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Validation of ITS3 Primer for the Plant *Vigna Radiata*

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Abstract

Legumes are the third largest flowering family and are the second only to cereals in their importance for human nutrition. Mung bean and common bean are legumes which are rich in nutrients such as proteins, oils and carbohydrate. DNA barcoding, a system for fast and accurate species identification will make ecological system more accessible. Plant DNA barcoding have a huge role in conserving endangered species and in assessment of biodiversity hotspots and also to monitor the international trade of the rare species apart from the routine identification. In this preliminary study, the ITS primers were evaluated and found to be the best for the selected plant *Vigna radiata* (L.). ITS primers 3 was found out to be a suitable barcode.

Keywords: Mung bean; PCR; Barcoding; ITS; BLAST; Sequencing

Introduction

Legumes (Fabaceae) are the third largest flowering family and are the second only to cereals in their importance for human nutrition [1]. Legumes are inexpensive, nutrient dense and high quality sources of protein that can be substituted for dietary animal protein. Mung bean has been widely cultivated in the Indian and in adjacent region for several thousand years and is known to have spread to other Asian countries and northern Africa [2,3]. Mung bean is an erect plant with several branched and having trifoliate leaves like other legumes [4].

DNA barcoding is a system for fast and accurate species identification that makes ecological system more accessible by short DNA sequence instead of whole genome and is used for eukaryotes [5]. The short DNA sequence is taken from standard region of genome to generate DNA barcode. The term DNA barcode is used to refer to a DNA sequence based identification system that may be constructed with one locus or several loci, which is used together as a complementary unit. The mitochondrial gene encoding cytochrome C Oxidase subunit 1 (CO1), is used as a DNA barcode in case of animals and even fungal species because of its variability and universality [6]. In case of plants, the mitochondrial genes and CO1 is not a suitable barcode because of their low mutation rate, rapidly changing structure of the genome their low nucleotide substitution rates and intra molecular recombination. The standard region used to generate DNA barcode is known as marker [7]. There are several markers used in this process such as ITS markers for coding nucleus regions such as rbcL and matK from the chloroplast genome which are recognized as barcode markers for plants [8].

The procedure of DNA barcoding has following three aspects:

- Molecularization *i.e.* the use of the variability in molecular markers as a discriminator.
- Computerization *i.e.* the transposition of the data using informatics support.
- Standardization *i.e.* the extending this approach to vast group of organisms.

Plant DNA barcoding have a huge role in conserving endangered species and in assessment of biodiversity hotspots and also to monitor the international trade of the rare species apart from the routine identification. Several factors are considered in selecting a plant DNA barcode, like

- Universal PCR condition
- Range of taxonomic diversity

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- Power of species differentiation.
- Dry lab analysis and application.

Gao, et al. evaluated ITS2 region as an effective marker for use in authentication of the fabaceae which has important medicinal plants. The reliability of DNA barcoding of *Dalbergia* specimens was assessed. The specimen identification was performed using four barcode (ITS, matK, rbcL, psbA-trnH) and their combinations [9-12].

Wojciechowski, et al. have used the matK regions to construct the phylogeny of legumes. The identification of the mediterranean leguminous crops using a method based on their morphological characteristics has proved tricky and even impossible when working with commercial products [13]. Madesis, et al. evaluated the use of 2 chloroplast regions, trnL and ITS2 for their efficiency in barcoding the main mediterranean plants. Nicole, et al. focuses on the application of DNA barcoding to cultivated bean germplasm as a new tool for discrimination among *Phaseolus* spp. and most of all, for identification of *Phaseolus vulgaris* (L.) varieties. Raveender, et al. evaluated the efficacy of the proposed plant barcoding loci, matK, along with ITS2 for the barcoding of *Vigna* species. In this current study the evaluation of ITS primers to be the suitable markers for the selected plants *Vigna radiata* (L.) and *Phaseolus vulgaris* (L.) [14-17].

Materials and Methods

Plant materials

The seeds were collected from different pulses research institutes located at Kanpur (Indian institute of pulses research), Gujarat (Junagadh agricultural university) and Tamil Nadu (national pulses research centre, Vamban). The plants were grown using the seeds obtained. The leaves and seeds were used to isolate DNA [18].

Isolation of genomic DNA from leaves

Isolation of DNA was performed for both leaves and seeds as suggested by Agbagwa, et al. and Gupta, et al. The leaves of the plant were collected, washed and dried. The leaves were ground well with freshly prepared extraction buffer and CTAB buffer for better cell wall disruption. The ground solution was incubated at 65°C for 15-20 minutes. The phenol: Chloroform: Isoamyl alcohol (25:24:1) was added and centrifuged at 10,000 rpm for 5-7 minutes. To the supernatant obtained isopropanol was added and left overnight at 4°C for better DNA precipitation. The DNA threads were spooled out and washed with ethanol. The washed DNA was stored in 1X TE buffer at -20°C. The DNA was quantified using nanodrop. The quality of the DNA was checked by 1% agarose gel electrophoresis [19,20].

Isolation of genomic DNA from seeds

The seeds were soaked overnight in the distilled water at 4°C. The seeds were ground using extraction buffer and CTAB buffer along with beta mercaptoethanol was added. The ground mixture was incubated at 65°C for an hour. To the ground mixture phenol: Chloroform: Isoamyl alcohol (25:24:1) was added to the incubated mixture. The above mixture was centrifuged at 12,000 rpm for 30 minutes. To the supernatant isopropanol was added and left overnight at 4°C for better precipitation. The DNA threads were spooled out and washed with ethanol and stored in 1X TE buffer at -20°C. The DNA was quantified using nanodrop. The quality of the DNA was checked by 1% agarose gel electrophoresis.

PCR conditions

The PCR amplification reactions were carried out in a 25 microlitre reaction volume which contained the following PCR mix master mix (7.0 microlitre), forward primer (ITS3) (0.7 microlitre), reverse primer (ITS4) (0.7 microlitre), template DNA (5 microlitre) and distilled water (11.6 microlitre).

The PCR experiments for mung bean were performed using the thermocycler with an initial denaturation step of 5 min at 94°C; followed by 35 cycles of 30’s at 94°C, 1.10 min at 54°C or 58°C and 1.20 min at 72°C; followed by 7 min at 72°C and held at 4°C.

The PCR experiments for legumes were using the thermocycler with an initial denaturation step of 3 min at 94°C; followed by 30 cycles of 20’s at 94°C, 20’s at 54°C and 20’s-40’s at 72°C; followed by 2 min at 72°C and held at 4°C.

Results and Discussion

Isolation of genomic DNA

The DNA isolated from the seeds and leaves were quantified using nanodrop and qualified using 1% agarose gel. The concentrations of DNA obtained from seeds were in the range 1500 ng/microlitre-2200 ng/microlitre. The purity (A260/280) was in the range in 1.8-2.0. The concentration of DNA obtained from the leaves was in the range 180 ng/microlitre-200 ng/microlitre. The purity (A260/280) was in the range in 1.3-1.6. The DNA quality of seeds was better than DNA of leaves after 1% agarose gel when viewed. This can be due to the absence of the pigment which is retained even after the purification when (DNA) isolated from leaves. The soaking of seeds is a step where the seed coat is removed hence pigment present in the seed coat can be removed.

PCR conditions

Multiple set of PCR conditions were applied and conditions mentioned Madesis, et al. produced maximum results. PCR conditions mentioned below produced one band for one species of mung bean (Figure 1). ITS primer 3 was observed to provide better results.

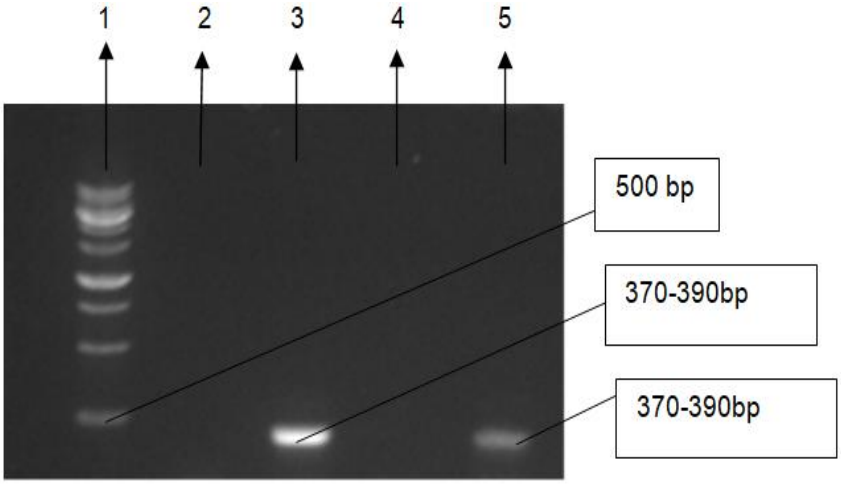

FIG. 1. **Photograph showing amplified product of DNA isolated from mung bean (lane 1-1kb ladder, lane 2-blank, lane 3 DNA with ITS3 and ITS4, lane 4 blank, lane 5-DNA with ITS3 and ITS4).**

Sequence and blast

The ITS region was amplified for the two samples. This resulted in a length ranging from 370 bp-390 bp. ITS3 were found out to be amplifying the regions of the samples.

The BLAST results for the plants mung bean BGG showed similarities with *Vigna radiata* cultivar Tainan (98.89%) and mung bean IPM-2 showed similarities with *Vigna radiata* voucher IPM99-125 (90.31%). This preliminary study show that ITS primer 3 can also be utilized for barcoding these plants.

Conclusion

In this preliminary study, 3 mung bean varieties were collected from agriculture institutes present in Gujarat, Tamil Nadu and Uttar Pradesh. The DNA was isolated from both seeds and leaves of both the plants. DNA isolated from the seeds were taken for amplification process and bands were observed in sample 1 and 2 respectively. The amplifications obtained were a result of ITS3, band size ranging from 370 bp-390 bp. The blast analysis was conducted and the species identity was obtained. Out of the three samples obtained, 2 were barcoded successfully which states that ITS 3 primers can also be utilized as a primer for barcoding of the plant *Vigna radiata*. Further studies can be done for authentic results.

Conflict of Interest

Conflict of interest declared none.

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