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Development of SNP-Based Dcaps Markers for Identifying *Oryza Officinalis*

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ABSTRACT

A single nucleotide po]ymorphic sequence (SNP) in the matK gene of chloroplast DNA that discriminate *Oryza officinalis* and its related species has been found. The derived cleaved amplified polymorphic sequence (dCAPS) primers were designed based on the SNP and the genomic DNA was used for amplification. The amplicons were digested by restrict enzyme to convert the SNP into dCAPS markers which can be applied in genetic map construction, gene mapping, germplasm identification, genetic diversity research and other fields.

Keywords: SNP; *Oryza Officinalis*; dCAPS

Abbreviations: SNP: Single Nucleotide Polymorphic Sequence; dCAPS: Derived Cleaved Amplified Polymorphic Sequence; SNPs: Single Nucleotide Polymorphisms; bp: Base Pairs; PAGE: Polyacrylamide Gel Electrophoresis

Introduction

Derived cleaved amplified polymorphic sequence (dCAPS) is a molecular labeling method produced by the combination of PCR reaction and enzyme digestion [1,2]. It is further improved on the basis of CAPS markers. The basic principle of CAPS technology is to firstdesign a set of specific PCR primers with the DNA sequence of known sites, and then amplify them, and then use a specific restriction enzyme to digest the amplified products and perform RFLP analysis; DCAPS technology is to introduce mismatched bases into amplification primers to generate new restriction endonuclease sites that can be combined with SNP sites. After endonuclease digestion, it produces polymorphisms similar to CAPS markers. DCAPS molecular marker technology is mainly applied to the research of plant gene location, map based cloning, typing and variety identification [3-6].

Oryza officinalis is a perennial herb of Oryza. As one of only three wild rice species native to China, it serves as a vital genetic reservoir, harboring valuable attributes such as resistance to pests and diseases, environmental stress tolerance, high photosynthetic efficiency, and superior yield and quality [7-8]. It is a perennial diploid plant with CC genome. It grows strongest among 22 species of Oryza genus, more than 20 times as high as common cultivated rice, and its growth height can reach 3 m. It is distributed in tropical and subtropical regions of Asia, Africa and Australia [9]. In China, it is mainly distributed in Yunnan, Guangdong, Hainan and Guangxi. In 1999, medicinal wild rice was listed as an endangered species under second- class national protection in the list of national key protected wild plants (the first batch). In 2013, it was included as a critically endangered plant in the red list of biodiversity in China. It has high academic status and scientific research value.

Accurate identification of *O. officinalis* is a prerequisite for its conservation, germplasm utilization, and molecular breeding. Traditional identification methods primarily rely on morphological characteristics, however, these are often influenced by environmental factors and developmental stages. Consequently, such methods suffer from low accuracy and make it difficult to distinguish *O. officinalis* from closely related Oryza species, particularly at the seedling stage or within mixed germplasm collections. Molecular markers have emerged as robust tools for species identification due to their stability, accuracy, and independence from environmental conditions. Among these, single nucleotide polymorphisms (SNPs) represent the most abundant and stable genetic variations in genomes, offering high resolution for distinguishing closely related species and genotypes. This study aims to address the lack of specific molecular markers for *O. officinalis* identification, thereby facilitatin the sustainable utilization of this valuable genetic resource and contributing to the genetic improvement of cultivated rice.

Material and Methods

The plant materials and their sources are shown in Table 1.

Table 1: Plant materials and their sources.

No.	Species	Material sources
1	*Oryza meyeriana*	Chinese academy of agricultural crops research institute
2	*Oryza officinalis*	Chinese academy of agricultural crops research institute
3	*Oryza officinalis*	Chinese academy of agricultural crops research institute
4	*Oryza officinalis*	Chinese academy of agricultural crops research institute
5	*Oryza longistaminata*	Chinese academy of agricultural crops research institute
6	*Oryza latifolia*	Chinese academy of agricultural crops research institute
7	*Oryza alta*	Chinese academy of agricultural crops research institute
8	*Oryza rufipogon*	Chinese academy of agricultural crops research institute
9	*Oryza sativa* cv. Nihonbare	Chinese Academy of Quality and Inspection Testing
10	*Oryza sativa* cv. Peiliangyou 210	Chinese Academy of Quality and Inspection Testing
11	*Oryza sativa* cv. Jinyou 207	Chinese Academy of Quality and Inspection Testing
12	*Oryza sativa* cv. Minghui 63	Chinese Academy of Quality and Inspection Testing
13	*Oryza rufipogon*	Chinese academy of agricultural crops research institute
14	*Oryza rufipogon*	Chinese academy of agricultural crops research institute
15	*Oryza rufipogon*	Chinese academy of agricultural crops research institute
16	*Oryza eichingeri*	Chinese academy of agricultural crops research institute

DNA Extraction

The DNA was extracted from sample materials using DNA Quick Plant System (TIANGEN BIOTECH Co.; Ltd; Beijing; China). The extracted DNA was dissolved in 50μL sterile water and then was stored in -20°C for further use.

Primer Design of the DCAPS Marker

The matK gene of *O. officinalis* (Genebank accession No. KF359910.1, OX397922.1, MT726930.1, NC027463.1) were compared with its related species (Genebank accession No. MT731950.1, MF401450.1, KP121861.1, KP864527.1, KF359901.1, MT726928.1, NC034762.1, OV049801.1, HG996592.1, OV050001.1). To effectively distinguish the single sequence variation of 'A/T' between *O. officinalis* and related species, a dCAPS marker was developed. The upstream primer was designed by software the web version of dCAPS finder 2.0 (http://helix.wustl.edu/dcaps/), and the downstream primer was designed by primer 3 (Table 2).

Table 2: Primers sequences and enzyme for the matK of *O. officinalis*.

Primers (5′-3′)	Enzyme	Sequences
OP-m-f	*Alu*I	GAAATCCCATTCTCGCTACGAGAGC
OP-m-r		ATTGAAGGAGTTGAAGC

PCR Specificity Amplification and Enzyme Digestion

PCR reactions were performed using Pfu DNA Polymerase (TIANGEN BIOTECH Co.; Ltd; Beijing; China) in 25 μL of reaciton systems. After preheating at 94°C for 3 min, 35 PCR cycles (94°C for 30 sec, 56°C for 30 sec and 72°C for 30 sec) were performed. The last cycle was followed by a final 10 min extension at 72°C. The amplification products were verified by 1.0% agarose gel electrophoresis. For dCAPS markers, aliquots (10 μL) of the PCR products were digested for 3 h in 20 μL total volume with 5-15 units of the AluI restriction enzyme (New England Biolabs, USA). After restriction enzyme digestion, the digested products were separated by 4.0% agarose gel electrophoresis.

Results and Discussion

The CondonCode software was used to determine whether the SNP could be converted into a dCAPS marker. Finally we found a SNP to convert into a dCAPS marker (Figure 1). By adding 1 bp mutation (A-G) in the forward primer, a AluI recognition site (-AGCT-) was introduced into the forward primer OP-m-f to convert the SNP into dCAPS marker. As a result, only the PCR product from *O. officinalis* could be digested by AluI, whereas the related species of *O. officinalis* PCR products remained undigested. To confirm the specificity of dCAPS, sixteen previously collected Oryza species were subjected to PCR using the pairs of dCAPS marker primers. The PCR products were

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then digested with AluI according to previously established test steps. The products were detected by 4% agarose gel electrophoresis. All PCR products of *O. officinalis* were found to be cleaved by AluI (Figure 2). These results were completely consistent with our expectations.

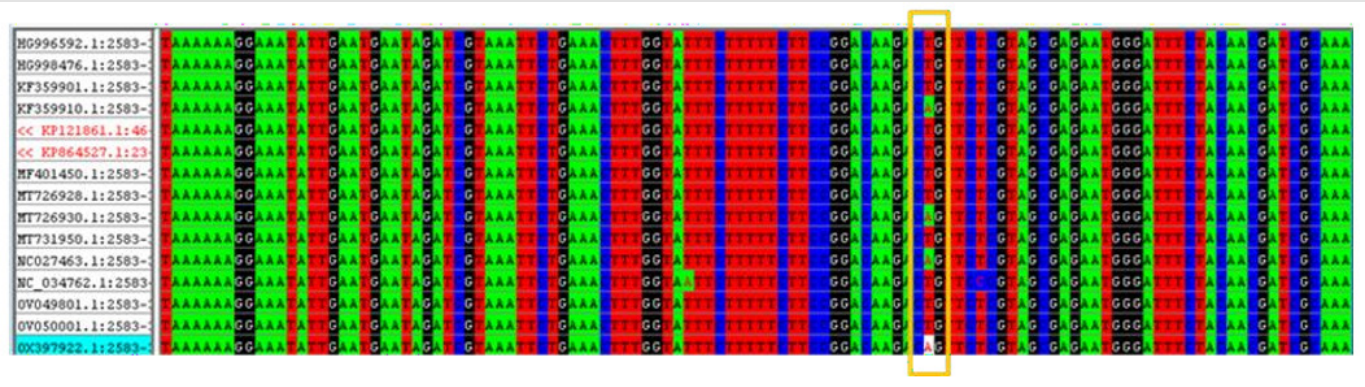

Figure 1: SNP exsiting in *O. officinalis* and its related species.

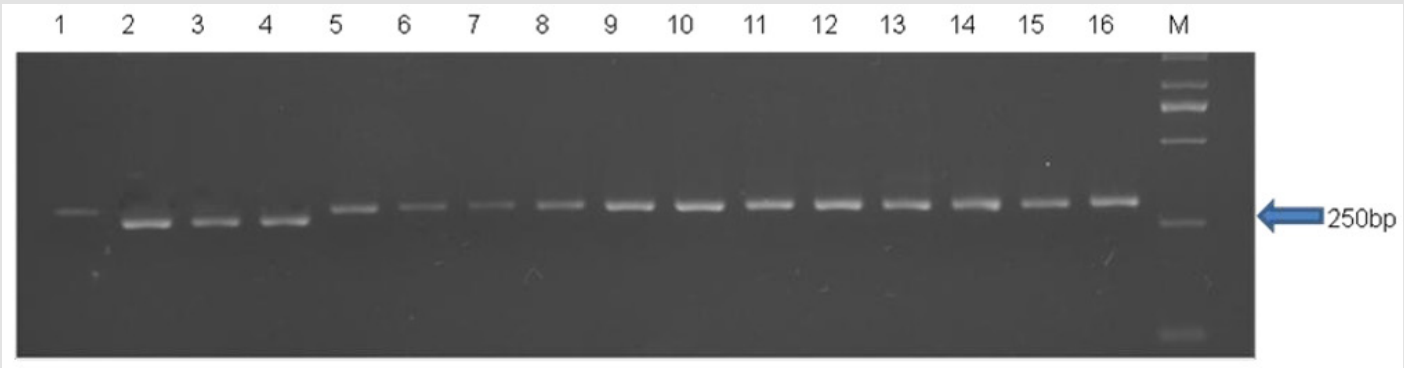

Figure 2: Validation of dCAPS marker usage:

1. *Oryza meyeriana*
2. *Oryza officinalis*
3. *Oryza officinalis*
4. *Oryza officinalis*
5. *Oryza longistaminata*
6. *Oryza latifolia*
7. *Oryza alta*
8. *Oryza punctata*
9. *Oryza sativa* cv. Nipponbare
10. *Oryza sativa* cv. Peiliangyou 210
11. *Oryza sativa* cv. Jinyou 207
12. *Oryza sativa* cv. Minghui 63
13. *Oryza rufipogon*
14. *Oryza rufipogon*
15. *Oryza rufipogon*
16. *Oryza eichingeri* M: Marker DL2000.

The matK gene, located within the chloroplast genome, is characterized by maternal inheritance and a lack of genetic recombination, rendering it a valuable marker for elucidating organismal evolutionary history [10-11]. This gene, which encodes the maturase K protein, spans approximately 1500 base pairs (bp) and is embedded within the intron of the trnK gene [12]. Due to its high efficacy, conserved

mutation rate, and superior resolution compared to other loci, matK is extensively utilized for plant identification. [13]. Finally we found a SNP by comparing the matK gene sequences of *O. officinalis* and its related species, and developed a set of dCAPS markers. Additionally, the primer set used in this study was designed to make the fragment differences after enzymatic digestion significant and easy to observe. This enables the rapid identification of *O. officinalis* via 4% agarose gel electrophoresis, obviating the need for polyacrylamide gel electrophoresis (PAGE) or sequencing, thereby significantly enhancing detection efficiency.

Conclusion

In conclusion, we described the development of dCAPS markers based on singlc nucleotide polymorpbic scquence (SNP) of the matK gene to discriminate between *O. officinalis* and its related species. The dCAPS-based method developed in this study is simple, cost-effective, and capable od distinguish *O. officinalis*, making it a valuable tool for genetic map construction, gene mapping, germplasm identification, and genetic diversity studies.

Disclosure Statement

No potential confilict of interest was reported by the authors.

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