution, Sequencing, Primer Design and PCR Analysis

AFLP fragments were eluted from the gel using a GFX™ PCR DNA and Gel Band purification kit (GE Healthcare), re-amplified using the corresponding AFLP primer combination and cloned using a pGEM-T Easy Vector System (Promega). The clones were sequenced on an ABI Prism 3730 genetic analyzer (Applied Biosystems) using the ABI PRISM BigDye Terminator Cycle Sequencing Ready Reaction Kit, version 3.0.

Primers for PCR amplification of AFLP sequences were designed according to the method of Shirasawa *et al.* (2004). In short, the AFLP sequences were aligned with rice genomic sequences following homology searches in the National Center for Biotechnology Information (NCBI) database, and primers were designed to sequences flanking the AFLP sequences using the software FastPCR (Kalendar *et al.*, 2009). We scanned approximately 300 bp of sequence flanking both ends of the AFLP sequence to locate a suitable site for primer design, but with the expected amplicon size not exceeding 900 bp including AFLP sequence. The primer combinations were evaluated *in silico* using FastPCR. The designed primers were custom synthesized by Sigma (Sigma Genosys, Bangaluru, India). Homologous sequences retrieved following BLAST searches using AFLP sequence queries in the NCBI database were annotated using the Rice Genome Annotation Project, Release 7: Genomic sequences (http://rice.plantbiology.msu.edu/index.shtml).

Each PCR was carried out in a total volume of 10 µL containing 15 ng of genomic DNA, 0.01 µM each of forward and reverse primers, 1X PCR buffer, 2.5 mM $MgCl_2$, 0.5 mM dNTPs, and 1 unit *Taq* DNA polymerase (AmpliTaq gold, Applied Biosystems). The PCR cycles were as follows: 94 °C for 5 min, followed by 40 cycles of 94 °C for 30 s, 55 °C for 30 s and 72 °C for 30 s, and a final extension at 72 °C for 7 min in a thermocycler (Mastercycler gradient, Eppendorf). The PCR products were fractionated on 2% agarose gels.

SCAR Assay

The SCAR assay was performed using the methodology described by Schuelke (2000) for microsatellite analysis. This method performs allele detection by using a forward primer linked with an 18-nucleotide M13 tail (5'-tgtaaaacgacggccagt-3') in combination with a single fluorescently (6–FAM) labeled M13 primer. The alleles were separated by capillary electrophoresis on an ABI Prism 3730 genetic analyzer. Allele sizing was performed by using the software Genescan 3.1.2 (Applied Biosystems) by comparing with the internal size standard ROX 400 (Applied Biosystems). Allele calling and sorting was performed using the software GeneMapper V. 4 (Applied Biosystems) and binning was done manually. Alternatively, SCAR alleles were detected by electrophoretic separation of the PCR products on 15 cm long 8% polyacrylamide gels for 14 h at 50 V. The gels were stained with 0.5 µg/mL ethidium bromide solution and the alleles were scored against 50-bp molecular size standards (New England Biolabs).

RESULTS AND DISCUSSION

The nine predominantly Njavara-specific AFLP fragments (E-AA x M-CAC_{210}, E-AA x M-CAC_{358}, E-AA x M-CTA_{312}, E-AC x M-CAG_{184}, E-AC x M-CAG_{410}, E-AC x M-CAT_{278} and E-TA x M-CTT_{235}, E-TA x M-CTT_{275} and E-TG x M-CTA_{149}) identified

earlier (Sreejayan *et al.*, 2011) were analyzed in this study. Of the nine primer pairs tested, primers designed to sequences flanking E-AA x M-CAC$_{358}$ (forward: 5'-gcgacaagaattatggtcaggtttg - 3'; reverse: 5'- atgactgaagtcgagattgaggaga - 3') consistently yielded a single conspicuous fragment of approximately 900 bp, but was monomorphic between Njavara accessions and traditional cultivars on agarose gels. The other primer pairs yielded either no amplification or multiple faint bands or inconsistent results.

AFLPs mostly detect InDels and SNPs (Shirasawa *et al.*, 2004). As agarose gel electrophoresis cannot resolve length polymorphisms of short InDels (InDels < 5 bp) and SNPs (Shen *et al.*, 2004; Liu *et al.*, 2013), we sequenced the 900-bp fragment in a few samples each of Njavara and traditional cultivars and performed a multiple alignment comparison of the sequences generated together with rice genomic sequences. The consensus sequence was 923 bp and located between nucleotide positions 7793761 and 7794683 of rice chromosome 3, within an expressed protein locus (LOC_Os03g14310.1). The 923-bp sequence comprised 217 bp of exon and 706 bp of intron regions of this locus (Figure 1). The 358-bp *Mse*I- and *Eco*RI-bordered AFLP fragment lies within the intron region (Figure 1). The multiple sequence alignment revealed a 4-bp InDel at nucleotide position 7794026 that is polymorphic between Njavara accessions and traditional cultivars. Two motifs of an AT repeat were inserted at this region in the Njavara accessions but not in the traditional cultivars (Figure 1).

Sequence analysis of 266 independently amplified 923-bp fragments (NCBI accession numbers: KF731340-KF731605) recovered from 73 Njavara accessions and 193 cultivars (the latter including 80 cultivars from Kerala and 20 improved hybrid varieties) (Supplementary Table S1) confirmed the polymorphism between the Njavara accessions and the other cultivars. Multiple sequence alignment of 266 sequences revealed altogether 47 polymorphic sites, comprising of 17 SNPs and 30 InDels within 748 bp of the aligned 923-bp sequences (Figure 2). Analyses detected eight distinct haplotypes in 266 sequences, and all Njavara accessions invariably belonged to haplotypes 1, 2 or 3 and no other cultivars shared these haplotypes (Figure 2 & Supplementary Table S1). While the genetic relationships among cultivars revealed by SCAR sequences were earlier reported to be inconsistent with known relationships in olive-trees (Bautista *et al.*, 2002), the genetic relationship revealed by the 923-bp sequence between the Njavara and traditional cultivars is consistent with the distinct relationship revealed between two earlier by microsatellite markers as well as morphological traits (Varghese *et al.*, 2013), indicating that this sequence harbors phylogenetic signatures that differentiate between Njavara and other cultivars.

The rice genome has one InDel every 953 bp (1050 InDels/Mb) on average, and 90% of the InDels can be used as molecular markers (Shen *et al.*, 2004). The genomic density of InDels outnumbers that of microsatellite markers (Liu *et al.*, 2013), and microsatellites and InDels are the markers of choice in most laboratories for the development of plant molecular markers (Shen *et al.*, 2004; Liu *et al.*, 2013). However, InDel assay by sequence analysis is time consuming and technically demanding; hence, it may not be commercially viable. InDel

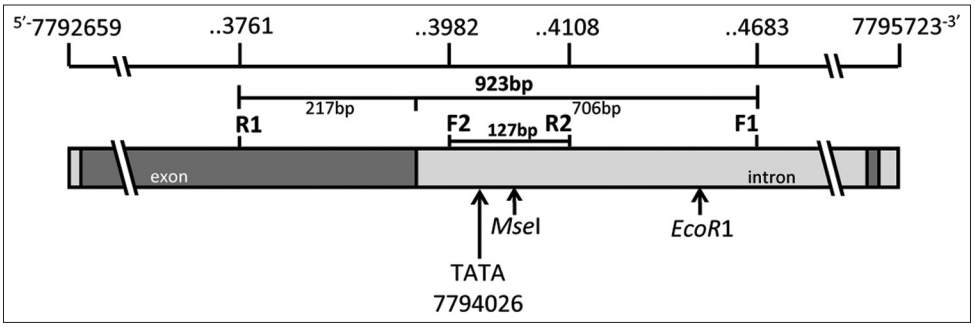

Figure 1: Map of the expressed protein-coding locus LOC_Os03g14310.1showing the location of the SCAR locus *Os*N$_{SCAR131}$on rice chromosome 3. Numbers at the top of the diagram indicate the nucleotide positions on chromosome 3. F1 and R1, and F2 and R2 represent the priming sites for amplification of 923-bp fragment and the SCAR locus *Os*N$_{SCAR131}$, respectively. Locations of the *Mse*I and *Eco* RI restriction sites bordering the original E-AA x M-CAC$_{358}$fragment and the Njavara-specific InDel ATAT are indicated

```
Hap_1   CAGCTCG-ATATCTCATGTCCAGTCAGTACTAAAT-----GGCAGAA
Hap_2   .......-.............................-----.A.....
Hap_3   ...-...-.............................-----.......
Hap_4   .....T.-----.....................G...-----.A.....
Hap_5   .......-----.....................G...-----.A.....
Hap_6   .....T.-----.....................G...-----.AT....
Hap_7   GGA.G.AT----AG-------------------GCCTTAG.A.CCC.
Hap_8   .......-----.....................G...-----TA....G
```

Figure 2: Eight haplotypes identified among sequences ofthe 923-bp fragment by analyzing 266 rice cultivars, including 73 Njavara individuals. The Njavara-specific InDels are shaded

markers can be assayed using the same separation and detection technologies as microsatellite markers (Liu *et al.*, 2013).

With the aim of being able to assay the 4-bp InDel in the manner of a microsatellite marker, we designed several primer pairs to the sequences flanking the 4-bp InDel in order to amplify fragments in the range of 100-160 bp. Each primer pair was then assayed as if testing a microsatellite marker using a few Njavara accessions and traditional cultivars following the method of Schuelke (2000), and alleles were separated by capillary electrophoresis. One of the primer pairs yielded a 149-bp (including the 18-nucleotide M13 tail linked to the forward primer) fragment in Njavara (Figure 3a) and a 145-bp fragment in traditional cultivars (Figure 3b). We validated the Njavara specificity of the 131-bp allele (excluding the 18-nucleotide M13 tail) using 286 cultivars, including the 266 cultivars used for assaying the InDel by sequencing (Supplementary Table S1). The locus lies between nucleotide positions 7793982 and 7794108 on chromosome 3 (Figure 1). We named this SCAR marker as *Os*$N_{SCAR131}$ after *O. sativa* landrace Njavara, and the primers amplifying this SCAR locus as *Os*$N_{SCAR131}$_F (5'-actactacgacacccaacatactt-3') and *Os*$N_{SCAR131}$_R (5'-gagatactaagatgtaactgactg-3'). Heterozygosity was recorded at the *Os*$N_{SCAR131}$ locus in six Njavara accessions (N34, N45, N50, N54, N7, N85) (Figure 3c & Supplementary Table S1), indicating the occurrence of genetic admixing events in some Njavara accessions. Several authors reported the occurrence of substantial intra-subspecies genetic admixture events in the evolution of rice (Zhao *et al.*, 2011; Santos *et al.*, 2019)

To facilitate the use of *Os*$N_{SCAR131}$-based authentication of Njavara grains in less equipped health food or pharmaceutical operations, we adapted the SCAR allele detection methodology to a polyacrylamide gel electrophoresis (PAGE) platform (Figure 3d). We also standardized the DNA isolation procedure (see Materials and methods) and SCAR allele detection from a single grain in order to be able to detect adulterants in the commercial sector, as the grains of Njavara and those of most other cultivars are indistinguishable based on morphology.

SCAR markers have been developed for the authentication of premium cultivars (Vidal *et al.*, 2000; Bautista *et al.*, 2002; Shirasawa *et al.*, 2004; Ohtsbo & Nakamura, 2007; Saengprajak, 2012; Qv *et al.*, 2024) and herbal drugs (Jain *et al.*, 2008; Kiran *et al.*, 2010; Moon *et al.*, 2010; Marieschi *et al.*, 2011). Our results support the earlier report that the success rate in the conversion of AFLPs into SCARs can be improved by designing primers to the flanking regions (Brugmans *et al.*, 2003; Shirasawa *et al.*, 2004). Codominant SCAR markers like *Os*$N_{SCAR131}$ have been reported previously in grapevines (Vidal *et al.*, 2000) and rice (Shirasawa *et al.*, 2004; Saengprajak, 2012). Codominant SCAR markers have an advantage over strictly dominant markers, because the former differentiate between experimental failures and null alleles whereas the latter do not. With the recent upsurge of interest among people in Njavara-based health

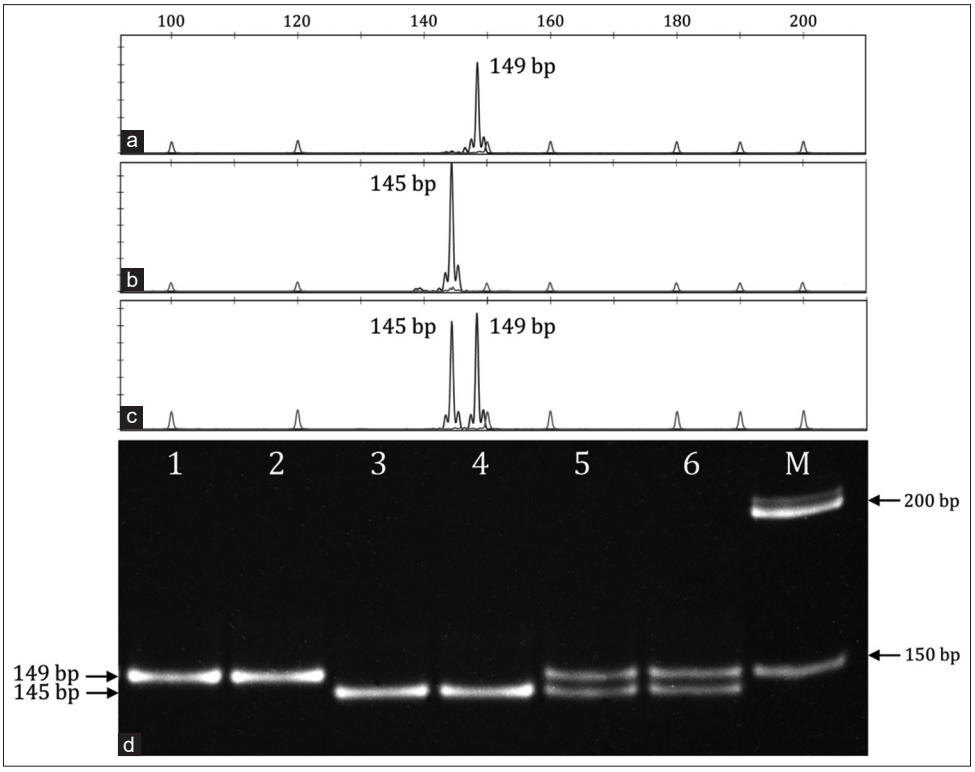

Figure 3: Representative chromatograms and an ethidium bromide-stained polyacrylamide gel showing allele detection at the SCAR locus *Os*$N_{SCAR131}$: a, b, c) alleles detected by capillary electrophoresis on an ABI Prism 3730 genetic analyzer in Njavara accession N2, traditional cultivar Bengi, and Njavara accession 45, respectively. Allele sizes are indicated and the smaller peaks represent the internal molecular size standard ROX 400 and d) alleles detected by PAGE in *lanes*: 1-Njavara accession N2, 2-Njavara accession N4, 3-traditional cultivar PTB8, 4- traditional cultivar Bengi, 5-Njavara accession N34, 6-Njavara accession N45 and *M*-50-bp molecular size standard.

food and healthcare systems, we believe that the SCAR marker developed by us will help expand the reach of Njavara-based applications by providing a scientific method for raw material identification and authentication.

Njavara rice is known for its distinct agronomically important traits like short duration (60 days maturity) and upland adaptation (Sreejayan *et al.*, 2011; Disket *et al.*, 2013; Rani & Sukumari, 2016). Nutritional superiority of njavara grains was reported elsewhere (Deepa *et al.*, 2008; Rao *et al.*, 2010; Mohanlal *et al.*, 2011; 2013; Lal preethi *et al.*, 2022). This makes njavara an ideal genetic resource for crop improvement in rice. The identification tool reported in this study will have applications in plant materials selection and other breeding strategies using njavara. Similar approaches using SCAR markers were reported earlier for hybrid rice identification (Li *et al.*, 2012), mutant selection (Sangwijit *et al.*, 2012), and specific trait based selection (Boopathi *et al.*, 2003), etc.

CONCLUSION

Present study developed a SCAR marker for authentication of the Indian health rice Njavara. The marker yields a 131-bp allele in Njavara and a 127-bp allele in other rice cultivars analysed. The marker is located on chromosome 3. A 4-bp InDel yields the polymorphism between Njavara and other cultivars. The marker can be assayed by capillary electrophoresis, like a microsatellite marker and also through PAGE gel assay. The SCAR marker developed here would be a valuable tool to authenticate the perceptually identified raw materials of njavara in the health food and pharmaceutical sectors.

ACKNOWLEDGEMENTS

G.T. acknowledges the Kerala State Council for Science Technology and Environment for funding (Research grant No. 024/SRSLS/2006/KSCSTE). D. R. R. acknowledges UGC-FDP Teacher Fellowship (No. FIP/11th Plan/KLKE002 TF03).

REFERENCES

Bautista, R., Crespillo, R., Canovas, F. M., & Claros, M. G. (2002). Identification of olive-tree cultivars with SCAR markers. *Euphytica, 129*, 33-41. https://doi.org/10.1023/A:1021528122049

Boopathi, N. M., Babu, R. C., Chandrakala, R., Mathews, S. C., Singh, A., Chezhian, P., & Shanmugasundaram, P. (2003). Identification of SCAR marker linked to root thickness in rice (*Oryza sativa* L.). *Tropical Agricultural Research, 15*, 61-67.

Brugmans, B., van der Hulst, R. G. M., Visser, R. G. F., Lindhout, P., & van Eck, H. J. (2003). A new and versatile method for the successful conversion of AFLP markers into simple single locus markers. *Nucleic Acid Research, 31*(10), e55. https://doi.org/10.1093/nar/gng055

Deepa, G., Singh, V., & Naidu, K. A. (2008). Nutrient composition and physicochemical properties of Indian medicinal rice-Njavara. *Food Chemistry, 106*(1), 165-171. https://doi.org/10.1016/j.foodchem.2007.05.062

Deepa, G., Singh, V., & Naidu, K. A. (2010). A comparative study on starch digestibility, glycemic index and resistant starch of pigmented ('Njavara' and 'Jyothi') and a non-pigmented ('IR 64') rice varieties. *Journal of Food Science and Technology, 47*, 644-649. https://doi.org/10.1007/s13197-010-0106-1

Deepa, G., Venkatachalam, L., Bhagyalakshmi, N., Shashidhar, H. E., Singh, V., & Naidu, K. A. (2009). Physicochemical and genetic analysis of an endemic rice variety, Njavara (*Oryza sativa* L.), in comparison to two popular south Indian cultivars, Jyothi (PTB39) and IR 64. *Journal of Agriculture and Food Chemistry, 57*(24), 11476-11483. https://doi.org/10.1021/jf902052p

Disket, J., Lukhmana, N., & Gupta, R. K. (2013). Significance of Njavara, a rice cultivar indigenous to Kerala (India): An Ethnomedicinal Perspective. *Significance, 4*(1), 14-19.

Jain, N., Shasany, A. K., Singh, S., Khanuja, S. P. S., & Kumar, S. (2008). SCAR markers for correct identification of *Phyllantus amarus*, *P. fraterns*, *P. debilis* and *P. urinaria* used in scientific investigations and dry leaf bulk herb trade. *Planta Medica, 74*(3), 296-301. https://doi.org/10.1055/s-2008-1034314

Kalendar, R., Lee, D., & Schulman, A. H. (2009). FastPCR software for PCR primer and probe design and repeat search. *Genes, Genomes and Genomics, 3*(1), 1-14.

Kethirun, L., Umpunjun, P., Chuenboonngarm, N., & Viboonjun, U. (2023). Development of a sequence-characterized amplified region (SCAR) marker for female off-season flowering detection in date palm (*Phoenix dactylifera* L.). *Journal of Plant Biotechnology, 50*, 190-199. https://doi.org/10.5010/JPB.2023.50.024.190

Kiran, U., Khan, S., Mirza, K. J., Ram, M., & Abdin, M. Z. (2010). SCAR markers: A potential tool for authentication of herbal drugs. *Fitoterapia, 81*(8), 969-976. https://doi.org/10.1016/j.fitote.2010.08.002

Lal preethi, S. S., Sabu, V., & Helen, A. (2022). Njavara rice (*Oryza sativa* Linn.) bran oil exerts anti-inflammatory effects through regulation of Notch-mediated T-cell receptor (TCR) activation in experimentally induced atherosclerosis. *Cellular and Molecular Biology, 68*(10), 21-29. https://doi.org/10.14715/cmb/2022.68.10.4

Li, S.-J., Xie, H.-W., Qian, M.-J., Chen, G.-H., Li, S.-Q., & Zhu, Y.-G. (2012). A set of SCAR markers efficiently differentiating hybrid rice. *Rice Science, 19*(1), 14-20. https://doi.org/10.1016/S1672-6308(12)60015-8

Liu, B., Wang, Y., Zhai, W., Deng, J., Wang, H., Cui, Y., Cheng, F., Wang, X., & Wu, J. (2013). Development of InDel markers for *Brassica rapa* based on whole-genome re-sequencing. *Theoretical and Applied Genetics, 126*, 231-239. https://doi.org/10.1007/s00122-012-1976-6

Marieschi, M., Torelli, A., Bianchi, A., & Bruni, R. (2011). Development of a SCAR marker for the identification of *Olea europaea* L.: A newly detected adulterant in commercial Mediterrranean oregano. *Food Chemistry, 126*(2), 705-709. https://doi.org/10.1016/j.foodchem.2010.11.030

Mohanlal, S., Parvathy, R., Shalini, V., Helen, A., & Jayalekshmy, A. (2011). Isolation, characterization and quantification of tricin and flavonolignans in the medicinal rice Njavara (*Oryza sativa* L.) as compared to staple varieties. *Plant Foods for Human Nutrition, 66*, 91-96. https://doi.org/10.1007/s11130-011-0217-5

Mohanlal, S., Parvathy, R., Shalini, V., Mohanan, R., Helen, A., & Jayalekshmy, A. (2013). Chemical indices, antioxidant activity and anti-inflammatory effect of extracts of the medicinal rice "Njavara" and staple varieties: a comparative study. *Journal of Food Biochemistry, 37*(3), 369-380. https://doi.org/10.1111/j.1745-4514.2011.00646.x

Moon, B. C., Choo, B. K., Cheon, M. S., Yoon, T., Ji, Y., Kim, B. B., Lee, A. Y., & Kim, H. K. (2010). Rapid molecular authentication of three medicinal plant species, *Cynanchum wilfordii*, *Cynanchum auriculatum*, and *Polygonum multiflorum* (*Fallopia multiflorum*), by the development of RAPD-derived SCAR markers and multiplex-PCR. *Plant Biotechnology Reports, 4*, 1-7. https://doi.org/10.1007/s11816-009-0114-7

Ohtsubo, K., & Nakamura, S. (2007). Cultivar identification of rice (*Oryza sativa* L.) by polymerase chain reaction method and its application to processed rice products. *Journal of Agricultural and Food Chemistry, 55*(4), 1501-1509. https://doi.org/10.1021/jf062737z

Patwardhan, B., Warude, D., Pushpangadan, P., & Bhatt, N. (2005). Ayurveda and Traditional Chinese Medicine: A Comparative Overview. *Evidenced-Based Complementary and Alternative Medicine, 2*, 465-473. https://doi.org/10.1093/ecam/neh140

Qv, M., Feng, G., Chen, S., Chen, H., Chen, C., Wang, F., Lv, S., Dai, L., Liu, H., Huang, B., Li, X., Su, Z., & Xu, C. (2024). The development and utilization of two SCAR markers linked to the resistance of banana (*Musa* spp. AAA) to *Fusarium oxysporum* f. sp. *cubense* race 4. *Euphytica, 220*, 69. https://doi.org/10.1007/s10681-024-03323-4

Rani, S., & Sukumari, P. (2016). Productivity and economics of medicinal rice Njavara (*Oryza sativa* L.) as influenced by different establishment techniques in lowland condition of Kerala, India. *African Journal of Agricultural Research, 11*(22), 1984-1989. https://doi.org/10.5897/AJAR2013.7566

Rao, A. S. V. C., Reddy, S. G., Babu, P. P., & Reddy, A. R. (2010). The antioxidant and antiproliferative activities of methanolic extracts from Njavara rice bran. *BMC Complementary and Alternative Medicine, 10*, 4. https://doi.org/10.1186/1472-6882-10-4

Reshmi, R., & Nandini, P. V. (2018). Medicinal Properties of Njavara Rice (*Oryza sativa* L.) cv. *International Journal of Advanced Engineering, Management and Science, 4*(3), 196-207. https://doi.org/10.22161/ijaems.4.3.10

Saengprajak, J. (2012). Development of a sequence characterized amplified region (SCAR) marker for identification of Thai jasmine rice (*Oryza sativa* L.) variety 'KDML 105'. *African Journal of Biotechnology, 11*(59), 12262-12268. https://doi.org/10.5897/AJB12.244

Sangwijit, K., Thangsunan, P., Cutler, R., & Anuntalabhochai, S. (2012). Development of SCAR marker for Thai fragrant rice (*Oryza sativa* L. var. *indica* cv. Pathumthani 1) mutants induced by low energy ion beam. *Chiang Mai Journal of Science, 39*(4), 545-553.

Santos, J. D., Chebotarov, D., McNally, K. L., Bartholomé, J., Droc, G., Billot, C., & Glaszmann, J. C. (2019). Fine scale genomic signals of admixture and alien introgression among Asian rice landraces. *Genome Biology and Evolution, 11*(5), 1358-1373. https://doi.org/10.1093/gbe/evz084

Sarwat, M., Nabi, G., Das, S., & Srivastava, P. S. (2011). Molecular markers in medicinal plant biotechnology: past and present. *Critical Reviews in Biotechnology, 32*(1), 74-92. https://doi.org/10.3109/07388551.2011.551872

Schuelke, M. (2000). An economic method for the fluorescent labeling of PCR fragments. *Nature Biotechnology, 18*, 233-234. https://doi.org/10.1038/72708

Shan, X., Blake, T. K., & Talbert, L. E. (1999). Conversion of AFLP markers to sequence-specific PCR markers in barley and wheat. *Theoretical and Applied Genetics, 98*, 1072-1078. https://doi.org/10.1007/s001220051169

Shen, Y.-J., Jiang, H., Jin, J.-P., Zhang, Z.-B., Xi, B., He, Y.-Y., Wang, G., Wang, C., Qian, L., Li, X., Yu, Q.-B., Liu, H.-J., Chen, D.-H., Gao, J.-H., Huang, H., Shi, T.-L., & Yang, Z.-N. (2004). Development of genome-wide DNA polymorphism database for map-based cloning of rice genes. *Plant Physiology, 135*(3), 1198-1205. https://doi.org/10.1104/pp.103.038463

Shirasawa, K., Kishitani, S., & Nishio, T. (2004). Conversion of AFLP markers to sequence-specific markers for closely related lines in rice by use of the rice genome sequence. *Molecular Breeding, 14*, 283-292. https://doi.org/10.1023/B:MOLB.0000047791.94870.40

Simi, C. K., & Abraham, T. E. (2008). Physicochemical rheological and thermal properties of Njavara rice (*Oryza sativa*) starch. *Journal of Agricultural and Food Chemistry, 56*(24), 12105-12113. https://doi.org/10.1021/jf802572r

Sreejayan, Suresh Kumar, U., Varghese, G., Jacob, T. M., & Thomas, G. (2011). Stratification and population structure of the genetic resources of ancient medicinal rice (*Oryza sativa* L.) landrace Njavara. *Genetic Resources and Crop Evolution, 58*, 697-711. https://doi.org/10.1007/s10722-010-9613-1

Varghese, G., Jose, M., Dinesh Raj, R., Bacianowski, J., Thomas, G., & Omanakumari, N. (2013). Quantitative and molecular analyses reveal a deep genetic divergence between the ancient medicinal rice (*Oryza sativa*) Njavara and syntopic traditional cultivars. *Annals of Applied Biology, 164*(1), 95-106. https://doi.org/10.1111/aab.12083

Vidal, J. R., Delavault, P., Coarer, M., & Defontaine, A. (2000). Design of grapevine (*Vitis vinifera* L.) cultivar-specific SCAR primers for PCR fingerprinting. *Theoretical and Applied Genetics, 101*, 1194-1201. https://doi.org/10.1007/s001220051597

Zhao, K., Tung, C.-W., Eizenga, G. C., Wright, M. H., Ali, M. L., Price, A. H., Norton, G. J., Islam, M. R., Reynolds, A., Mezey, J., McClung, A. M., Bustamante, C. D., & McCouch, S. R. (2011). Genome-wide association mapping reveals a rich genetic architecture of complex traits in *Oryza sativa*. *Nature Communications, 2*, 467. https://doi.org/10.1038/ncomms1467

Zheng, K., Cai, Y., Chen, W., Gao, Y., Jin, J., Wang, H., Feng, S. & Lu, J. (2021). Development, identification, and application of a germplasm specific SCAR Marker for *Dendrobium officinale* Kimura et Migo. *Frontiers in Plant Science, 12*, 669458. https://doi.org/10.3389/fpls.2021.669458